Title
Bacterial growth in the plant apoplast is limited by nutrient availability

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Publication Date
2010

Peer reviewed|Thesis/dissertation

Bacterial growth in the plant apoplast is limited by nutrient availability

by

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A dissertation submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

MICROBIOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA – BERKELEY

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Fall 2010

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Acknowledgements

I would first like to thank my advisor Steve, for guiding me through my PhD, through the high and low points a long process like this has. He is a true mentor for whose support I am very grateful.

Many thanks go to the Lindow labbers, past and present, for your friendship and for making every day life at the lab so much nicer; and to the undergrads that journeyed with me, especially Wesley and Paulina, their helping hands and cheerfulness made all the difference in the world.

My family, that never-ending source of wisdom and love deserves all the credit. I especially want to thank my parents, Isabel and Alejandro, for letting a little girl believe that anything can be achieved with willpower and hard work, and my sister Natalia, for her wise advice and loving help during tough times.

Finally to my husband Juan Pablo, a most special man, thanks for being so loving and supportive. “I couldn’t have done it without you” is an understatement.

Chapter 1
Introduction

Preface

There has been a great deal of effort into studying the mechanisms of survival and multiplication of bacteria in plants. The implications of such studies with respect to pathogenic and non-pathogenic bacteria are not only essential to create feasible disease management, biocontrol strategies, and/or the engineering of resistant crops but also can inform other eukaryotic host-prokaryote pathogen systems.

The present chapter introduces the main topics that will be relevant for both the ecological aspects that influence pathogenic and non-pathogenic bacterial growth in leaves (Chapter 2), and the search for the specific virulence determinants that allow pathogens to achieve high population levels (Chapter 3).

The ecological habitat provided by the aerial parts of plants (of which the leaves are the most dominant organs) is known as the phyllosphere (Last, 1955; Ruinen 1956), a term that emphasizes the three-dimensional nature of this habitat (Lindow and Brandl, 2003). The phyllosphere harbors a high diversity of bacteria, yeast and fungi that range in their interactions with the host plant from saprophytic to pathogenic; but bacteria are the main colonists of the phyllosphere. Most of our knowledge about plant-microbe interactions in the phyllosphere is biased towards the dominant species, coming from the studies on a few dominant genera among the aerobic culturable microorganisms; and of those mostly on studies on phytopathogenic bacteria.

The conditions for growth in and on leaves are particular to this habitat. It is thus important to analyze the environmental conditions present in leaves and know the adaptations to the different ecological niches it provides.

Epiphytic and Endophytic bacterial populations in the leaf

The microbes that live on the surface of the aerial parts of plants are called epiphytes (Leben, 1965; Hirano and Upper, 1983). Strictly speaking, the epiphytic population is composed of the microbial species (or a subpopulation of them) that multiply in the ecological niches provided outside of the waxy cuticle layer of the epidermal cells (Beattie and Lindow, 1995; 1999). The surface of a leaf, an enormous expanse on a bacterium's scale, is not homogeneously colonized (Leveau and Lindow, 2001; Monier and Lindow, 2004; Leveau, 2006). Studies with scanning electron microscopy have found bacteria located in the crevices of epidermal cells, at the base of trichomes, near stomatal openings, in the cell wall junctions of epidermal cells, along veins, in depressions of the irregular cuticle and near hydathodes (Beattie and Lindow, 1999). Using epifluorescence microscopy, *Pseudomonas syringae* pv. *syringae* B728a (hereafter *Pss* B728a) cells were observed mostly at the base of glandular trichomes and along veins but not associated with stomata on the leaves of bean plants (*Phaseolus vulgaris*) (Monier and Lindow, 2004). The majoritiy of the bacterial cells are also found within aggregates (Leveau and Lindow, 2001; Monier and Lindow, 2004). Conversely endophytes (from the greek: "endon"= whitin and "phyton"= plant) are those microbes that colonize the inside of leaves, including the substomatal cavities, intercellular spaces of the mesophyll, and the vascular tissues (Beattie and Lindow, 1995).

While saprophytic, non-pathogenic populations are more aptly described as generally being epiphytic in nature, they also sometimes attain a small but measureable endophytic population, usually in levels less than $100\text{-}10^5$ cells/g (Wilson et al., 1999). Plant pathogens, on the other hand, can have both an epiphytic phase followed by endophytic multiplication, which in turn will enlarge the epiphytic population as bacteria egress to the leaf surface (Fig. 1-1). The endophytic phase is the one directly associated with the onset of disease symptoms, as substantial endophytic growth needs to occur before a threshold level is reached for lesions to occur (Beattie and Lindow, 1995; Alfano and Collmer, 1996). The extent of each phase will depend largely on the type of pathogen and its particular lifestyle. For example, some pathogens like *Pss* B728a have an extensive epiphytic colonization phase before entering the leaf and multiplying endophytically. It is apparently common to find asymptomatic leaves that are heavily colonized by pathogenic

strains on the surface (Leben, 1965; Hirano and Upper, 1983; 1990). Thus, a large epiphytic population is a pre-requisite for a large endophytic population but it does not necessarily lead to one (Beattie and Lindow, 1995). For these types of pathogens like *Pss* B728a, the level of epiphytic growth will correlate with the probability of disease, as a large epiphytic population will increase the likelihood of entering the leaf to, in turn, multiply to high levels endophytically and produce disease.

Other pathogens like *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) and *Pseudomonas syringae* pv. *tomato* DC3000 (hereafter *Pto* DC3000) do not generate an initially large epiphytic population but tend to rapidly locate the interior of the leaf and multiply there (Beattie and Lindow, 1995; Feil et al., 2005). The egress of the bacteria may generate most of their epiphytic population in this case. For this kind of pathogen, the epiphytic population will only appear late in time and is therefore not well correlated with the probability of disease (Beattie and Lindow, 1999).

In functional terms, the distinction between epiphytes and endophytes is somewhat unclearly defined populations that nevertheless overlap with the aforementioned definitions: the bacteria that can be removed by sonication and washing constitutes the epiphytic population, while the remaining bacterial population after surface sterilization of the leaf defines the endophytic population. It is important to note that the total population for a given strain will not necessarily constitute the addition of these functionally defined epi and endophytic populations. Washings often times underestimate the number of bacteria living in the surface (either because of strong attachment and/or bacterial localization in "protected sites" on leaf surfaces and surface sterilization can potentially kill some bacteria living inside the leaf in sites that may be accessible to the sterilizing agent. Moreover, the method utilized to retrieve these populations will have different yields (i.e. UV surface sterilization is less efficient than H_2O_2 in killing surface bacteria) (Wilson et al., 1999). Thus, for the purpose of this work, we will refer as the endophytic population the bacterial cells left after surface sterilization using H_2O_2, and the total population being all the colony-forming units (CFUs) that can be enumerated in the unsterilized macerated leaf.

The conditions found in the phyllosphere can be stressful to bacteria. There are many physical, chemical and biological factors that may (un)favorably affect colonization and survival on the leaf surface: radiation and UV-light incidence, fluctuating temperatures, osmotic/matric stress levels, quantity and quality of carbon and other resources, presence of other resident bacteria, plant genotype, age of and site on the leaf, etc. Many of these factors are highly variable both spatially (among leaves, specific sites on leaves) and temporally (time of day, season, etc.). The survival strategies of pathogenic and non-pathogenic bacteria can thus be very different. Beattie and Lindow defined two main different strategies for dealing with environmental stress by phyllosphere bacteria: stress tolerance and stress avoidance. While non-pathogens living on the surface rely primarily on adaptations for stress-tolerance, pathogens likely have adaptations leading to both stress tolerance and avoidance since they are able to establish populations both outside and inside the leaf. The ability to invade the leaf apoplast provides pathogens with

a means of avoiding the harsh surface environment but also the advantage of colonizing a new niche with less competition and possibly a more stable habitat (at least in terms of temperature, light, wind, moisture, etc.). Bacteria may encounter a totally different habitat, with different growth-limiting conditions, inside leaves in terms of nutrient/water availability, type of plant defenses, etc. (Lindow and Brandl, 2003; Beattie and Lindow, 1995). I will thus further discuss some of the factors that limit bacterial growth in the phyllosphere, and the strategies bacteria employ to circumvent them, paying particular attention to those factors that are especially relevant to the current work, namely nutritional resources (especially carbon source), humidity and plant defenses.

Bacterial epiphytic growth is strongly influenced by nutritional demands

Surface populations of both "good epiphytic" pathogens such as *Pss* B728a and saprophytic non-pathogens such as *Pantoea agglomerans* and *Pseudomonas fluorescens* apparently both have stress tolerance adaptations. Some of these surface stress adaptations are, for example: pigment production (differentially present in most phyllosphere but not rhyzosphere bacteria); expression of enzymes for increased UV tolerance such as several catalases and *rul*AB genes encoding error-prone DNA polymerases to cope with potentially lethal UV-induced mutations; the ability to form aggregates and produce EPS for resisting desiccation stress, etc. (Lindow and Brandl, 2003; Feil et al., 2005).

A major factor that seems to determine bacterial fate on and in the leaves is the availability of nutritional resources. In particular, the abundance and distribution of carbon sources seems to be critical. The main source of carbon on leaves is the leachate from wounds, and natural openings in structures like stomata, trichomes and hydathodes that creates a pattern of heterogeneous distribution of resources (Leveau, 2006). Leaves leach mainly carbohydrates such as glucose, fructose and sucrose but also aminoacids, organic acids and plant waste products such as methanol and methylamines to the surface (Mercier and Lindow, 2000; Leveau, 2006). Sugars from uncolonized leaves can be recovered by washing (Tukey, 1970). The finding that bacterial aggregates follow a similar heterogenous pattern to where water and sugars collect lends credence to the idea that Carbon is the main limiting factor for bacterial growth on the surface of leaves, while other resources like nitrogen sources are only secondarily limiting in well-fertilized plants (Wilson and Lindow, 1994; Mercier and Lindow, 2000; Leveau, 2006).

For example, Mercier and Lindow found that the levels of epiphytic colonization of *Pseudomonas fluorescens* cells on bean leaves was correlated with the amount of sugars found on individual leaves (varying 28-fold and 25-fold respectively). They also determined that the amount of growth on different plant hosts could be correlated with the amount of sugars on the different plant species: pea and corn, the plants with the least amounts of sugars harbored smaller populations of epiphytic bacteria than bean and tomato (tobacco and cucumber were intermediate) (Mercier and Lindow, 2000). Similarly to other studies (Wilson and Lindow, 1994), they found no nitrogen limitation since unlike the addition of glucose, the addition of ammonium sulfate did not increase the amount of *P. fluorescens* cells. Monier and Lindow also found that the distribution of *Pss* B728a on leaves was not spatially uniform, that the majority of cells formed aggregates of at least

100 bacteria, and that these aggregates were mainly associated with wounded cells and certain foliar leaf structures such as glandular trichomes, veins, hooked trichomes and undifferentiated epidermal cells (in order of preference) (Monier and Lindow, 2003).

Studies with whole cell bacterial biosensors have been instrumental in showing the localization and heterogeneity in the availability of nutrients at epiphytic sites on a more relevant bacterial scale. Leveau and Lindow, utilizing a fructose-responsive biosensor based on a short-half life version of GFP (*P. agglomerans* (pPfruB-gfp[AAV])), determined that the distribution of bacteria that were consuming fructose on the foliar surface varied spatially and temporally. New bacterial immigrants to a previously uninhabited leaf rapidly engaged in the consumption of fructose (initially nearly all cells were reporting fructose-sensing activity) while by 24 hrs nearly all had stopped metabolizing fructose (only 11% of cells exhibited fructose-sensing activity by 7 hrs and by 24 hrs the percentage had dropped to only 1%) (Leveau and Lindow, 2001). In another study with a *P. agglomerans* sucrose-sensitive biosensor, it was determined that the average amount of sucrose on bean leaves was 20μM and that sucrose also seems to be spatially variable (Miller et al., 2001). Following this spatial pattern of nutrient availability, bacteria vary in size along leaves, where larger cells are found near glandular trichomes (where they also tend to form larger aggregates) than in other places (Monier and Lindow, 2003, Monier and Lindow, 2004). Thus after initial colonization is established (and most available sugars consumed) a pattern of a desert containing "oases" is established; most bacteria find themselves in "deserts" of sugars and only a few others are located in very nutrient-rich "oases" (Leveau and Lindow, 2001; Lindow and Brandl, 2003). These oases, site of nutrient leakage, are the sites where natural communities of different microorganisms form heteroaggregates and seem to thrive on leaves (Lindow and Brandl, 2003).

An interesting aspect of the availability of sugars on leaf surfaces is the fact that a significant amount of sugar is still present but somehow unavailable to bacteria after extensive colonization, and that subsequent leaching of sugars may be limited. For example, Mercier and Lindow found that as much as 20% of glucose can still be washed from heavily colonized leaves suggesting that some of the sugars on leaves are unavailable to the bacteria and that continual leakage might be more limited than originally thought (bacteria stop dividing after 20hpi) (Mercier and Lindow, 2000). Consistent with the idea of a limited availability of new sugars due to limited leaching after initial colonization, it was found that only small additional amounts of sugars can be washed from leaves in decreasing amounts (Lindow and Brandl, 2003; Tukey, 1970).

Several factors can affect foliar sugar distribution and availability. Some factors that have been proposed are: intrinsic differences in cuticular thickness and leakiness, (for example due to differences in waxy content and leaf age), photosynthesis rates (i.e due to position in the canopy) and the nutrient content of the plant as a whole (Ercolani, 1991; Lindow and Brandl, 2003; Mercier and Lindow, 2000; Fiala et al., 1990; Leveau, 2006).

The level of moisture on the foliar surface can be another growth-limiting condition, both directly due to desiccation stress, but also indirectly by decreasing the availability of nutrients. Epiphytes produce surfactants and other molecules that are able to increase the amount of superficial water on the leaf (Lindow and Brandl, 2003). The formation of aggregates, and other traits such as EPS production controlled by "quorum-sensing" are thought to at least in part alleviate water-stress conditions for *Pss* B728a in leaves. A quorum-sensing mutant (*ahlI*- and *ahlR*- double mutant) produced less EPS and had reduced fitness on dry leaves (Quiñones et al., 2005). The formation of aggregates is thought to provide protection from desiccation stress by encasing bacteria in EPS and aiding in retention of water and nutrients.

A study showed that the survival of *Pss* B728a cells under dry conditions was higher in bacterial clusters than for solitary cells (Monier and Lindow, 2003). In a later study and using different fluorescent dyes, a study on the survival of bacteria growing on bean leaves under dry conditions determined that the presence and type of resident bacterial aggregates on the leaves influenced the survival of solitary immigrant cells. For example, they found that *P. agglomerans* and *P. fluorescens* A506 were twice as likely to survive when landing in *P. agglomerans* aggregates than on uncolonized epidermal cells (Monier and Lindow, 2005).

Interestingly, there is also evidence that moisture levels in the environment outside of leaves affects the proportion of pathogenic cells (*Pss* B728a and *Pss* 9B1) that survive surface sterilization with H_2O_2 (Wilson et al., 1999). The proportion of viable cells found in locations protected from surface sterilization increased with dry conditions. Furthermore, growth in these H_2O_2-protected locations (mostly endophytic growth) was correlated with the total amount of growth on the leaves, suggesting that internal growth was chiefly responsible for the large population levels of the *Pss* pathogens. Because saprophytes (*P. agglomerans*, *Methylobacterium organophilum* and *Stenotrophomonas maltophilia*) and non-pathogenic *Pss* were unable to develop these internal populations, their total viable population decreased greatly under dry conditions (Wilson et al., 1999).

Several of these previous studies speculated that nutrients might be the main limiting factor and the driving reason for bacterial localization on leaves (Wilson et al., 1999; Leveau and Lindow, 2001; Monier and Lindow, 2003; 2005). In support of this view, another study that inoculated bean leaves with *P. agglomerans* (P_{proU}-GFP), a matric and osmotic stress biosensor strain, provided evidence that epiphytically growing cells likely do not suffer extreme water deprivation (Axtell and Beattie, 2002). Thus, perhaps the amount of water on the leaf might have indirectly a more dramatic effect on the availability of nutrients. Bacteria in turn can also modify their environment by altering cuticular permeability (Schreiber et al., 2005) or by surfactant production to stimulate wetting of the leaf surface (Bunster et al., 1989), and thus colonized leaves might provide limited amounts of sugars due to quick consumption of the released nutrients by the resident microbes (Leveau and Lindow, 2001). On the other hand, the formation of hetero-aggregates by resident microbes in colonized leaves can provide protection from desiccation stress to new immigrants and aid in nutrient retention, thus increasing the

survival ability of new foliar immigrants (Monier and Lindow, 2005; Lindow and Brandl, 2003).

What are the main factors affecting endophytic baterial growth in the apoplast?

While many factors may limit bacterial growth inside leaves, nutritional limitation may be a primary limitation. Information on the availability of nutrients to bacteria in the apoplast is very limited. However, there are reports that endophytic pathogens affect carbohydrate metabolism in infected tissues and modify the source-to-sink interaction in plants in order to grow. Leaves are source tissues that produce carbohydrates in excess and transport them mainly in the form of sucrose from the producing mesophyll through the phloem to the sugar-consuming sink tissues (growing meristems, developing leaves, fruits, seeds and tubers). Sucrose is transported symplastically through plasmodesmata toward the minor veins where it is uploaded apoplastically. Studies on infection with bacterial, fungal and viral pathogens in plants agree that pathogens in general down-regulate photosynthesis and likely induce accumulation of apoplastic nutrients, thus inducing the transformation of the normally carbon-source producing mesophyll into a carbon source-consuming sink. The source-to-sink transformation likely happens due to the dual action of sugar consumption by pathogens, and their activation of the plant cell wall-invertases (fungi even activate their own invertases upon infection) (Berger et al., 2004; Biemelt and Sonnewald, 2005). However, reports do not agree on the pattern of carbohydrate utilization by pathogens and it is possible that it may vary between different pathogens. Not withstanding, reports tend to agree that infection induces a higher hexose/sucrose ratio. For example, in a study where *Pto* DC3000 was used to infect an incompatible tomato host, sucrose levels decreased faster than total sugars but both sucrose and hexoses were significantly decreased after 3 days post infection (the hexose/sucrose ratio was increased within 24 hrs but increased even more with time) (Berger et al., 2004). Another study with brown rust fungus (*Puccinia hordeii*) in barley leaves also revealed a general reduction in sugar levels in the apoplast of infected tissue, with a concomitant increase in the hexoses/sucrose ratio. Infection reduced total sucrose and glucose but left fructose unaltered (Tetlow and Farrar, 1993). The authors proposed that this was consistent with an increase in apoplastic invertase activity followed by preferential uptake of glucose by the fungus. Because there are mainly sucrose, not hexose transporters, for uploading such sugars into the phloem, the higher concentration of hexoses in the apoplast created by a higher invertase activity would presumable provide a competitive advantage for the fungus over the plant.

Other studies with viral and powdery mildew infections showed instead of a decrease, an accumulation of sugars in infected tissues (Biemelt Sonnewald, 2005). It is possible that the differences might arise due to the fact that some pathogens might differentially induce utilization of starch reservoirs in mesophyll cells (in which case sugars might be observed to increase even though they are being actively consumed). Study of *Pto* DC3000 in an incompatible tomato host however showed that starch levels were quite stable (Berger et al., 2004).

Some interesting studies by Baker, Atkinson and co-workers suggested that changes in plasma membrane permeability of mesophyll cells are the cause of successful endophytic growth by pathogens. The authors postulate that there is a correlation between bacterial population levels of both compatible and incompatible pathogens and of saprophytes with the amount of H^+ influx/ K^+ efflux exchange that occurs between the plant cells and the apoplast (Baker et al., 1987; Atkinson and Baker, 1987a; 1987b; Baker et al., 1991). They argued that pathogens, but not saprophytes, could induce a H^+ influx into cells and a concomitant K^+ efflux to the apoplast (XR exchange) in infected suspension-cultured tobacco and bean cells. This XR exchange, they argued, effectively disrupted the normal trans-membrane electromotive force created by the plant's H^+/ATPase embedded in the plasma membrane which in turn promoted sucrose leakage out of plant cells and its accumulation in the apoplast to enable pathogenic growth.

The apoplast is composed mainly of air spaces and only a thin layer of liquid cover the plant cell walls. The composition and volume of this apoplastic liquid layer seems to be important for normal plant physiology. This compartment is highly sensitive to perturbations like flooding or osmolarity changes but not to other stresses like change in pH (Felle and Hanstein, 2002). In a very interesting work, different substances were fed into the cut petiole of *Vicia faba* leaves while the pH of the substomatal chamber was continually monitored by microelectrodes inserted through stomata. The authors found that when they infiltrated different buffers having similar apoplastic composition but ranging in pH from 4.9 to 8.5, the substomatal pH remained largely unaffected and within 30 min had returned to basal levels (even with increasing buffer strengths of 1 to 10mM, although higher concentrations made it more difficult for the plant to adjust). Responses to known elicitors of apoplastic pH change such as ABA (alkalinization) and fusicoccin (acidification) were normal, validating their system. Feeding suspensions of high osmolarity through the petiole induced alkalinization of the leaf apoplast. Interestingly, they also found the apoplast to be highly sensitive to both local flooding through the stomata and especially by vacuum infiltration flooding through the petiole. The local flooding induced a very large 1.5 pH-unit change (from pH=5 to 6.5) that returned nonetheless to basal levels within an hour. The major infiltration through the petiole, though, took up to 5 hours to recover, likely due to the much higher volume of liquid the leaf had to reabsorb. During this period, responses to "light-on" and "light-off" were strongly hindered (Felle and Hanstein, 2002).

The apoplastic environment can also be influenced by the fact there is no waxy cuticle in the cell walls of mesophyll cells as in epidermal cells. The availability of water in the apoplast thus could be very different from the surface of leaf. There is evidence that some pathogens induce water congestion in the apoplast, perhaps to alleviate water stress but more likely to overcome a nutritional limitation. For example Rudolph found in 1978 that the concentrated sterilized exudate from water-soaking lesions of *Pseudomonas syringae* pv. *phaseolicola* (*Pph*) could induce a persistent cell-free water-soaking in susceptible but not resistant bean cultivars. These water-soaked lesions were more visible when plants were bagged (under 100% relative humidity) or when treated with highly concentrated exudate but disappeared quickly in resistant beans or non-host chards. The

author proposed that infection sites in natural infections probably cause water-soaking under conditions of low relative humidity as well (Rudolph, 1978).

Support for the idea that water-soaked lesions likely alleviate some other growth-limiting stress but not necessarily a desiccation stress, came from experiments by Beattie and co-workers. A study using a water-stress responsive biosensor based in the *proU*-promoter (P_{proU}) (Axtell and Beattie, 2002) demonstrated that only incompatible (HR-inducing) *Pto* DC3000 strains suffered extreme desiccation stress in the apoplast of *Arabidopsis* leaves. *P. fluorescens* A506, *hrcC*⁻ *Pto* DC3000 and the compatible pathogen *Pto* DC3000 all experienced higher water potentials that were suitable for bacterial multiplication (Wright and Beattie, 2004). Thus this study concluded that under normal conditions bacteria should encounter enough apoplastic water to divide unless the plant mounts a strong HR-based defense reaction.

The role of plant defenses in the apoplastic growth of bacteria has been a central topic of research for several decades. Many TTSS effectors have been implicated in this role and thus the analysis of the relevance of TTSS and its effectors for endophytic growth is highly pertinent.

Type III secretion (TTSS) in plant pathogens

Many Gram-negative bacteria utilize the type III-secretion system (TTSS) for the secretion and translocation of protein effectors directly from the bacterial cytosol to the host cell. Bacteria with pathogenic, commensal or symbiotic lifestyles utilize their TTSS to modify the conditions of growth in their eukaryotic host. Intracellular and extracellular animal pathogens like *Salmonella*, enteropathogenic *Escherichia coli* and *Vibrio cholerae*; plant pathogens (all extracellular), opportunistic pathogens like *Pseudomonas aeruginosa* and *Burkholderia* spp., symbionts like *Rhizobium* sp. NGR234, *Mesorhyzobium loti*, and other more obscure bacteria like *Verrucomicrobium spinosum* that may interact with algae have been shown to carry one or more TTSS (Pallen et al., 2005). Many bacterial species of the genus *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Erwinia* and *Pantoea* are plant pathogens capable of growing in the intercellular spaces of leaves. They all share the presence of the TTSS, which is encoded by the *hrc*/*hrp* cluster of *hrp* (hypersensitive response (HR) and pathogenicity) and *hrc* (hrp conserved) genes. The *hrc*/*hrp* gene cluster encodes the conserved TTSS proteins that form the needle-like TTSS pilus. The TTSS pilus is assembled in the bacterial envelope, upon induction by plant diffusible signals that vary depending on the type of pathogen but may include low pH, scarcity of complex nitrogen sources, certain carbon sources, etc. Utilizing *Pto* DC3000 expressing *hrpA*:GPF, Boureau and coworkers found that expression of the TTSS starts inside the leaf within 3 hrs after being infiltrated into tomato leaves (Boureau et al., 2002).

The TTSS pilus is formed by monomers of HrpA protein and spans the bacterial inner membrane, the periplasm and the outer membrane and reaches to contact the cell wall and plasma membrane of the plant host where it forms a structure called translocon. This forms a conduit through which bacterial virulence proteins called TTSS effectors travel and directly reach the cytoplasm of the host cell. Together all the translocated TTSS

effectors will eventually transform the host into an environment suitable for bacterial multiplication (Alfano and Collmer, 2004; Alfano and Collmer, 1996).

Some TTSS effector genes are found next to the *hrp*/*hrc* cluster in loci that have been named the conserved effector locus (CEL) and exchangeable effector locus (EEL) for their conserved presence in other phyto-pathogens or their unique characteristics. Many other effectors (i.e. about half in *Pto* DC3000 and almost all the rest in *Pss* B728a) are spread across the genome, and are thought to have been individually acquired through horizontal gene transfer (Alfano et al., 2000).

Both the TTSS effectors and the pilus assembly proteins are transcriptionally coregulated by the product of the *hrpL* gene, an alternative sigma factor that allows the RNA polymerase to transcribe these genes. Under inducing conditions (*hrp* inducing media or growth in the apoplast) characterized by a low pH, absence of complex nitrogen sources, etc., the products of the *hrpRS* regulatory operon are transcribed. HrpR and HrpS are members of the NtrC family of two-component response regulators and they positively regulate expression of the *hrp* genes, since they are required for the σ^{54}-dependent transcription of the *hrpL* gene (Brencic and Winans, 2005). HrpV is a known negative regulator of TTSS *hrp* genes since its overexpression downregulates *hrp* gene expression. Conversely, in a *hrpV* mutant the *hrp* genes including *hrpA*, encoding the TTSS pilin subunit are upregulated.

In pathogenic interactions, the TTSS is required for multiplication and virulence in susceptible hosts, since TTSS mutants are not able to achieve high cell densities in plant tissues and, therefore cannot induce lesions (Hirano and Upper, 2002). The TTSS is also involved in eliciting a type of localized programmed cell death defense response called the hypersensitive response (HR) in resistant and non-host plants (incompatible interactions). The loss of TTSS is also accompanied by the inability to induce the HR in incompatible plant hosts (Gabriel, 1999).

Non-pathogenic fluorescent Pseudomonads can carry genes encoding a TTSS that may be functional or cryptic. Interestingly, TTSS *hrp* genes encoding homologs for most of the *P. syringae hrp*/*hrc* cluster genes and TTSS effector AvrE were found in the rhizosphere bacterium *P. fluorescens* SBW25 (SBW25) (named *rsp*/*rsc* cluster genes and effector RpoE) (Preston et al., 2001). TTSS expression in *Pf* SBW25 seems to be constitutive, as *hrp* expression under different growing conditions (minimal media, *hrp* inducing media, LB, leaf apoplast) were similar. TTSS expression however, was under the regulation of a HrpL homolog they named RspL. Upon overexpression of *rspL in trans* they were able to show that *Pf* SBW25 could cause an AvrB-dependent HR in *Arabidopsis* (with a 10-fold higher inoculum dose than *Pto* DC3000) (Preston et al., 2001).

TTSS and TTSS effectors are important for pathogenicity

When pathogens like *Pss* multiply in a susceptible host (compatible interaction), population levels rise swiftly, reaching very high levels (up to 10^9 cfu/leaf), with necrotic lesions forming only late in infection when endophytic populations are maximal (Alfano

and Collmer, 1996; Beattie and Lindow, 1995). When the pathogen is multiplying inside a resistant plant cultivar or non-host plant (incompatible interaction), a series of plant defense responses occur that block further bacterial growth. However, until these responses prevent further multiplication, the pathogen can sustain substantial cell division and at a similar rate as that in a compatible interaction (Fig. 1-2). On the other hand, strains that do not have a functional TTSS are unable to multiply inside plant tissues. This is the case of *hrp* mutants of pathogenic strains and also of leaf saprophytes like *Pantoea agglomerans* and *Pseudomonas fluorescens*. Thus, the TTSS and its effectors mediate the ability to multiply inside the host. Saprophytic bacteria and *hrp* mutants are unable to multiply endophytically due to the absence of a functional TTSS and its effectors.

Experiments with beans in the field performed by Hirano and coworkers confirmed that genes encoding the TTSS are essential for growth in the bean phyllosphere but not on seeds or seedlings in field and chamber experiments (Hirano et al., 1999). Interestingly they also found that the *hrp* mutants were able to occationally cause brown spot disease on primary bean leaves. Brown spot lesions were found at an incidence of 4.4% under certain field growth-promoting conditions (high relative humidity and temperatures) or in growth chamber experiments when high inoculum levels of *hrp* mutants were used. These results suggest *hrp* mutants normally do not acheive the threshold population level for lesion formation, but they still have some ability to cause disease. At least for *Pss* B728a, the TTSS is necessary and sufficient for establishing a high population level in the phyllosphere but not for lesion formation, the later being under control of GacS/A (Hirano et al., 1999; Hirano and Upper, 2002).

TTSS effectors of plant pathogens

When a pathogen fails to cause disease in a certain host plant, the interaction is called incompatible. The pathogen is thus an incompatible or “avirulent” pathogen and the plant host is called “resistant”. Effectors recognized by plant resistance proteins (R) were originally denoted Avr proteins to denote the avirulence phenotype that ensued from their expression in a resistant host. A pathogen-plant interaction where the pathogen is able to multiply to high levels and cause disease is referred to as a compatible interaction, where the pathogen is “virulent” and the host is “susceptible”. Effectors that were found on secretion assays were denoted as Hop proteins (for hrp outer proteins) (Alfano and Collmer, 2004). It is now recognized that Avr and Hop proteins are all TTSS effectors that may have both virulent and avirulent activities depending on the particular host. A unified nomenclature was generated to address this problem, and new TTSS effectors are denoted as “hop” proteins. The new nomenclature also unifies the families of related effectors that are represented in more than one genome, where the effector name denotes the family it belongs to followed by the name of the bacterium in which it is found given in subscript (Lindeberg et al., 2005).

TTSS effectors have different relative biochemical activities and cellular targets as different as the PM, mitochondria, chloroplast, nucleus, vesicular traffick, etc. Many effectors have been discovered in different plant pathogens, especially in *P. syringae* pv. *tomato* DC3000 (*Pto*). They were found based on shared features such as association in

pathogenicity islands in the genome, presence of a common transcriptional regulatory element called the "hrp box", abnormal G+C content, amino-acid bias in secretion signals, association with chaperone genes, and by homology-based sequence comparison in newly sequenced organisms (Guttman et al., 2002; Collmer et al., 2002; Fouts et al., 2002; Petnicki-Ocwieja et al., 2002; Chang et al., 2004; Schechter et al., 2006). However, the molecular function and role in virulence for most of these proteins is still an area of active research. One of the reasons why it has been difficult to discover their function is because effectors are thought to be redundant and/or to have small additive effects that contribute to virulence. Thus, single effector mutants frequently attain similar population levels in the plant as the wild type bacterium and do not exhibit a distinguishable macroscopic phenotype. Some progress, at least in *Pto* DC3000 has been made with the simultaneous knock out of multiple effectors to uncover their common function and a reduced fitness of the mutants in the plant (Kvitko et al., 2009).

There are three main functional categories by which plant pathogen effectors are thought to alter the host environment: 1) suppressing plant defenses, 2) aiding in bacterial dispersion, and 3) providing nutrients and water to the pathogen (Chang et al., 2004). The role of effectors in modulating plant defenses has been by far the most studied. Some effectors target components of the defense response pathways of the plant. For example, $HopX1_{Pto\ DC3000}$, $AvrPtoB_{Pto\ DC3000}$, $HopE1_{Pto\ DC3000}$ and $HopF1_{Pto\ DC3000}$ were found to decrease expression of the *PR1* gene by targeting the salicylic acid plant defense pathway to prevent an HR (Jamir et al., 2004). In another study, $AvrPto1_{Pto\ DC3000}$, was shown to target the innate cell wall-based defense or pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) of the plant by preventing papillae formation at the site of infection and by altering the expression of secreted plant defense molecules (Hauck et al., 2003). Some are even able to suppress or mask the strong defense reponse elicited when another effector is recognized by the plant surveillance system (effector-triggered immunity or ETI) (Jamir et al., 2004; Mackey et al., 2002). Other types of effector proteins, like AvrB6 and PthA from *Xanthomonas* spp., induce water-soaking lesions and cell enlargement, respectively, allowing bacteria to escape to the plant surface when the leaf tissue collapses and thus aid in dispersion to new sites of infection (Yang et al., 1994).

Limited attention has been given to the role of TTSS in altering nutrient levels in planta (Biemelt and Sonnewald, 2005) and has not been studied in *Pseudomonas syringae*. A study with *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) suggested a TTSS-dependent control of cell wall-invertase production in peppers. While *Xcv hrp* mutants induced cell wall invertase activity, *Xcv* wild type exhibited a more complex pattern of initial suppression of cell wall invertase activity in the first 24 hrs followed by a subsequent induction of activity that the authors proposed would contribute to meet the growing demands of the actively multiplying pathogen (Biemelt and Sonnewald, 2005). These results suggest that within the first 24 hrs the pathogen would consume the carbon sources already present in the apoplast, and only later would need to induce cell wall inveratase activity to sustain more endophytic growth.

Another proposed way that nutrient limitation could be abrogated by pathogens is the formation of water-soaking lesions that some pathogens induce in plant tissues (like bean pathogen *Pseudomonas syringae* pv. *phaseolicola* and *Pss* B728a in bean pods) (Rudolph, 1978). Such liquids might mobilize nutrients to the site of bacterial growth.

Plant defense resistance against bacterial pathogens

Bacterial pathogens can be broadly classified as necrotrophs and biotrophs. Necrotrophic pathogens feed on dead tissue and rely heavily on the production of degradative enzymes to kill plant cells early in infection (i.e. *Pectobacterium carotovorum* and the fungi *Botrytis cinerea* and *Alternaria brassicicola*) jasmonic acid (JA) pathway and sometimes also the ethylene (ET) defense pathway (Alfano and Collmer, 1996; Glazebrook et al., 2005). Biotrophic pathogens, on the other hand, rely on live tissue to obtain their nutrition (i.e. *Peronospora parasitica*). Some microbes might have one or the other strategies depending on the environmental conditions, and yet some other microbes have strategies somewhere in between like *Pseudomonas syringae*, which is considered a hemibiotroph (basically a biotroph that eventually kills its host tissue in later stages of infection). Plant defense strategies against biotrophs and necrotrophs employ different defense pathways in the plant. Because biotrophs depend on live tissue for sustenance, plant defenses against them involve the salicylic acid (SA) pathway and generally plant cell death (CD). On the other hand, since necrotrophs obtain their nutrition from dead cells, plant defense strategies against them do not lead to CD and generally depend on the jasmonic acid (JA) pathway and sometimes also the ethylene (ET) defense pathway (Glazebrook et al., 2005).

Plant defenses against hemibiotrophs: PAMP-triggered immunity (PTI) and Effector-triggered immunity (ETI)

Basal plant defense resistance to microbial attack has been shown to have great similarity to innate immunity in animal defense. However, they probably arose independently in each lineage (Ausubel, 2005). Unlike in vertebrates, all resistance mechanisms in plants are “innate” and none are adaptive. However, interestingly the same microbial targets are recognized by both animal and plant immune systems (i.e. flagella). Similar PAMP-pattern recognition receptors (PRRs) are used in both cases that carry extracytoplasmic leucine-rich repeats (LRRs) for epitope recognition linked to cytoplasmic kinases (RKs) for signal transduction (such as flagellin receptors FLS2 and TLR5 in *Arabidopsis* and humans, respectively). Finally, they also share similar signal transduction cascades after PAMP-PRR recognition takes place (Nürnberger and Kemmerling, 2009; Ausubel, 2005).

There is a consensus among plant pathologists to classify plant defenses to hemibiotrophic microbes in two levels, PAMP-triggered immunity or PTI and effector-triggered immunity or ETI, although there is considerable overlap in the responses. PTI is considered a first level of defense, thought to be the first barrier for growth especially for saprophytes and other non-TTSS bacteria. In this “basal defense” the plant recognizes conserved molecules or patterns that are widely present in both pathogenic and non-pathogenic groups of microbes but non-existent in plants. This defense

has historically been called "pathogen-associated molecular pattern (PAMP)-triggered immunity" or PTI. Because PAMPs are actually common to all microbes whether pathogenic or not, there have been recent proposals to rename them MAMPs for microbe-associated molecular patterns (Ausubel, 2005; He et al., 2007). Some PTI elicitors are derived from bacterial surfaces, such as flagella, LPS and peptidoglycans; and chitin, glucans, mannans and ergosterol from fungi. Other PTI elicitors, however, are cytoplasmically-derived such as methylated DNA, elongation factor-Tu (EF-Tu) and cold shock protein (CSP) in bacteria. Finally, there are plant-derived patterns that also act as PTI elicitors. They are produced by mechanical or pathogen-induced injury to the host tissues and are thus regarded as "danger"-related molecules (damage-associated molecular patterns or DAMPs). Some DAMPs are for example fragments of the plant cell wall (cellodextrins, oligogalacturonide) or cuticle (cutin monomers) (Nürnberger and Kemmerling, 2009). Thus, PTI provides a mechanism for self versus non-self recognition in plants, or, perhaps more accurately, of a "healthy" or intact self versus a damaged self or non-self. They detect "danger" as evidenced by the presence of an extraneous organism or its activity due to the penetration of barriers and tissues (Jones and Dangl, 2006; Nürnberger and Kemmerling, 2009). A pattern-recognition receptor (PRR) embedded in the plant membrane recognizes a/some PAMP/s in a microbe, and activates a series of plant defense responses collectively known as PTI that include activation of mitogen-activated protein kinase (MAPK) signaling cascades, changes in Ca^{2+} cytoplasmic levels, production of phytoalexins, reactive oxygen species, nitric oxide species, and reinforcement of the cell wall through deposition of callose (He et al., 2007; Nicaise et al., 2009; Ausubel, 2005).

The best understood example of PRR is FLS2 which is one of the abundant LRR-RLK discovered in *Arabidopsis*. FLS2 recognizes a specific conserved epitope in the amino terminal region of flagellin (flg22). FLS2 and flagellin interact and enter the cell through endocytosis. Then, through further interaction with a recently discovered signal transducer BAK1 (which also converges the activation signal by EF-Tu), it activates PTI (Heese et al., 2007; Schwessinger and Zipfel, 2008; Nishimura and Dangl, 2010).

Even though several PAMP targets exist, there is evidence that plants recognize individually only a few of these; and that some PAMPs are more broadly perceived by plants than others. For example, flagellin is recognized by several plant species, but EF-Tu perception is limited to the Brassicas (Schwessinger and Zipfel, 2008; Nicaise et al., 2009). There is also evidence that PAMPs rendundantly activate most of the same defense pathways. Similar sets of genes are targeted in the PTI generated by Ef-Tu and flagellin in *Arabidopsis* (Schwessinger and Zipfel, 2008; Zipfel et al., 2006). Also, a *Pto* DC3000 TTSS mutant, with all its PTI-eliciting activity unprotected due to the absence of PTI-suppressing effectors, induces basically the same transcriptional response in *Arabidopsis* as flg22 alone (Jones and Dangl, 2006). However, flagellin alone may account for most of PTI perception in *Pto* DC3000, at least as measured by the output of callose deposition (Guo et al., 2009). The loss of flagellin in a *hrcC*- *Pto* DC3000 background results in a ~52% reduction of callose deposited in *Arabidopsis* Col-0 (Guo et al., 2009).

Studies on PTI and PAMP perception are still unfolding and in many cases the biological relevance of a given PAMP has been questioned, either because high concentrations of PAMPs were used in plant studies or because of the presence of possible contamination with more biologically active PAMPs (Zipfel and Felix, 2005). Indeed, not all PAMPs seem to be equally good at triggering PTI. For example, while 1μM flg22 was used to induce PTI in *Arabidopsis* Ler-0 (Zipfel et al., 2004) sensitivity to Ef-Tu is in the picomolar range, and doses of LPS 1000-fold greater than the LPS concentration needed to obtain a reaction in animals were used in other studies in plants (Zipfel and Felix, 2005).

PTI induction by flg22 was carefully studied in *Arabidopsis* Ler-0 and *Arabidopsis fls2* mutants, which have lost the PRR receptor for flagellin (Zipfel et al., 2004). The authors found that mutant *fls2* plants are as susceptible as wild type plant when *Pto* DC3000 are infiltrated into leaves, supporting the same growth of compatible *Pto* DC3000 and even avirulent bacteria as wild type *Arabidopsis*. However, plants were more susceptible to *Pto* DC3000 when the pathogen was sprayed onto the leaf surface, suggesting that flagellin perception restricts bacterial invasion but does not play an important role in the apoplast (Zipfel et al., 2004). Since PTI is thought to be the major plant defense response to non-pathogens, and PTI perception of flagellin, the main eubacterial PAMP, is thought to act before bacteria reach the apoplastic compartment, this raises the possibility that PTI might not be restricting non-pathogenic growth in the apoplast.

A careful examination of the literature related to bacterial PAMP molecules shows that many studies monitor ROS, Ca^{2+} signaling changes and/or PR gene induction in cell suspension cultures to assess the effects of their molecules (i.e. CSP in Felix and Boller, 2003; LPS in Zeidler et al., 2004) but information on how a particular PAMP perception restricts bacterial growth is often lacking. When bacterial population studies are done, they seldom address the role of PTI on non-pathogenic strains and the focus is mostly on the effect on virulent strains that often times involve activation of stronger defense responses (Zipfel et al., 2004; Zeidler et al., 2004; Stefani et al., 1994). Information on how particular PAMPs affect non-pathogens, particularly saprophytes, is much more limited (Jakobek and Lindgren, 1993; Lindgren, 1997). Moreover, when bacterial growth assays were performed to test the effects of non-flagellin PAMPs, a reduction in growth of the bacteria was shown in spray inoculation experiments only, and thus the possibility exists that these PAMPs would not restrict growth in the apoplastic compartment (similarly to what occurs with flagellin).

Interestingly, genes activated upon PRR-PAMP recognition include both positive and negative MAPK regulators of PTI, suggesting that PTI is under a self-limiting negative loop regulation (see Schwessinger and Zipfel, 2008 for a model). An interesting idea that arises from this observation is the possibility that PTI might normally prime or reinforce a stronger and longer lasting defense response specifically for pathogens, perhaps sparing a potentially wasteful full blown immune reaction to harmless bacteria (Schessinger and Zipfel, 2008; Zipfel et al., 2004; Ausubel, 2005). Indeed, defense response reactions must be tightly regulated because they can be damaging and costly to the plant. Plant defense

responses are not only energetically costly but also because there is a cost associated with the need for tight regulation of defenses against biotrophic pathogens, since the mere existence of such defenses necessarily weakens antagonistic defenses against necrotrophs (Kliebenstein and Rowe, 2008). We can thus speculate that PTI, as a branch of plant defense responses against pathogens, might have a more relevant role in priming defenses against virulent bacteria but could be more limited for non-pathogenic bacteria that do not constitute a threat. Generally since non-pathogens do not multiply in the apoplast, they might not adequately induce PTI (Ausubel, 2005). Alternatively, if results that PTI perception is lacking in the apoplast (Zipfel et al., 2004) are confirmed by other studies, non-pathogenic bacteria might be limited in growth in the apoplast by factors other than plant defenses.

Effectors likely evolved independently in several lineages and at least in part to overcome PTI (Jones and Dangl, 2006; Chisholm et al., 2006). To counteract this, plants likely co-evolved a second line of defense, effector-triggered immunity, or ETI, forever locking plants and phytopathogens in a perpetual arms-race (see the zig-zag-zig model in recent reviews by Jones and Dangl, 2006; Chisholm et al., 2006 and the evolutionary model by Alfano and Collmer, 2004). This second level of immunity in plants specifically recognizes intracellular changes associated with pathogen activity and produces a defense response that often includes an HR. ETI is initiated when either an effector or molecular pattern resulting from the effector/s' biochemical activity are detected by NB-LRR (nucleotide-binding domain leucine-rich repeat domain) receptors within the plant cell. A subsequent rapid amplification of the signal induces a strong resistance response that generally culminates in an HR and growth arrest of the pathogen. Thus, specific plant genotypes that can recognize a given effector (a dominant phenotype) then become resistant to normally disease-causing compatible pathogens (Chisholm et al., 2006; Jones and Dangl 2006).

The "guard hypothesis" (Dangl and Jones, 2001), a more inclusive paradigm that emerged after the gene-for-gene hypothesis (Flor, 1971) explains how similarly to "danger molecule" recognition (DAMP) in PTI, ETI monitors the presence of a "modified self" by recognizing modified patterns in certain host targets commonly attacked by pathogen effectors (Chisholm, 2006; Jones and Dangl, 2006). PTI and ETI have some overlap in signal transduction cascades and output traits but importantly, PTI elicitors do not normally induce an HR. It has been proposed that PTI might not reach a certain threshold to trigger the HR in most cases, while ETI can. The difference between the two thus could be more quantitative than qualitative (Schwessinger and Zipfel, 2009, Jones and Dangl, 2006; Yao and Greenberg, 2006). Thus ETI could function by enhancing activation of pathways already regulated in PTI and/or de-repressing negative PTI regulators. In this manner, ETI could constitute a "second hit" signal for a stronger defense response proposed by F. Ausubel (Schessinger and Zipfel, 2008; Jones and Dangl, 2006; Ausubel, 2005).

The Hypersensitive Response (HR) in non-host resistance

Broadly defined as a type of localized plant cell death (CD) that takes place upon pathogen attack; the HR can occur in response to incompatible pathogens in both non-host plants and in cultivars of a host plant that are resistant to particular race/s of a given pathogen (cultivar-specific resistance, or host resistance). TTSS effectors can be recognized in both non-host and cultivar-specific resistant hosts, eliciting ETI and resulting in a HR. On a microscopic level, individual plant cells die at the site of pathogen attack resulting in a microscopic HR. This is apparent macroscopically as a region of tissue collapse when the plant is challenged with a high concentration of an incompatible pathogen (generally at least 10^7 CFU/ml). Very few pathogen cells are sufficient to induce an HR. Turner and Novacky, studying the interaction of *Pseudomonas syringae* pv. *pisi* with the non-host tobacco, determined that there is a quantitative relationship of 1:1 between the number of incompatible bacteria in the inoculum and the number of individual plant cells that are dead within 6 hrs after inoculation. Microscopic plant cell death (PCD) started at 1-2 hpi and was always maximal at 6 hpi. If at that time the number of dead plant cells was at least 25% of those in the tissue, a confluent macroscopically visible HR would subsequently develop presumably due to tissue collapse from such extreme damage (Turner and Novacky, 1974).

Although previously hypothesized to be the main reason for pathogen growth arrest, the HR is generally associated with the ETI but is not required for plant resistance to occur. For example, in disease assays some *Arabidopsis* accessions are resistant to *Pto* DC3000 expressing the EEL effector HopA from *Pss* 61; even though they do not develop a typical macroscopic HR when infiltrated at high inoculum levels ($2x10^7$cfu/ml) (Gassman, 2005). What is more, since the ETI defense and cell death signaling cascades are under two different pathways (Zhou et al., 1998), it is unknown what the function of PCD might be, and what the real cause of bacterial growth arrest is. It has been proposed that PCD might exist only to provide a specific signal response that alerts neighboring cells of pathogen attack (Heath, 2000; Nürnberger and Kemmerling, 2009). The true cause of arrest of bacterial cell division was recently proposed to be extreme desiccation produced by the rapid and localized cessation of vascular transport of water to the area undergoing incompatible pathogen attack (Freeman and Beattie, 2009).

Defenses against non-host pathogens can often be associated with HR but not always. There is not a stereotypical plant reaction to non-host pathogens and it is likely that diverse plant pathways are activated in different pathogen-nonhost interactions. In some cases non-host resistance is dependent on single TTSS effectors being recognized by the non-host plant and thus the mutation of these effectors renders the plant suceptible to pathogen growth and lesion formation (Lindeberg et al., 2009). According to a recently proposed model by Mysore and Ryu, non-host resistance responses have been classified as type I (non-HR causing interactions) and type II (HR-causing interactions). Type I non-host resistance is the least characterized even though it is probably the most common type. In some cases the pathogen cannot overcome “passive” or pre-formed plant defenses such as antimicrobials (i.e. saponins in oats against some fungi), the actin cytoskeleton (i.e cytochalasins inhibit actin polymerization and prevent non-host resistance of several plants

against penetration by a plethora of fungi). Even a few cases of non-HR-mediated gene-for-gene resistance are known (i.e. *Pseudomonas syringae* pv. phaseolicola in *Arabidopsis*) (Mysore and Ryu, 2004). On the other hand, type II non-host resistance produces an HR response in much the same way and involves the same genes required for cultivar resistance. For example, an NHO1 *Arabidopsis* mutant loses its ability to resist non-host *Pseudomonas syringae* and *Botrytis cinerea*, but is colonized by its compatible pathogen *Pto* DC3000. Interestingly, the mutant is not compromised in interactions with other non-host pathogens, which reinfoces the idea that different players are involved in different non-host interactions (Mysore and Ryu, 2004; Lindeberg et al., 2009).

Two *Pseudomonas syringae* pathovars to study host (compatible) and non-host (incompatible) interactions with bean

Bacterial plant pathogens engage in one of two types of pathogenic strategies for nutrient aquisition: necrotrophy and biotrophy. Necrotrophs such as *Proteobacteria atrosepticum, Dickeya dianthicola* and fungus *Botrytis cinerea* rely on the production of hydrolytic enzymes that degrade the host's tissues to obtain nutrients, whereas biotrophs rely on the acquisition of nutrients from living tissue. *Pseudomonas syringae* pv. *syringae* B728a (hereafter *Pss* B728a) like other *Pseudomonas syringae* pathovars, *Xantomonas campestris* pathovars and *Ralstonia* are hemibiotrophs, essentially biotrophs that nevertheless eventually kill tissues in their infected host (Collmer et al., 2009; Alfano and Collmer, 1996).

In the present work, we have used *Pss* B728a in most experiments as a model phytopathogen in both compatible interactions with bean and *N. benthamiana*, and incompatible interactions with *Arabidopsis thaliana* to explore the conditions that affect endophytic growth of pathogens and later to identify TTSS effectors from this pathogen that might induce an increased nutrient/water availability in the apoplast. This strain has some relevant specific characteristics that separate it from the rest of the *Pseudomonas syringae* pathovars.

Pss B728a is a Gram negative, γ-Proteobacterium that was originally isolated from a bean plant in Wisconsin (Loper and Lindow, 1987). As the causal agent of bacterial brown spot of snap bean (*Phaseolus vulgaris*), it produces necrotic lesions and water-soaking in both leaves and pods. It is a well-studied plant pathogen both for its agricultural importance and as a model organism to study epiphytic colonization and pathogenicity; and its fully annotated genome sequence is available (Feil et al., 2005).

This strain is particularly successful in the colonization of the leaf surface and has evolved many mechanisms for stress tolerance. However, as part of its survival and strategy of "stress avoidance" in the phyllosphere, *Pss* B728a can also invade and readily multiply endophytically in the apoplast of leaves (Beattie and Lindow, 1995; Feil et al., 2005). *Pss* B728a is equipped to continue growing endophytically even under normally growth-restricting surface dry conditions, a trait that likely constitutes an ecological advantage for *Pss* B728a (Wilson et al., 1999). Apart from its well known host, snap beans, *Pss* B728a is also a compatible pathogen of *N. benthamiana* as it has been shown

recently to multiply to very high levels in this plant and to cause disease (Vinatzer et al., 2006).

Pto DC3000 is our choice for a prototypical incompatible pathogen of beans. The lifestyle of *Pto* DC3000 would place it on the other end of the epiphyte-endophytic spectrum (Feil et al., 2005) since its epiphytic fitness is low as compared to *Pss* B728a. *Pto* DC3000 normally occurs in an extensive endophytic phase in plants with only a later phase of bacterial cell egress to the foliar surface through stomata (Boureau et al., 2002). *Pto* DC3000 causes bacterial speck of tomato and *Arabidopsis*; and with the single effector gene deletion of *hopQ1-1*, it can also cause disease in *N. benthamiana* (Wei et al., 2007). It was the first Pseudomonad to be sequenced (Buell et al., 2003) and is a model organism for the study of TTSS and plant immunity. It has a relatively large genome of 6.5Mb as compared to *Pss* B728a 6.1Mb but shares 4/5 of its genes with *Pss* B728a. However, each strain's unique genes probably encode traits that are important for their different lifestyles (i.e. *Pss* B728a has a more pronounced epiphytic phase) (Feil et al., 2005). *Pto* DC3000 produces the toxin coronatine (a methyl-JA acid mimic that is involved in JA-pathway activation to antagonize SA-dependent defenses against *Pto* DC3000) (He et al., 2004), has more transport and regulatory genes, and a larger TTSS-repertoire (28+ effectors) than *Pss* B728a including 2 effector-rich plasmids that *Pss* B728a lacks (Schechter et al., 2006; Feil et al., 2005). On the other hand *Pss* B728a has a higher UV-resistance and osmotolerance than *Pto* DC3000. *Pss* B728a produces the phytohormone IAA, the toxins syringomycin and syringopeptin and ice nucleation protein for inducing frost-damage (Feil et al., 2005; Beattie and Lindow, 2003).

The large TTSS repertoire of *Pto* DC3000 is considered firmly established after extensive genome mining by bioinformatic approaches as well as functional studies, and is composed of 28 actively secreted and translocated effectors, 12 pseudogenes disrupted in their coding or regulatory sequences and seven weakly-expressed genes (Schechter et al., 2006; Lindeberg et al., 2006; 2009). Most of the functional effector genes of *Pto* DC3000 are clustered, where only 10 out of the total 28 effectors are present as single gene operons in the genome (Kvitko et al, 2009).

The TTSS repertoire of *Pss* B728a is smaller (15 confirmed effectors) and has been determined previously by homology to known effectors in other pathogens (see Feil et al., 2005). Functional TTSS effectors have, however, been determined by translocation assays using whole effector protein fusions to AvrRpt2$^{101\text{-}255}$ (Vinatzer et al., 2005; Vinatzer et al., 2006), and their CD-inducing activities have been determined with *Agrobacterium*-mediated transient expression (Agroinfiltration) studies (Vinatzer et al., 2006). The list of effectors is likely not exhaustive, though.

The smaller TTSS repertoire of *Pss* B728a is attractive for the search of effectors that produce an increase in apoplastic nutrient availability in a bacterium that is well adapted to growing in host and non-host plants. This creates the possibility that it may have effectors that function in different hosts, and the smaller effector repertoire increases

the likelihood of finding a given effector that can increase nutrient/water availability, since they include a plethora of effectors involved in plant defense suppression.

There has previously been no screening for effectors that can increase the nutrient and water leakage in the apoplast. Since nutrients are a clear limiting factor in the phyllosphere (especially C source) (Leveau and Lindow, 2001) we here hypothesize they will be in limiting amounts in the apoplast as well. Our main hypothesis is that there is a subgroup of TTSS effector proteins that function to directly or indirectly alleviate an apoplastic carbon source-mediated growth limitation through increased concentration and/or accessibility of carbon sources (most likely sugars). Since nutrients and/or water are probably limiting in the apoplast, then the second hypothesis is that a subset of these effectors are also responsible (at least partially) for the increased population densities that pathogens achieve in plant tissues.

We expect that the mechanism(s) underlying the effector-induced alleviation of nutrient limitation should be well-conserved among phyto-pathogens and therefore their target(s) and mechanistic function in both host and non-host plants should be similar as well. The question of how a pathogen is able to modify the host environment to its own benefit is central to pathogenesis, and the mechanisms of nutrient acquisition by bacterial pathogens are likely an important piece of the puzzle. This knowledge can prove important for developing disease prevention and biocontrol strategies in the field.

Fig. 1–1 –Schematic diagram of the endophytic multiplication of bacteria.
Growth of bacteria inside leaves depends on the type of strain. Pathogenic bacteria multiplying in its host (compatible interaction) multiply extensively before virulent lesions appear. Pathogens growing in resistant or non-host plants (incompatible interaction) also multiply until detected by the plant defense surveillance system, often associated with a type of plant cell death (CD) called the hypersensitive response (HR). Non-pathogenic strains generally do not multiply endophytically. Adapted from Alfano and Collmer, 1996.

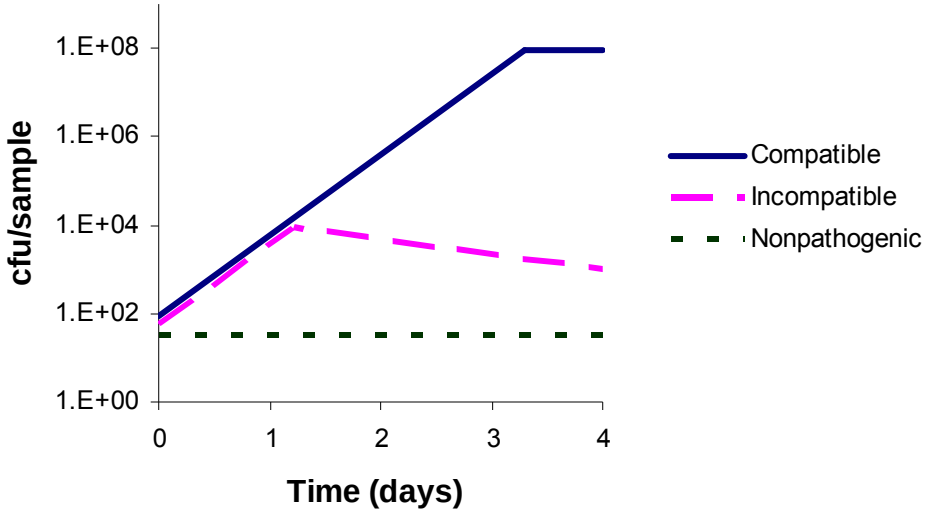
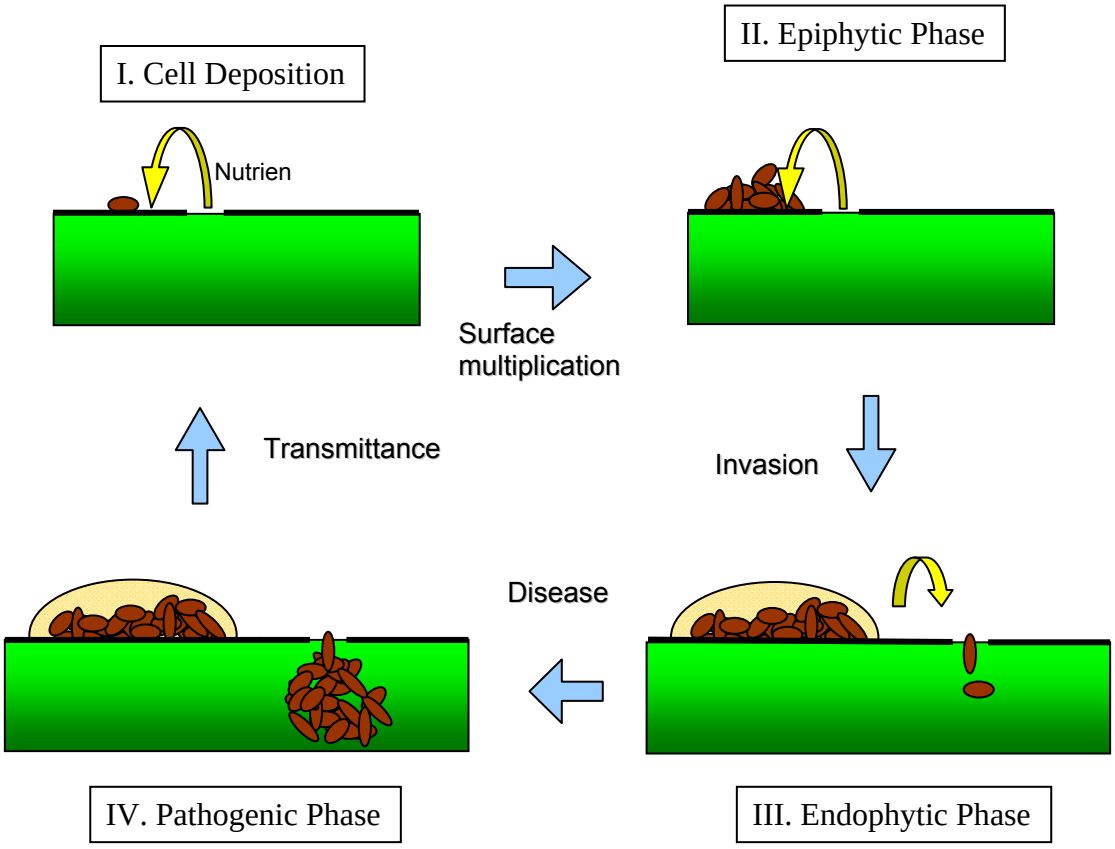


Fig. 1–2 –Schematic diagram o the life cycle of a foliar pathogen.
Pathogens such as *Pss* B728a arrive on leaves and establish a surface population (epiphytic phase). Subsequently, bacteria can gain access to the intercellular spaces inside leaves and further multiply (endophytic phase). The endophytic phase is associated with pathogenicity and the onset of lesion formation (pathogenic phase). Bacteria then can complete the cycle and re-surface for transmittance to other leaves/plants. Adapted from Beattie and Lindow, 1999.



Chapter 2
Bacterial growth in the apoplast is limited by nutrient availability

Preface

Chapter 2 describes the experiments that determined the growth-limiting factors for bacteria multiplying endophytically. For this purpose, in planta growth assays were performed in different host plants.

Abstract

Bacterial ability to multiply endophytically (inside leaves) is an intrinsic trait of bacterial plant pathogens such as *Pss* B728a and *Pto* DC3000. Type III secretion system (TTSS)-possessing pathogens multiply endophytically to large population sizes ($>10^8$-10^9cfu/g). Non-pathogenic strains such as the saprophytes *Pantoea agglomerans* and *Pseudomonas fluorescens*, and *hrp/hrc* cluster mutants (TTSS mutants) mostly fail to multiply endophytically. The growth-restricting activity of the plant defense surveillance system is thought to restrict growth of non-pathogens inside the leaves through a series of responses that induce deposition of callose, reactive oxygen species production, and production of a variety of antimicrobials. This process is collectively known as PTI (pathogen-associated molecular pattern-triggered immunity). However we found several treatments that induce high sugar availability in the apoplast to elicit a dramatic growth of various non-pathogenic strains. Saprophytes and *hrp* mutants growing in both host and non-host plants multiplied up to 10,000-fold either when co-infiltrated into plants with utilizable (i.e. metabolized substrates in vitro) carbon sources, infiltrated with water alone while water-soaking conditions maintained or when co-infiltrated with a TTSS-proficient pathogen. Pathogens were not limited in growth by carbon sources since they carry TTSS effectors that they can deploy to provide them with needed resources. We thus propose that similarly to bacteria growing in the surface of leaves, availability and distribution of sugars are the main limiting factors for endophytic bacteria, and while TTSS-proficient pathogens can alter the plant to obtain apoplastic nutrients, non-pathogenic strains cannot.

Introduction

Many factors are involved in the successful bacterial colonization of the phyllosphere, the environment formed by the aerial parts of plants. Bacteria living on the surface of leaves are called epiphytes while endophytes colonize the interior of leaves. Bacterial survival and colonization in a habitat dominated by highly fluctuating environmental conditions hinge on the presence of stress-tolerance and/or stress-avoidance strategies (Beattie and Lindow, 1995). Pathogenic strains such as *Pseudomonas syringae* are capable of a great degree of stress-avoidance by being able to access the interior spaces of leaves (the apoplastic compartment) and often develop a large endophytic population there that is subsequently associated with the onset of disease symptoms (Beattie and Lindow, 1995; Alfano and Collmer, 1996). On the other hand, saprophytic strains such as *Pantoea agglomerans* and *Pseudomonas fluorescens* are unable to multiply endophytically and their multiplication is restricted to the surface of the leaf (epiphytic growth). These bacteria generally have good stress-tolerance strategies to deal with solar/UV radiation, changing temperatures and moisture conditions, etc. (Lindow and Brandl, 2003; Beattie and Lindow, 1995).

In the present study we seek to understand the growth-limiting conditions for bacteria growing endophytically in leaves. The ability to multiply endophytically in the intercellular spaces of leaves is intrinsic to plant pathogens such as *Pseudomonas syringae* pv. *syringae* B728a (*Pss* B728a) (Feil et al., 2005; Beattie and Lindow, 1995); although a few non-pathogenic bacteria can also achieve some endophytic growth. The endophytic growth of both saprophytes and *hrp* mutants of phyto-pathogens, (lacking the ability to secrete proteins into plant) is minimal (Alfano and Collmer, 1996, Beattie and Lindow, 1995). The type-III secretion system (TTSS) and the virulence proteins that it injects into plants presumably modify the apoplastic environment to allow extensive multiplication of pathogenic strains. Lacking a functional TTSS or the effector proteins secreted by the system prevents non-pathogenic strains from colonizing the apoplast (Alfano and Collmer, 2004). For example, in *Pss* B728a, TTSS genes are essential for growth in the phyllosphere of field grown bean plants (Hirano et al., 1999); and only high relative humidity (RH) and temperatures in the field, or high inoculum levels in growth chamber experiments allowed *hrp* mutants of *Pss* B728a to reach sufficiently high population levels to produce brown spot lesions (Hirano et al., 1999, Hirano and Upper, 2002).

The growth-limiting factors in the apoplast of leaves are less clear than those affecting epiphytic populations on the surface of leaves. The availability of nutrients and water has been proposed to be essential for bacterial multiplication on the surface of leaves. Epiphytic multiplication is primarily carbon-source limited on plants receiving normal fertilization and secondarily limited by other resources such as nitrogen (Wilson and Lindow, 1994; Mercier and Lindow, 2000; Leveau, 2006). The distribution of bacteria on a collection of leaves matches the distribution of the amount of sugars that leach from those leaves. Nutrient loss is thought to be through wounds and natural openings (Leveau and Lindow, 2001; Monier and Lindow, 2004; Mercier and Lindow, 2000). Thus, bacteria tend to form aggregates next to leaf anatomical structures that apparently constitute sources

of nutrients such as glandular trichomes, wounded epidermal cells, veins, etc. (Monier and Lindow, 2003; Lindow and Brandl, 2003; Leveau, 2006).

Surface water availability has also been proposed to limit bacterial growth and several bacterial traits have been implicated in water-stress tolerance such as EPS and surfactant production and the formation of bacterial aggregates (Lindow and Brandl, 2003; Beattie and Lindow, 1995; Leveau, 2006). Water on the surface of leaves might not be as unavailable as previously thought, as shown in a study using a desiccation-responsive biosensor (Axtell and Beattie, 2002); however when scarce, it may reduce the accessibility of nutrients. A similar situation might be occurring inside the leaf, where water itself might not be a strongly limiting resource for most bacteria (Freeman and Beattie, 2009; Wright and Beattie, 2004) but, its low abundance may restrict the distribution and/or accessibility of carbon source substrates to bacteria.

Previous studies addressing pathogen-induced changes in leaves have shown that diverse pathogens can cause accumulation of sugars inside leaves (Biemelt Sonnewald, 2005). Pathogens can also increase water availability as evidenced by the occurrence of water-soaked lesions in leaves and pods infected with some *P. syringae* pathovars (Rudolph, 1978). Since the intracellular flooding caused by water infiltration of leaves may alter ion and carbon source mobility (Felle and Hanstein, 2002) it is possible that such an induction of water congestion inside leaves may also provide sugars to growing pathogens.

We propose that the main growth-limiting factor for endophytic bacteria is the same as that for epiphytic bacteria: the availability and distribution of carbon. Due to the essential nature of the TTSS and its effector proteins for endophytic growth of pathogens, we propose that a subgroup of effectors directly or indirectly relieve nutrient limitation by enhancing the abundance and/or availability of nutrients/water in the apoplast. We thus will study what we term to be the Enhanced Nutrient Availability (ENA) effect of pathogens. We propose that these ENA effectors are responsible for the increased population densities that pathogens achieve in plant tissues. The effectors may act directly by increasing nutrient leakage into the apoplast but also may alter the water content of the apoplast thereby affecting the availability of leaf nutrients. Thus, we imagine that either a nutrient and/or a water-releaser could alleviate carbon source limitation. We propose that factors or treatments that alleviate carbon source limitation should allow both non-pathogenic saprophytes and *hrp* mutants of phytopathogens to achieve high apoplastic population densities. We also hypothesize that the mechanism(s) underlying the effector-induced ENA activity should be well-conserved among phyto-pathogens and therefore their target(s) in plants should be similar as well. It has frequently been proposed that plant defenses are the reason why non-pathogenic and avirulent pathogens are unable to multiply endophytically in plants (Alfano and Collmer, 2004). Recognition of common bacterial molecular patterns (PAMP-triggered immunity or PTI) has been proposed to limit the growth of non-pathogenic bacterial strains such as saprophytes and *hrp* pathogens. On the other hand effector-triggered immunity or ETI (and its frequently associated hypersensitive response or HR) is the main growth arresting factor for TTSS-proficient pathogens that are recognized by the plant surveillance system (both in resistant host

cultivar interactions and non-host interactions). The ETI and its associated severe desiccation stress during the HR may be the main limiting factor for avirulent pathogens (Freeman and Beattie, 2009). However, it is not clear that PTI is the main limiting factor for colonization of non-pathogenic strains and likely constitutes a branch of the plants response that is restricted to pathogenic attack. While we find evidence that water-congestion can rescue the HR-dependent growth arrest of non-host *Pseudomonas syringae* pv. *tomato DC3000* (*Pto* DC3000) in an incompatible host beans, we find that a PTI against non-pathogens might not occur in the apoplastic compartment (Zipfel et al., 2004) or might not be strong enough to prevent the bacteria from multiplying extensively once the carbon source limitation has been lifted.

The PTI and ETI branches of the plant defense surveillance system activate similar pathways and have been proposed to differ mainly quantitatively rather than qualitatively (Yao and Greenberg, 2006). In this sense PTI seldom induces strong responses such as the HR, and others have proposed that PTI is negatively self-regulating and likely needs co-activation to overcome this negative regulation (Schwessinger and Zipfel, 2008). In this model plants may be relatively unresponsive to non-pathogenic strains and full-blown responses might only be produced when concomitant recognition of several bacterial patterns occurs (i.e co-activation of ETI by recognition of TTSS-deployed effectors) (Ausubel, 2005).

We present data here supporting the hypothesis of the availability of carbon sources as main limiting factor for endophytic growth of bacteria. We found that when we infiltrated non-pathogenic saprophytes and/or *hrp* mutants of both host and non-host pathogens in the presence of a) exogenous carbon sources that bacteria can consume, b) maintained apoplastic water-congestion, or c) a TTSS-proficient pathogenic strain, population levels rise dramatically, reaching in many cases the levels a wild type pathogen achieves in its host. The ability to rescue the endophytic growth of non-pathogenic strains was clearly seen in several host and non-host plant species and with different bacterial strains, supporting the conjecture that the underlying mechanisms of growth restriction are similar in different plants.

Materials and Methods

Bacterial strains

All of the strains used where isolated from leaves, except for *Pf*01 which is a soil bacterium. The pathogens are the causal agent of spot of bean (*Pss* B728a) and bacterial speck in tomato and *Arabidopsis* (*Pto* DC3000). Some TTSS mutants of the two pathogens were also utilized (Table 2-1); the *Pss* B728a HrpJ$^-$ (*hrpJ*::ΩSpc) mutant carried a polar mutation in *hrpJ*, the first of five genes in the *hrpJ* operon. One or more of the genes in the operon appears to function as an inner membrane component(s) of the secretion system (Hirano and Upper 2000; Hirano et al., 1999). The *Pto* DC3000 HrcC$^-$ mutant has a mutation in the *hrcC* gene, a member of the PulD/pIV superfamily of outer membrane proteins (Alfano and Collmer, 1997). Antibiotics were used at the following concentrations (in μg/ml): chloramphenicol (Cm) 20, natamycin (Nat) 21.6, nalidixic acid (Nx) 20, Rifampicin 100, Spectinomycin (Sp) 20, tetracycline (Tc) 50.

Carbon source utilization

Several carbon sources were tested for their use as a sole carbon source in agar plates. The carbon sources to test were mainly identified in previous studies as discriminating in utilization by different groups of Pseudomonads (Stanier 1966). Those and other carbon sources were tested on M9 salts agar plates that were prepared with noble agar (Difco) and amended with 0.4% of carbon source. Plates were scored for growth often after at least 3 days growth at 28C (Table 2-2).

Plant inoculations

Bacterial strains were recovered from glycerol stocks at -80C and streaked onto Luria Bertani (LB) agar amended with appropriate antibiotics. *Pseudomonas fluorescens* strains were incubated at 28C for 15-18 hrs, while *Pseudomonas syringae* strains were incubated at 28C for 30-35 hrs and *Pantoea agglomerans* was incubated overnight at 37C. Each strain was then resuspended in 10mM KPO_4 buffer (pH=7) and their concentration was estimated by turbidity (OD_{600}) and subsequently adjusted by dilution to 10^5 or 10^6 CFU/ml (for syringe inoculations) (Wilson et al., 1999).

Plant hosts used included beans (*Phaseolus vulgaris* cv. Bush Blue Lake 274), *Nicotiana benthamiana* and *Arabidopsis thaliana* ecotype Col-0. Beans were established (4-6 per pot) from seed and utilized at about 2 weeks old when trifoliate leaves were barely emerging or very small; for all other plants used one plant was established per pot. All plants were grown in a greenhouse setting.

Plant infiltrations were performed using one of the following two methods: vacuum infiltration of entire leaves as described previously (Willis et al., 1990) was used for most experiments or local infiltrations using a needleless blunt syringe. For vacuum infiltrations 2L of bacterial suspension of 10^5 or 10^6 cfu/ml were added to a beaker to enable full submergence of all the leaves in the bacterial suspensions. The plants were inverted in the beaker containing the inoculum, but the roots and soil were excluded (cotton and tape was used to prevent excessive soil from falling into the inoculum). The submerged leaves were then introduced into a chamber and a vacuum was applied for 2-5 min, to remove air from the air-filled spaces of the plant. Upon release of the vacuum bacterial cell suspension was

forced into the leaves. Only fully infiltrated leaves (evidenced by complete water-soaking) were sampled. When needed, vacuum was reapplied when too many leaves were partially infiltrated. Partially infiltrated leaves were marked or removed to avoid their sampling. Time 0 was taken after all signs of water-soaking disappeared in all plants (1.5-2 hrs later). When maintaining water-soaking conditions, the plants were sealed in transparent plastic bags immediately after infiltration, before the water-congested leaves were allowed to dry, thus maintaining moisture in the leaf apoplast.

For syringe infiltrations, a small nick cut was made on either side of the midvein with a razor blade, and a needleless syringe loaded with inoculum was then applied with pressure to infiltrate the nicked site. The water-soaked area of infiltration was then marked with a permanent marker for future sampling. After water-soaking disappeared, 0.5cm diameter discs were cut out from the infiltrated area of each leaf.

Bacterial population measurement

Whole leaves (vacuum infiltration) or discs (syringe infiltration) were then sampled and macerated to determine total viable bacterial cells. Generally four biological replicates were assessed per treatment (for timepoint 0 sometimes only three replicates were sampled due to the low initial variability in cell concentrations). When estimating endophytic bacterial population sizes, sampled leaves for the same treatment were pooled together in plastic boxes and treated for 5 min. with a 15% solution (vol/vol) of H_2O_2 to achieve surface sterilization. For vacuum infiltrated plants, leaves were rinsed three times with autoclaved distilled water before being macerated with a mortar and pestle in 5 ml 10mM phosphate buffer to determine the surviving population (endophytic bacteria per leaf). For syringe-infiltrated plants, the leaves were rinsed in the same manner as described for vacuum infiltration and a single disc per leaf was cut out with an eppendorf tube cap and macerated in 0.5ml of 10mM phosphate buffer. Further 10-fold dilutions were made of some samples and plated with a spiral plater onto appropriate selective agar plates to obtain bacterial colony-forming units (CFUs) per sample.

Bacterial co-inoculation experiments

The inoculum for co-inoculation experiments was prepared as above except *P. agglomerans* 299R and *Pss* B728a were mixed in a beaker immediately before plant infiltration at a final concentration of 10^6 CFU/ml by OD_{600} for a 1:1 ratio and 10^6 and 10^5 cells/ml for a 10:1 ratio respectively.

Hypersensitive response (HR) assays

For macroscopic visualization of the HR we performed syringe infiltrations of bacterial suspensions at high inoculum concentrations (10^8 and 10^9 CFU/ml) as determined by OD_{600}. Bacterial suspensions were infiltrated on either side of the leaf midvein and the area of infiltration was delineated with a water-proof marker. Plant cell death (CD) was recorded within 24 hrs post infiltration and the quantity and quality of the lesion was scored.

Ice nucleation reporter assays

Ice nucleation activity (INA = Log [Ice nuclei/cell]) we determined by using the whole-cell sucrose-responsive strain *P. agglomerans* 299R (p61RYIce). The strain carries the promoter of the sucrose porin gene *ScrY* fused to the promoterless *inaZ* reporter gene encoding an ice nucleation protein. *P. agglomerans* 299R (p61RYIce) detects sucrose concentrations as low as 10^{-6} M and to a lesser extent it also detects fructose (100-fold less sensitive) (Miller et al., 2001). Sucrose-dependent INA was directly measured in the apoplast by co-infiltrating the biosensor strain with the appropriate treatment/s using vacuum infiltration as described above. Since *P. syringae* pv. *syringae* B728a is a naturally ice-nucleating strain, for INA determination we used *Pss* LK2, a derivative of *Pss* B728a with an *nptII* insertion in the *inaB* gene that renders it Ice^{-}. Samples were collected about 3 hpi to allow for the expression, maturation and folding in the outer membrane of the reporter InaZ ice nucleation protein. After maceration we performed serial 10-fold dilutions of the leaf macerate in sterile 10 mM KPO_4 buffer. Fourty 10 μl drops of a given dilution were spotted onto paraffin-coated aluminum foil boats and placed on an ethanol bath at - 9°C for 5 min. The number of frozen drops was counted and appropiate dilutions of plant macerate were plated as described above to determine CFU/leaf. Each dilution plate was incubated both at 28C (to determine combined population size of *Pss* B728a and *P. agglomerans*), and at 37C (to determine the population size of biosensor *P. agglomerans* only). The number of ice nuclei was then normalized with the CFU data (37C) to calculate the INA for each sampled leaf.

Results

1) Pathogens induce better growth conditions for endophytic bacteria

Although the ability of TTSS-proficient pathogens to rescue the growth of non-pathogens in plants has been known for some time, we wanted to determine if this phenomenon also occurred in our particular pathosystem of *Pss* B728a in snap bean. We performed co-infiltration studies where the non-pathogen *Pantoea agglomerans* 299R (*Pa* 299R) was co-infiltrated into bean plants in the presence of either a virulent compatible pathogen (*Pss* B728a) or another non-pathogenic strain (*Pf*55) (Fig. 2-1a). Little endophytic growth of *Pa* 299R was observed, typical of non-pathogens inside plant tissues (Alfano and Collmer, 1996). However, in the presence of *Pss* B728a, *Pa* 299R reached an endophytic population size that was more than 1000-fold higher by 48 hpi, indicating that the strain benefitted from apoplastic changes introduced by the pathogen. These growth promoting changes were not, however, induced by the saprophyte *Pf*55 (Fig. 2-1a).

In an attempt to explain the mechanism behind this non-pathogenic population rescue, we performed INA assays with a whole-cell sucrose biosensor strain of *P. agglomerans* (*Pa* 299R (p61RYIce)) to see whether we could detect a higher concentration of apoplastic sucrose in the presence of the pathogen (Fig. 2-1b and c). The growth and INA of the sucrose biosensor *Pa* 299R (p61RYIce) in bean leaves was determined both in the presence and absence of the *Pss* B728a Ice$^-$ derivative. The Ice$^-$ derivative, *Pss* B728a (LK2) is otherwise isogenic to the wild type pathogen. Again, *Pa* 299R (p61RYIce) multiplied extensively in the presence of *Pss* B728a LK2 (by two orders of magnitude more than when infiltrated alone), but unexpectedly, the INA detected was approximately the same both in the presence and absence of the *Pss* B728a LK2 pathogen. At 48 hpi *Pa* 299R (p61RYIce) was highly active (approximately INA= -1), corresponding to a sucrose concentration of ~10^{-3} M (Fig. 2-1d) both when infiltrated alone and when co-infiltrated with the pathogen (Fig. 2-1c). This experiment was performed twice with similar results.

2) Non-pathogens are nutrient-limited in the bean apoplast

Pss B728a HrpJ$^-$, a non pathogenic TTSS-deficient derivative of *Pss* B728a was found to multiply endophytically to higher levels when co-infiltrated into bean plants with 2g/L of fructose than when infiltrated with buffer control (Fig. 2-2). In a different experiment, we found that *Pss* B728a HrpJ$^-$ also exhibited substantial endophytic growth when co-infiltrated with sucrose (Fig. 2-3).

To investigate the scope of this interesting growth response to an exogenously added carbon-source further, we determined the total population size of two types of non-pathogenic strains: the leaf saprophyte *Pf*55 and the TTSS-deficient mutant *Pss* B728a HrpJ$^-$, in the presence of increasing concentrations of fructose. At three days post infiltration, the endophytic population size of the non-pathogen *Pf*55 had increased in a dose-dependent (but non-linear) manner with increasing fructose concentrations (Fig. 2-4). While the population size of each of the non-pathogenic strains increased about 1000-fold at the highest fructose concentration used (8g/L) as compared to when infiltrated with water alone, *Pss* B728a exhibited similarly high population sizes. Moreover, in the case of

the *hrp* mutant of *Pss* B728a, the increase in population size conferred by the addition of 4 g/L of fructose was such that 4 g/L of fructose was enough to increase it to that of the wild type *Pss* B728a (Fig. 2-4). Large increases in endophytic population sizes with other common foliar non-pathogenic saprophytes such as *Pa* 299R and *P. fluorescens* 55 (*Pf*55) occurred in response to the presence of fructose (data not shown).

3) Multiplication *in planta* is mediated only by carbon sources that can be metabolized by the bacteria

We tested a panel of several pathogenic and non-pathogenic bacterial strains for their ability to grow in minimal M9 media plates supplemented with an extensive collection of different carbon sources (the results are shown in Table 2-2). Because several *Pseudomonas fluorescens* strains such as *Pf*55 and *Pf*A501 cannot grow in M9 containing sucrose as the sole carbon source, we also tested whether this sugar would allow these non-pathogens to multiply *in planta* to the extent seen in the presence of metabolizable fructose. The total population size of *Pf*A501 increased more than 100-fold in the presence of fructose whereas it failed to increase in the presence of sucrose (Fig. 2-5), the latter being a sugar the strain cannot utilize as sole carbon source on agar plates. A similar endophytic growth response to fructose and lack of response to sucrose was obtained in separate experiments with *Pf*55, another strain incapable of metabolizing sucrose (data not shown).

Besides fructose and sucrose, we tested other substrates that can be utilized as sole carbon sources, including some that are generally preferred by *Pseudomonas* strains (i.e. sugars, Krebs cycle organic acids) (Fuhrer et al., 2005; Huynh et al., 1989), for their ability to enable increases in endophytic population size in different non-pathogenic bacteria. Thus, representatives of chemically different compounds (succinate, glucose and proline) were added to the inoculum of several strains infiltrated individually into bean. For example, in one such experiment in bean, *Pf*55 population developed a larger size when the amino acid proline was added to the inoculum, but not when non-metabolizable sucrose was added (Fig. 2-6). In summary, we found that growth of non-pathogens in the apoplast could be rescued with appropriate carbon sources that are metabolized by the bacteria.

4) Preferred carbon sources completely rescue *in planta* growth of both virulent and HR-causing type-III secretion mutants of plant pathogens

Pss B728a HrpJ$^-$ normally grows very little in bean leaves as compared to wild type *Pss* B728a; it multiplies to a population size about 10-fold higher than initially inoculated, but fails to actively multiply endophytically as wild type *Pss* B728a. However, in the presence of 4 g/L of added fructose in the inoculum infiltrated into bean the TTSS-deficient strain increased its population size to levels comparable to the wild type *Pss* B728a strain (see Fig. 2-4).

Wild type *Pto* DC3000 is subject to a hypersensitive response (HR) in the non-host bean and thus grows to a significantly lesser extent than the compatible pathogen *Pss* B728a (Fig. 2-7). A metabolizable carbon source added to the inoculum was not only capable of rescuing the growth of a *hrp* mutant of a normally virulent, compatible

pathogen such as *Pss* B728a HrpJ$^-$ in bean, but interestingly, metabolizable carbon sources such as succinate and glucose could restore the growth of *Pto* DC3000 HrcC$^-$, a TTSS-deficient mutant of an incompatible pathogen in bean, to levels similar to that exhibited by B728a itself (Fig. 2-7). On the other hand, it became evident that the extent of growth of virulent TTSS-proficient strains was not significantly altered in the presence of exogenously added carbon sources. As we had seen before with *Pss* B728a in bean inoculations, this lack of growth response was evident in a compatible pathogen-host interaction (Fig. 2-4). We thus wanted to establish whether bacteria having an incompatible, HR-causing interaction with bean would also not respond to added nutrient sources. Therefore, we syringe infiltrated wild type strains *Pto* DC3000 and *Pss* B728a in solutions of 5g/L of fructose, a concentration we had found generally gave a strong population growth increase in bean (Fig. 2-8a). While *Pto* DC3000 exhibits a slower growth rate in bean than did *Pss* B728a, neither the rate of growth nor the final population size was altered in either strain upon addition of fructose to the inoculum.

The ability of a saprophyte (*Pf*55), a compatible host pathogen (*Pss* B728a) and two non-host incompatible pathogens (*Pto* DC3000 and *Pseudomonas syringae* pv. *tabaci* 11528 (*Pta* 11528)) to induce an HR in bean when infiltrated with high (10^9 CFU/ml) inoculum was determined. The strains were infiltrated with and without 5 g/L of fructose to determine if the sugar addition could affect the non-host HR that is produced in bean with the *Pto* DC3000 and *Pta* 11528 pathovars (Fig. 2-8b). The fructose control and saprophytes exhibited no HR symptoms as expected, and the necrosis that was seen with *Pss* B728a and *Pto* DC3000 was similar in plants with and without added fructose. There also seemed to be no obvious delay in the response. In some bean leaves inoculated with *Pta* 11528, necrotic lesions seemed worse in the presence of fructose (fig. 2-8b), but in none of the cases did it seem that the addition of fructose diminished the HR necrosis.

5) Maintaining water-soaking conditions in the leaf is sufficient to induce a large population size increase by non-pathogenic bacteria

We also estimated bacterial population sizes in plants in which water-soaking conditions were maintained in the leaves after inoculation. As it had been established before that HR-inducing apoplastic bacteria can be subject to water-stress (Wright and Beattie, 2004), we wanted to determine if we could rescue growth by alleviating water stress by maintaining apoplastic water-congestion. This was achieved by keeping the vacuum-infiltrated plants covered with a transparent plastic bag that would retain moisture inside while at the same time allowing light to pass through. Since water could not escape the plastic bag, the intercellular spaces of the leaves remained water-congested throughout the experiments. As seen in Fig. 2-9, TTSS mutant *Pss* B728a HrpJ$^-$ does not exhibit much growth by 48 hpi in bean. However, in the presence of added fructose, water-soaking conditions or both, it attained a similarly high population sizes (100,000-fold increase) as the wild type strain *Pss* B728a. When inoculum of *Pss* B728a HrpJ$^-$ was both amended with fructose and plants subsequently kept water-congested this strain grew as much as when amended with fructose or water-congested alone. This suggests that either fructose addition or water-soaking conditions are each capable of alleviating the same growth limitation.

In a similar experiment, maintenance of water congested conditions also allowed the non-pathogenic strain *Pf*55 to increase its population size up to 1000-fold with added fructose, with water-soaking alone or when both were infiltrated (Fig. 2-10a). Similarly to what had been observed previously with the addition of appropriately metabolizable carbon sources, the maintenance of water soaking of the apoplast does not provide any added benefit for a virulent compatible pathogen (see *Pss* B728a with and without water-soaking treatment, or fructose and water-soaking treatments, Fig. 2-10b). *Pto* DC3000 inoculated without added fructose did not grow much in bean in this particular study, although when fructose was added to the inoculum it increased somewhat, and multiplied most in the presence of water-soaking conditions (Fig. 2-10c). Overall change in population size upon fructose addition or water-soaking was much less for *Pto* D3000 compared to the non-pathogenic strain *Pf*55, but both were more substantial than for *Pss* B728a.

Because evidence had been accumulating that both the water congestion and carbon source treatments were both probably alleviating the same apoplastic limitation, we next wanted to determine if we could rescue the growth of a TTSS-deficient mutant of an incompatible non-host pathogen of beans with water congestion. Indeed, *Pto* DC3000 HrcC$^-$ reached a population size similar to wild type *Pss* B728a when water-soaking conditions were maintained inside the leaves by bagging the plants (Fig. 2-11a). These results were very similar to previous experiments where the large population size increase of a TTSS mutant of a compatible pathogen (*Pss* B728a HrpJ$^-$) was observed in bean under these water congested conditions. We also observed that under conditions of water-soaking, wild type incompatible (HR-causing) *Pto* DC3000 achieves a population size comparable to *Pss* B728a in bean.

6) TTSS mutants can produce lesion-like necrosis under conditions inducing extensive multiplication

We often observed in our experiments the formation of extensive necrotic areas, sometimes accompanied by chlorosis, that increased in area and severity during the course of several days after bacterial growth was assessed. Disease-like symptoms not only occurred as expected with the virulent wild type *Pss* B728a in beans, but also with plants inoculated with TTSS mutants co-infiltrated with utilizable carbon sources and was especially evident in the case of TTSS mutants maintained under water-soaking conditions in bean (Fig. 2-11 pictures b, c, d and e) and *N. benthamiana* (data not shown).

Pss B728a HrpJ$^-$ exhibited 100 to 1,000 fold growth in *N. benthamiana* when water-soaking was maintained after inoculations (fig. 2-12). The plants were allowed to dry out at 48 hrs after inoculation at which point some necrotic lesions had started to appear in the *Pss* B728a HrpJ$^-$ bagged but not the un-bagged leaves. After un-bagging the pots, symptoms either appeared or completely developed in the following days. Thus symptoms developed after an extensive endophytic bacterial multiplication had already occurred. This restored virulence effect seen with *hrp* mutants was especially clear in primary leaves of bean and was apparent in TTSS-deficient mutants of both compatible and incompatible pathogens. However, these lesions appeared different from normal

symptoms induced by pathogens on plants that were not water-soaked, and the development of normal appearing lesions had a later onset. The ability to cause lesions was observed with only pathogenic strains (with or without a functional TTSS). Even though saprophytic strains achieved a high population size in plants with supplemental carbon sources and/or subjected to water-soaking, they never produced necrotic lesions.

7) Increased population size of non-pathogens are also achieved in *Arabidopsis thaliana* and *N. benthamiana*

It remained a question whether supplementation with carbon sources or water soaking would increase growth of bacteria in other plants besides bean. Since *Arabidopsis thaliana* Col-0 (hereafter *Arabidopsis*) is the most well-studied model for plant-microbe interactions, we performed experiments using the *Arabidopsis-Pseudomonas syringae* pathosystem and used wild type *Pto* DC3000 as the virulent compatible strain and its TTSS-deficient mutant *Pto* DC3000 HrcC$^-$ as a non-pathogenic variant.

We performed several syringe co-infiltrations of non-pathogens and different carbon sources into *Arabidopsis* and also in *N. benthamiana* plants. Syringe infiltrations, unlike vacuum infiltrations (where the whole plant is thoroughly infiltrated), result in localized areas of each leaf where a small volume (less than 10μl) of bacterial suspension is infiltrated. Fructose, glucose, and succinate can be utilized as a sole carbon source in M9 agar plates by both *Pto* DC3000 and *Pss* B728a. Thus, similarly to previous bean experiments, our expectation was that these carbon sources would induce vigorous apoplastic growth of non-pathogens in these hosts. Surprisingly, whenever we used the syringe infiltration method, none of these substrates induced apoplastic growth. In one experiment, we measured the endophytic population size of *Pto* DC3000 HrcC$^-$ coinoculated with fructose (fig. 2-13a) and in another we measured the population sizes of *Pto* DC3000 HrcC$^-$ and *Pss* B728a HrpJ$^-$ treated with glucose and succinate (Fig. 2-13b). In all cases, the bacteria coinoculated with carbon sources maintained the same population size as in leaves inoculated with the bacteria alone, while wild type *Pto* DC3000 multiplied substantially. When similar experiments were attempted in *Arabidopsis* but using instead the vacuum infiltration method, we found that co-inoculation with appropriate substrates and maintenance of water-soaking conditions after inoculation produced substantial growth of non-pathogenic *hrp* mutants (fig. 2-14). This supports our previous results found in beans. Interestingly, soil bacterium *Pseudomonas fluorescens* 01 (*Pf*01) behaves similarly to epiphytic strains of this species and exhibits a large increase in population sizes in response to infiltrated fructose (data not shown) but not to succinate (Fig. 2-14). Succinate is a substrate that is normally preferred by Pseudomonads over other compounds including sugars (Fuhrer et al., 2005, Huynh et al., 1989). Interestingly, succinate is not metabolized by *Pf*01 as a sole carbon source in M9 plates. Nevertheless, maintenance of water-soaking in *Arabidopsis* after inoculation of plants with either *Pto* DC3000 HrcC$^-$ or *Pf*01 resulted in similarly large increases in the population size of these strains (Fig. 2-14). It is worth noting that the results of this experiment should be interpreted with some caution since *Arabidopsis* plants were adversely affected by the 72 hr water congestion conditions. The same experiment should be conducted with shorter incubation times (i.e. 24-48 hrs). In an experiment where both *Arabidopsis* and *N. benthamiana* plants were successively

infiltrated with the same beaker of glucose-containing bacterial inoculum we were able to demonstrate it could enable growth of *hrp* mutants in *N. benthamiana* but not in *Arabidopsis* (Fig. 2-15 a and b). This strongly suggests that *Arabidopsis* may reabsorb carbon sources faster than other plants like beans and that the added carbon source does not remain available for consumption by the bacteria.

Growth of non-pathogenic bacteria in the presence of either exogenous nutrients or water-soaking conditions was also assessed in other plant hosts. In *N. benthamiana* we observed substantial multiplication and the restoration of lesion formation by the *Pss* B728a HrpJ$^-$ mutant if water congestion was maintained (see Fig. 2-12). Growth of both *Pto* DC3000 and *Pss* B728a *hrp* mutants could also be observed upon the addition of high concentrations of glucose to the inoculum even in the absence of subsequent water-soaking when vacuum infiltrated (Fig. 2-15a).

Discussion

The absence of a TTSS and its co-regulated and secreted effectors is universally seen as the main cause for the poor apoplastic multiplication of non-pathogenic bacteria. Therefore, at least some mechanism/s of altering the normal plant cell function presumably using such effectors is/are an absolute requirement for endophytic growth. There is the general perception that TTSS and its effectors evolved mainly to subvert the plant defense pathways that can inhibit bacterial multiplication. The underlying assumption being that beyond the need to suppress plant defenses, bacteria require little else for growth in what is considered a nutrient-rich environment. Furthermore, the model thus predicts that non-pathogenic bacteria do not normally grow endophytically because of their lack of TTSS effectors to subvert plant defenses. As a consequence, virulent bacteria rendered non-pathogenic by the deletion of a core component of the TTSS apparatus (*hrp* mutants) would encounter the same hurdle to multiply in the nutrient-rich apoplast as saprophytes. Thus, this model predicts that the main factor restricting growth of bacteria in the apoplast is the ability to overcome the host plant defenses. The results presented in this work, however, seem to challenge this model, instead favoring availability of nutrients as the main factor limiting bacterial growth in the apoplast.

We have performed vacuum and syringe infiltration of different strains of pathogenic and non-pathogenic bacteria directly into the intercellular spaces inside the leaf (apoplastic space). These methods for direct apoplastic inoculation circumvent the normal route of infection where phyllosphere bacteria first establish an epiphytic population before invasion and initiation of endophytic growth inside the leaf. Thus, the initial endophytic populations we establish by infiltration might seldom be attained by non-pathogenic strains under normal conditions and in the case of pathogens, would probably only occur after extensive surface growth. The endophytic population and the environmental and biological conditions that limit their growth are the factors we analyze here.

Our data confirms that saprophytes are poor endophytic colonists as previous studies have concluded (Wilson et al., 1999). Likewise, we also find that *hrp* mutants of phytopathogens (Alfano and Collmer, 1996) grow poorly in the apoplast, although growth varies depending on whether the *hrp* mutant is growing in a host or non-host plant. A group of saprophytic bacteria from the phyllosphere (*Pf*55, *Pf*A501 and *P. agglomerans* 299R) and a soil bacterium (*Pf*01) all failed to grow endophytically in any of the three plant hosts tested. The only exception appeared to be *P. agglomerans*, which seemed to consistently multiply about 10-fold in in beans (see Fig. 2-1b). *hrp* mutants of phytopathogens do not multiply appreciably inside non-host plants (see Fig. 2-7, 2-11a for example) but in their hosts they can multiply between 10-fold (*Pss* B728a HrpJ$^-$ in beans) and 100-fold (*Pto* DC3000 HrcC$^-$), while both multiplied well in *N. benthamiana*, (1,000-fold growth). On the other hand, pathogens are known to exhibit at least some growth in host, non-host and resistant host plants (Wilson et al., 1999 and references therein) but to be able to establish populations several orders of magnitude higher in their compatible hosts. For example wild type *Pss* B728a multiplies 10,000-fold in bean (Fig. 2-3 and Fig. 2-5) and 100,000-fold or more in *N. benthamiana* (Fig. 2-12 and Fig. 2-15a). When multiplying in their respective hosts, TTSS mutants are still able to grow

substantially more than the saprophytes *Pf*55 and *Pf*01 (compare population levels of *Pss* B728a HrpJ$^-$ and *Pf*55 in fig. 2-4; and *Pto* DC3000 HrcC$^-$ versus *Pf*01 at 72hpi in Fig. 2-14). This suggests that they express other virulence traits that saprophytes may lack, and that those traits are TTSS-independent. However, such endophytically important TTSS-independent traits in most cases contribute little to the total apoplastic growth. For example, *Pss* B728a HrpJ$^-$ exhibits only up to ~0.1% of the population growth of wild type TTSS-proficient *Pss* B728a in 48-72 hrs, clearly reflecting the dominance of the TTSS and its effector repertoire to endophytic (and total) growth of this pathogen in its host. Interestingly, the contribution of the TTSS to the overall *Pto* DC3000 population growth in its host *Arabidopsis* might not be as substantial as in the *Pss* B728a-bean pathosystem (compare *Pss* B728a HrpJ$^-$ versus *Pss* B728a growth in Figs. 2-2, 2-3 or 2-9 and *Pto* DC3000 HrcC$^-$ versus wild type strain growth in *Arabidopsis* in Fig. 2-14 and 2-15b). Thus the contribution of other non-TTSS related virulence factors in *Pto* DC3000 (for example, coronatine) are of a larger relative importance for the endophytic growth in this pathosystem.

To our knowledge, this is the first report showing the complete growth rescue of a *hrp* mutant of an incompatible pathogen such as is the situation of hrcC$^-$ *Pto* DC3000 in bean plants (see Fig. 2-7).

If the pathogen is able to utilize TTSS-dependent traits (e.g. TTSS effectors) to obtain nutrients in the apoplast, we reasoned that its presence would make the apoplast more resource-rich for itself and for any other neighboring bacterium. In pathogen-saprophyte co-infiltration studies, plant pathogens are able to induce a very large increase in the population sizes of saprophytes. In our experiments, only in the presence of *Pss* B728a but not with other saprophytes tested did *P. agglomerans* multiply 100 to a 1000-fold after co-inoculation. The high population size that *P. agglomerans* achieves is similar to the 1000-fold growth of *Pf*55 and *Pss* B728a HrpJ$^-$ induced by addition of 8 g/L fructose *Pf*55 (Fig. 2-4), and is also comparable to the population size that addition of 10 g/L fructose conferred on *P. agglomerans* in other experiments (data not shown). The maximum attainable population size each strain can achieve appears to occur in co-infiltrations with pathogenic *Pss* B728a. It is noteworthy that when *Pss* B728a and *Pf*55 are co-infiltrated the growth of *Pf*55 is as high as when either high amount of carbon resources are inoculated or water-congestion is retained.

P. agglomerans clearly benefited from having a virulent pathogen in the vicinity in the apoplast; however, the nature of the beneficial changes introduced by the pathogen is unknown. We thus tried to measure the levels of sucrose, the most abundant carbon source in the apoplast (Deverall, 1967), utilizing a sucrose-responsive *P. agglomerans* biosensor strain. Working under the assumption that increased apoplast growth would be associated with an increase in sugars such as sucrose, we hypothesized that *P. agglomerans* would have access to higher amount of sucrose when co-infiltrated with *Pss* B728a compared to when alone in the apoplast. We could not, however demonstrate higher apoplastic sucrose content in the presence of the pathogen (fig. 2-1). Instead, the biosensor was highly induced both in the presence and absence of *Pss* B728a. The high sucrose-dependent INA

is probably due not to bacterial-induced sucrose release but instead to the fact that the infiltration process itself is able to make pre-existing sugars in the apoplast available to the biosensor (Felle and Hanstein, 2002). Due to the stable nature of the ice nucleation protein, even a brief initial exposure to high concentrations of sucrose would result in induction to INA that would not disappear once the normal apoplastic conditions are restored, especially if the bacterium had not grown. Little growth of the biosensor would be expected if it was inoculated individually into the apoplast. Consequently, we believe we could not obtain a true measure of the apoplastic sucrose level in non-water-congested leaves. Moreover, the problem of interpreting INA data in this experiment is aggravated with the fact that the biosensor is actively consuming the substrate that is being "sensed". An alternative biosensor strain that is unable to metabolize the sugar is more desirable. Alternatively, an unstable reporter protein such as short half-life GFP would enable estimates of recent sucrose availability to be made after initial water congestion was relieved.

Support for the model of apoplastic nutrient limitation came nonetheless from the experiments performed with the co-infiltration of non-pathogenic strains with exogenous carbon sources. The addition of an appropriate carbon source to the apoplast allowed all non-pathogenic bacteria tested to multiply extensively, implying that bacteria are limited for nutrients. This finding is in agreement with other reports that show that epiphytic bacteria on bean are limited by carbon sources that leach onto the surface of the leaf and their spatially heterogenic distribution is similar to that of the epiphytes themselves (Mercier and Lindow, 2000; Leveau and Lindow, 2001). Our choice for substrates to be utilized in these experiments was based on the assumption that mostly sugars would be released into the apoplast as these are the most abundant photo-assimilates produced by the mesophyll cells and uploaded and transported by the phloem. Sucrose, fructose and glucose constitute about 90% of the carbon on leaves, with amino acids, organic acids and other compounds much less abundant (Deverall, 1967; Leveau, 2006). Thus, most of our carbon source addition experiments were done with the most common leaf sugars sucrose, fructose and glucose. However, we also exploited the fact that different bacteria have diverse carbon source utilization profiles by adding varying substrates in our infiltration experiments to determine if they could all yield higher population sizes of non-pathogens. In particular, we wanted to determine if the different carbon sources directly impacted growth (through bacterial consumption), or whether instead the impact on growth was indirect (through modifying the plant host). Direct bacterial consumption would lend support to our model of apoplastic carbon source limitation, as opposed to the carbon sources having a more indirect effect by perhaps affecting the plant's ability to induce a normal defense response. We thus co-infiltrated non-pathogenic strains with those carbon sources that would be informative, that is, that were differentially metabolized in vitro by some but not all non-pathogenic strains (Table 2-2).

For example, while *Pss* B728a HrpJ$^-$ grows *in planta* whith either fructose or sucrose (see Figs. 2-2 to 2-4), (two substrates it can readily metabolize in plates), *Pseudomonas fluorescens* strains achieved higher population sizes only after addition of fructose but not sucrose which they are unable to consume in vitro (see *Pf*A501, Fig. 2-5

and *Pf*55 Figs. 2-4 and 2-6). Several carbon sources of different chemical nature are able to produce a striking increase in growth of non-pathogenic bacteria in beans and *N. benthamiana*. This induction was restricted to those compounds that bacteria could consume. The substrates sucrose and succinate were especially informative because they lacked the ability to induce multiplication *in planta* for the *Pseudomonas fluorescens* strains since they are unable to metabolize them, thus showing that the growth benefit they produce is through direct bacterial consumption and not through impacting the plant host.

Much higher concentrations of added apoplastic nutrients were required to augment bacterial growth than would be expected if they were all consumed by the bacteria. A concentration of 8 g/L of fructose was necessary to induce growth of *Pss* B728a HrpJ$^-$ and yielded ~10^8 cfu/bean leaf (Fig. 2-4 and 2-9), while smaller amounts of fructose yielded lesser growth of *Pss* B728a HrpJ$^-$. Our data shows a clear dose-response effect to fructose and sucrose addition and bacterial growth that is, albeit not directly proportional (fig. 2-4). The amount of sugar in the apoplast probably gets reabsorbed by the plant at a rate that depends on concentration and the nature of the particular sugar transporters. The growth rate of bacteria is largely independent of substrate concentration above a certain minimum level. Thus, the addition of different amounts of carbon compound to the apoplast probably resulted in different amounts of time that the compounds would have remained available in the apoplast. Thus, at the highest concentrations of added carbon compound an "excess" would have persisted longest, allowing the most growth of bacteria. It is also likely that the higher amount of added compound would have slowed the rate of removal since the source strength was so large that it may have increased the concentration of the compound in the "sink" tissues of the leaf.

It is worth noting that lesser responses were seen in apoplastic growth when the carbon sources were introduced with syringe infiltrations than with whole leaf vacuum infiltrations. In the more localized sites of syringe infiltration it is conceivable that the absorption of sugars by the plant was much faster than when the entire leaf was treated. The time available for bacteria to utilize and thus grow in response to the compound might be very short in these experiments (data not shown). This, however, points at the fact that different rates of reabsorption of sugar in the apoplast of the plants can account for different responses. Induced growth of non-pathogens by water congestion was apparent in all three plants tested but the results with the addition of carbon sources varied more. Addition of carbon sources induced larger population growth responses in beans than in the other two plants. In *N. benthamiana* up to 6 g/L of glucose was sufficient to see a growth increase of 10-fold of *Pss* B728a HrpJ$^-$ and 100-fold of *Pto* DC3000 HrcC$^-$ while these populations remained unchanged in *Arabidopsis* in experiments where the same inoculum and conditions were used. *Arabidopsis* might thus be more efficient at absorbing the carbon source added (compare Fig. 2- 15a and b).

Maintaining apoplastic water-soaked conditions is sufficient to entirely abolish the growth limitation of non-pathogenic population strains, saprophytes and *hrp* mutants of compatible and incompatible pathogens alike in the apoplast. We believe that maintaining water-soaked conditions inside leaves produce an increased availability of nutrients for

endophytic bacteria. The availability of water could be, by itself, a limiting factor however, we do not think non-pathogenic strains suffer severe apoplastic water limitation for several reasons. First, studies using a water-stress responsive biosensor based in the P_{proU} promoter demonstrated that non-pathogenic saprophytes and *hrp* mutants are not water-limited in the apoplast (Wright and Beattie, 2004). Second, while we think it is likely there is some apoplastic water accumulation induced by the higher osmolarity triggered by the introduced carbon sources (and indeed some degree of leaf wrinkling occurred in bean plants infiltrated with sugar concentrations higher than 8g/L), this effect alone cannot account for endophytic bacterial growth. The fact that only consumable substrates were able to induce the large population increase effect, would argue that relieving a desiccation stress is not the main reason for bacterial growth. Instead, both addition of carbon sources and water-soaking conditions are likely alleviating an apoplastic carbon source limitation.

In order to discern between water-soaked conditions relieving a water versus a carbon source limitation we varied both conditions at the same time. If the main limiting factor in the apoplast is the availability of carbon source, then by adding a high concentration of it (i.e. enough to completely aleviate this limitation), then the further imposition of the water-soaking condition should not provide any further benefit. This is, in fact, what we saw in the experiments where we treated bean plants with exogenous 8 g/L fructose addition as well as maintained apoplastic water-congestion. We observed that each of the treatments by themselves produced about the same level of endophytic growth of non-pathogens which seemed the maximum population size the strains could reach (see Fig. 2-9 and 2-10a). For example, *hrp* mutants *Pss* B728a HrpJ$^-$ and *Pto* DC3000 HrcC$^-$ reached populations similar to wild type compatible strains themselves while for *Pf*55 the maximum population size achieved was much lower (see Fig. 2-12), thus suggesting that they are in fact alleviating the same limitation: a nutrient limitation.

In the process of infiltration we produce a flooding of the intercellular spaces inside leaves (Fig. 2-16). The flooding probably makes apoplastic resources more locally bioavailable to bacteria by solubilizing and dispersing any available nutrients, which will then start dividing. The flooding then begins to dry and over the course of 1.5-2 hpi probably completely disappears, coincident with loss of visual water-soaking. Most bacteria would then stop multiplication because only the nutrient deposited locally might be available. However, if normal drying is prevented by covering the plants with bags, then apoplastic bacteria are able to continue multiplying, unhindered by nutritional limitation. A similar process would take place when the infiltration introduces additional substrates. Upon drying, higher amounts of nutrient are probably deposited proximal to the bacteria. If those substrates can be consumed by the bacteria then division can continue even after the flooding recedes. If, instead the receding water leaves behind substrates that cannot be catabolized, then no bacterial multiplication ensues.

Population sizes of *Pss* B728a did not increase upon introduction of either water or nutrients in any of the experiments performed in beans, *Arabidopsis* or *N. benthamiana* hosts (see for example Fig. 2-8a and 2-10b). In the incompatible interaction of *Pto* DC3000 with the non-host bean the results vary in different experiments (Fig. 2-8a, 2-10c

and 2-11a), but in all cases there was a much smaller response to the nutrient-alleviation treatments than any of the non-pathogens tested. Thus the TTSS-proficient strains are less responsive to addition of either water or nutrient in the apoplast. TTSS-proficient pathogens apparently do not need the extra resources provided by the exogenous addition of carbon source or water-soaking treatments to grow due to their possession of a functional TTSS and effectors. Our data is consistent with the model that TTSS effectors have a role in making nutrients more accessible to pathogenic strains, independent of the host plant they are multiplying in.

It is possible that water-soaking might be alleviating apoplastic desiccation stress in *Pto* DC3000 in the non-host bean. It was shown before that strains of *Pto* DC3000 carrying AvrRpt2 and AvrRpm1, two effectors that induce an ETI and HR response in *Arabidopsis*, are subjected to low water availability (Wright and Beattie, 2004). Likewise, during the HR caused by wild type *Pto* DC3000 in non-host bean, bacteria might experience a similar desiccation stress. Consequently, in the case of an HR-inducing incompatible pathogen, water limitation could be a major hurdle for multiplication; such limitation could then be relieved by maintenance of water-congestion (for example see Fig. 2- 11a and compare *Pto* DC3000, *Pto* DC3000 + bag and *Pss* B728a treatments).

The main focus of our work was to determine the conditions that would affect endophytic growth of bacteria. However, during the course of our experiments we observed that some non-pathogens were able to induce necrosis in plants. While saprophytic bacteria never caused lesion-like symptoms, even when reaching very high population levels, *hrp* mutants of *Pto* DC3000 and *Pss* B728a were observed to produce such symptoms in treatments that induced increases in their endophytic population sizes such as addition of exogenous carbon sources and maintainance of water-soaking. This restoration of virulence of TTSS pathogens is intriguing and suggests that *hrp* mutants still have the traits that enable them to cause necrosis whenever environmental conditions allow them to multiply extensively. These results corroborate previous results by Hirano and Upper with HrpJ$^-$ and HrcC$^-$ *Pss* B728a. Growth-conducive conditions revealed the intact lesion-forming ability of *Pss* B728a HrpJ$^-$ (under the GacS/A regulon in *Pss* B728a), (Hirano and Upper, 1999; Hirano and Upper, 2002).

The experiments presented suggest that bacteria that arrive in the apoplastic compartment, either naturally by entering through stomata, wounds and other plant structures, or by introduction directly by infiltration, likely need a mechanism for active acquisition of resources. TTSS proficient pathogens seem not to be nutrient limited, especially in compatible interactions whereas non-pathogens seem unable to obtain the carbon sources they need for endophytic multiplication.

Experiments performed in beans, *Arabidopsis* and *N. benthamiana* showed that under conducive conditions, endophytic populations of non-pathogens dramatically increase. The conditions we imposed, either directly provided metabolizable carbon sources in the apoplast (carbon source addition treatments) or indirectly aided in the availability and/or accumulation of sugars in the apoplast (apoplast flooding by infiltration

followed by persistent water-soaking). Since this endophytic growth response was seen in all non-pathogens tested (saprophytes and *hrp* mutants alike), all presumably having unprotected PAMPs (i.e. lacking TTSS effectors to mask their recognition) we feel that plant defense responses are not the main factor limiting bacterial growth in the apoplast. Support for this view also comes from recent experiments with the PTI elicitor flagellin; the best characterized PAMP and likely one of the most important in *Pseudomonas*-plant interactions. A study of flagellin perception suggested that PTI might not play a crucial role in the apoplastic compartment but would instead restrict motility on and invasion of the plant (Zipfel et al., 2004). We also believe that normal plant defense responses are not impeded in our experiments, as we have seen non-host HR and growth arrest of *Pto* DC3000 in beans occur in a normal fashion irrespective of the growth-inducing treatments. However, PTI is the main branch of the plant surveillance system that has been implicated in responses to non-pathogens. It is also weaker than ETI and thus we need to consider the possibility that our treatments could have blocked more subtle growth arrest responses. It is possible that carbon sources might differ in suppressing the plants' abilities to mount a defense response against non-pathogens. For example, hexose levels seem to be monitored by the surveillance system of plants (Biemelt and Sonnewald, 2005). High sucrose levels may conversely decrease the hexose/sucrose ratio and may thus down-regulate plant defenses. However we have observed population increases of non-pathogens with the hexoses fructose and glucose, as well as with sucrose, and also with non-sugar carbon sources such as succinate and proline. Therefore we believe that the direct consumption of those substrates is responsible for the apoplastic non-pathogenic growth. Nevertheless, direct experiments addressing the possibility that the ENA treatments presented here (carbon source addition and water-soaking maintenance) may down-regulate plant defenses will be addressed. The experiments proposed involve testing the ENA treatments in PTI knockout (or knockdown) plants and also in plants expressing PTI-suppressing effectors such as AvrPto1 and AvrPtoB1. We also plan to directly compare expression of key PTI genes with and without ENA treatments in *Arabidopsis*.

Similar to what has been done in the surface, we could use biosensors to show direct consumption of carbon compounds. Direct consumption of sugars was shown utilizing GFP-based biosensor strains on the surface of the leaf and they could be useful here to report on bacterial sugar consumption inside the leaf. Also, generation of non-pathogenic mutants unable to utilize plant sugars can be useful to test the hypothesis of direct consumption as the cause of growth of the non-pathogens in the apoplast. The generation of fructose and glucose-catabolizing mutants can be complex due to their central role in bacterial metabolism and the number of enzymes involved. However, a *Pss* B728a HrpJ$^-$ ScrY$^-$ sucrose-utilization double mutant has been generated and its endophytic growth in the presence of sucrose should be decreased compared to *Pss* B728a HrpJ$^-$ if our model is correct.

Table 2-1 – List of bacterial strains and plasmids used in this work.

Strain or plasmid	Characteristics*	Reference or source
P. syringae pv. *syringae* B728a	Wild type isolated from *Phaseolus vulgaris* (snap bean)	Loper and Lindow (1987)
Pss B728a HrpJ-	hrpJ::ΩSpec; T3SS-deficient	Hirano *et al*. (1999)
P. syringae pv. *tomato* DC3000	Wild type; spontaneous Rif^r	Cuppels 1986
P. syringae pv. *tomato* DC3000 $HrcC^-$	*hrcC* mutant defective in Type III secretion, Cm^r	A. Collmer / J. Alfano
Pseudomonas fluorescens 55	Nal^r	M. Sasser
Pseudomonas syringae pv. *tabaci* 11528	Isolated from *N. tabacum*	Brian Staskawicz
Pf01	Cm^r	JH Chang
PfA501	Rif^r	Lindow, 1985
PfA506	Rif^r	Lindow, 1985
PfA510	Rif^r	Lindow, 1985

* Nal^r, Rif^r, Cm^r, Sp^r, Km^r = resistant to nalidixic acid, rifampicin, chloramphenicol, spectinomycin and kanamycin.

Table 2-2 –Growth of *Pseudomonas* strains in M9-salts minimum media with 0.4% of carbon sources listed below.

C-source	*P. fluorescens* 55	*P. syringae* B728a LK2	*Pf*55 / Pf (pLN18)	*Pss* B728a / HrpJ-	*Pto* DC3000	*Pa* 299R	*Pf*55	*Pf* A501	*Pf* A506	*Pf* A510	*Pf* Cd32	*Pf* 1256
Succinate	+++	+++										
Citrate	+++	++										
D-sorbitol	++	++										
D-ribose	++	+										
D-galactose	+++	+										
Mannitol	+++	++										
L-proline	+++	++										
L-arginine	++	+/-										
Triptophan	++	-										
Sucrose	+/-	++										
Fructose	++	++	+++	++	+	+++	++	+++	+	++	+/-	+/-
M9 salts (-ctrol)	-	-										
LA (+ ctrol)	+++	++										
LA Km Nat	N/A	+++										
LA Nx_{10} Nat	+++	N/A										
Glucose			+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Sucrose			-	+++	+++	+++	-	-	++	-	++	-

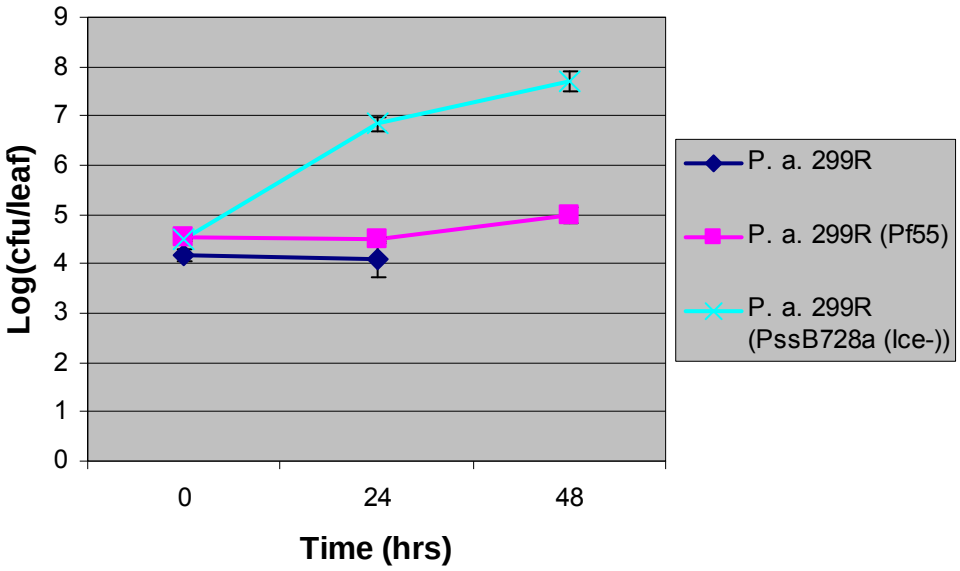
Table 2-2 –(cont.)

C-source	*P. fluorescens* 55	*P. syringae* B728a LK2	*Pf*55 / Pf (pLN18)	*Pss* B728a / HrpJ-	*Pto* DC3000	*Pa* 299R	*Pf*55	*Pf* A501	*Pf* A506	*Pf* A510	*Pf* Cd32	*Pf* 1256
D- Fucose			-	-	-	-	-	-	-	-	-	
D- Xylose			++	++	++	+++	+	++	++	+/-	+	+
D-Arabinose			-	-	-	-	+/-	-	-	-	-	-
Mesaconate			-	-	-	-	-	-	-	-	-	-
L(+) Tartaric ac.			-	-	-	-						
Adonitol			+++	-	-	-	++	-	++	-	-	-
meso-Erythritol			+++	++	-	-	+	-	-	-	-	-
Glycerol			+++	+++	++	++	+++	+++	+++	+++	+++	+++
Xylitol			+++	-	-	-	+++	-	+	-	-	-
KB			+++	+++	+++	+++						
LA			+++	+++	++	+++						
M9 salts			-	-	-	-	-	-	-	-	-	-

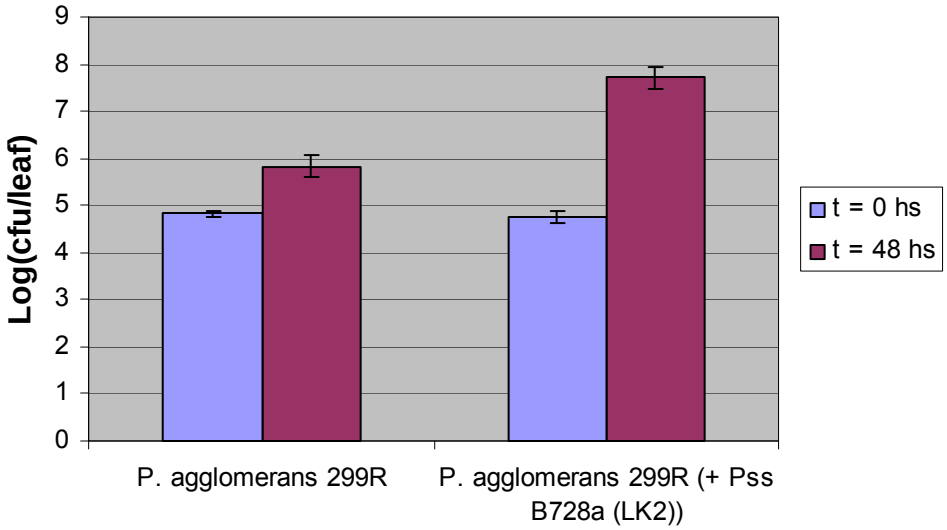
- No growth
+/- Little growth of streak only
+ growth of streak, no single colonies
++ growth of streak and single colonies (relatively small)
+++ growth of streak and single colonies comparable to KB.

Fig. 2–1 –Pathogens induce growth of non-pathogens inside leaves.
a) Endophytic population of *P. agglomerans* 299R is higher when co-infiltrated with an Ice^{-} derivative of wild type *Pss* B728a (*P.a.* 299R (PssB728a (Ice-)) (Xs), but not when co-infiltrated with *Pf*55 (P.a. 299R (Pf55)) (squares) or alone (P.a. 299R) (diamonds). Bean plants were vacuum-infiltrated and incubated under mist chamber conditions often after congestion disappeared. Vertical bars represent two standard errors of the mean. **b)** *Pantoea agglomerans* 299R (p61RYIce) endophytic population size alone (P. agglomerans 299R) or in the presence of *Pss* B728a (LK2) and **c)** Sucrose-dependent ice nucleation activity (INA) in bean plants. **d)** Sucrose dependent INA calibration curve (taken from Miller et al., 2001). Bean plants were vacuum infiltrated, and inoculated in a mist chamber after water soaking disappeared. Vertical bars represent two standard errors of the mean.

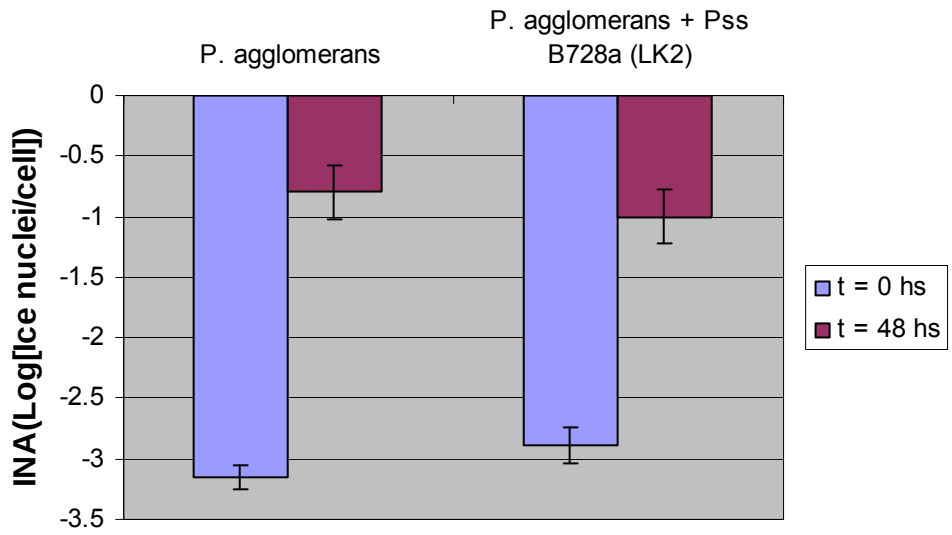
a)



b)



c)


P. agglomerans
P. agglomerans + Pss B728a (LK2)
INA(Log[Ice nuclei/cell])
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
t = 0 hs
t = 48 hs

d)

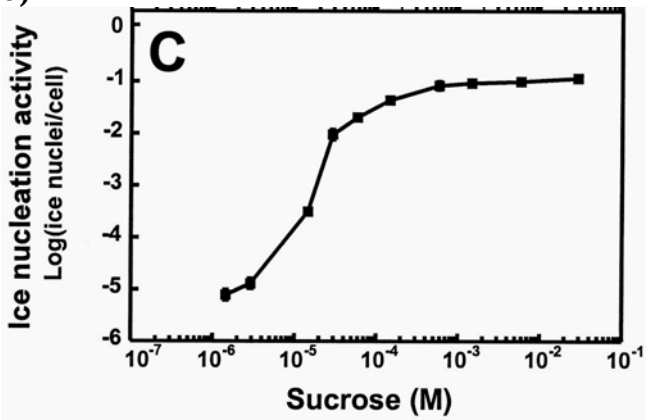

C
Ice nucleation activity
Log(ice nuclei/cell)
0
-1
-2
-3
-4
-5
-6
10-7
10-6
10-5
10-4
10-3
10-2
10-1
Sucrose (M)

Fig. 2–2 –Endophytic growth of *Pss* B728a HrpJ$^-$ with fructose addition.
Pss B728a (LK2) (X's) and *Pss* B728a HrpJ$^-$ (hrpJ-) (squares) were infiltrated alone or co-infiltrated with 2g/L of fructose (triangles and diamonds, respectively) into bean leaves. Vertical bars represent two standard errors of mean log population sizes.

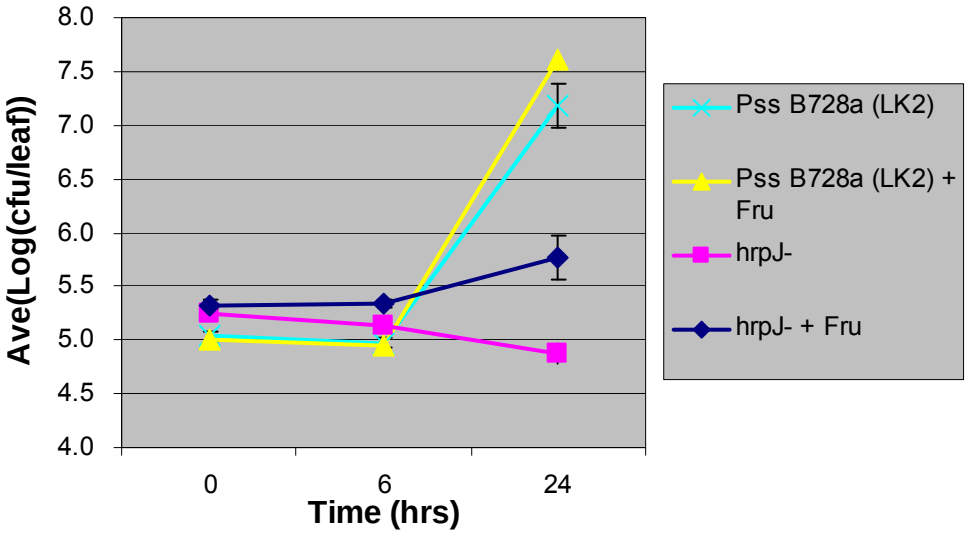


Fig. 2–3 –Response of *Pss* B728a HrpJ- to different amounts of exogenous sucrose upon infiltration into bean leaves.
Population size of *Pss* B728a HrpJ$^-$ alone (HrpJ-) (squares) or in the presence of 1g/L of sucrose (HrpJ- + 1 g/L Suc) (triangles) or 10 g/L of sucrose (HrpJ- + 10g/L Suc) (Xs) and *Pss* B728a (diamonds). Vertical bars represent two standard errors of mean log population sizes.

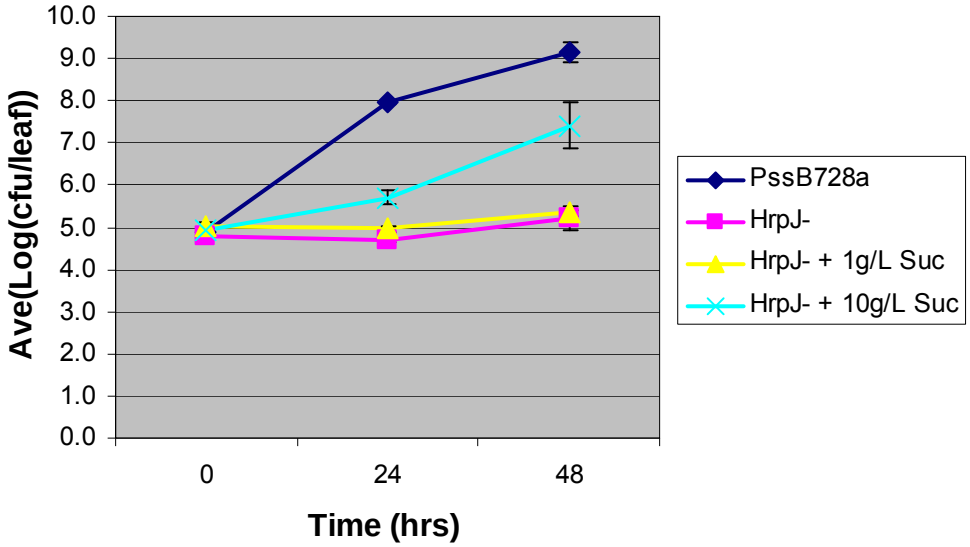
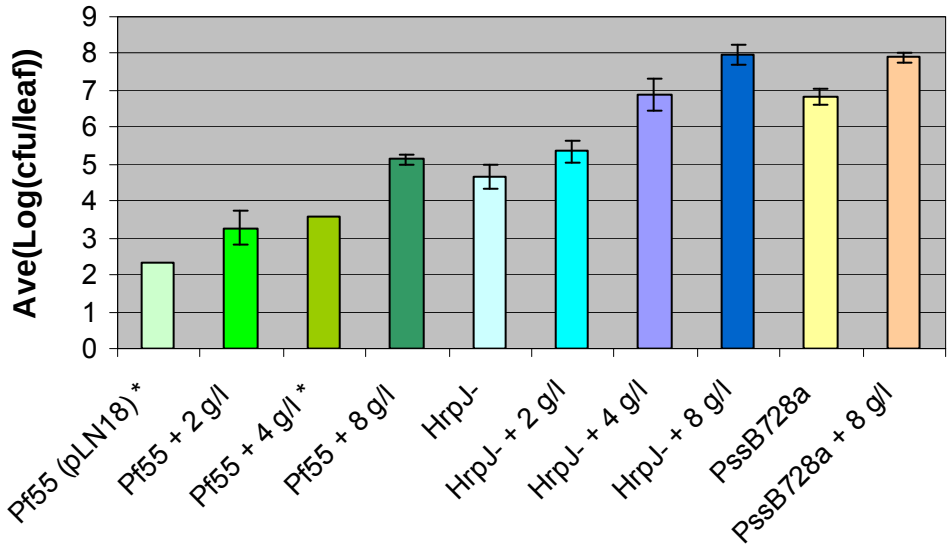


Fig. 2–4 –Endophytic population sizes of non-pathogenic strains saprophyte *Pf*55 and *hrp* mutant *Pss* B728a HrpJ⁻ when infiltrated with increasing concentrations of fructose.

Population growth of *Pss* B728a HrpJ⁻ is rescued to wild type levels. Growth of *Pf*55 (pLN18) carrying the TTSS *hrp*/*hrc* cluster from *Pss*61; TTSS mutant *Pss* B728a HrpJ⁻ alone or in the presence of 2, 4 and 8 g/L of fructose; and *Pss* B728a alone or in the presence of 8g/L fructose. Vacuum infiltration of beans, total population bacterial counts of individual sampled leaves, dry growth conditions. Vertical bars represent two standard errors of mean log population sizes.



* average of 2 and 3 measurements

Fig. 2–5 –Fructose but not sucrose rescues growth of *Pseudomonas fluorescens* A501 (*Pf*A501) in the apoplast.
Total population size of *Pf*A501 alone, in the presence of 4 g/L of sucrose (Pf + Suc) and in the presence of 4 g/L of fructose (Pf + Fru); and population size of *Pss* B728a Ice$^-$ strain (Pss B728a (LK2)). Vertical bars represent two standard errors of mean log population sizes.

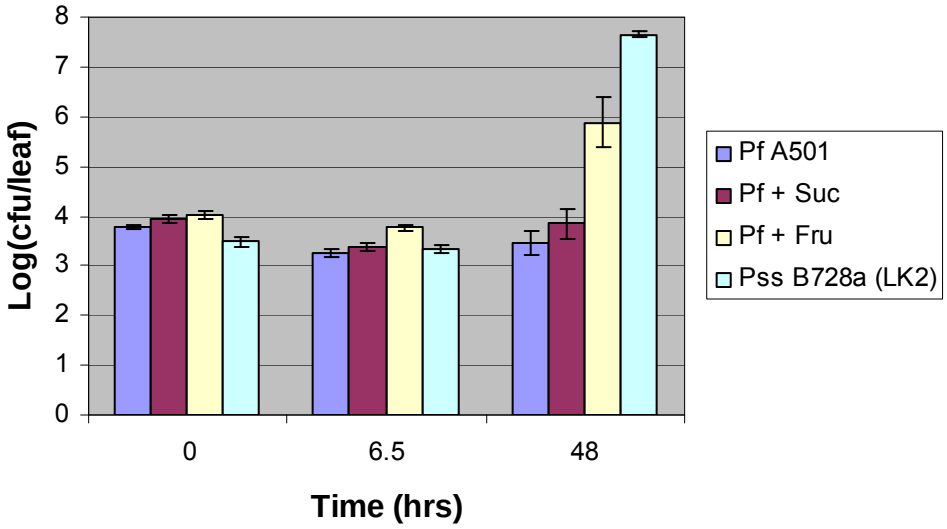


Fig. 2-6 –Endophytic population size of *Pf*55 and *Pss* B728a HrpJ⁻ infiltrated into bean leaves with sucrose and proline.
Proline but not sucrose can rescue the endophytic growth of both *Pf*55, and *Pss* B728a HrpJ⁻ *Pf*55 alone or in the presence of 5 g/L of sucrose (Pf55 + Suc) or proline (Pf55 + Pro); *Pss* B728a HrpJ⁻ alone (HrpJ-) or in the presence of 5g/L of proline (HrpJ- + pro); and *Pss* B728a LK2 alone or in the presence of 5 g/L of proline (Pss B728a (LK2) + pro) were infiltrated into bean plants. Vertical bars represent two standard errors of mean log population sizes.

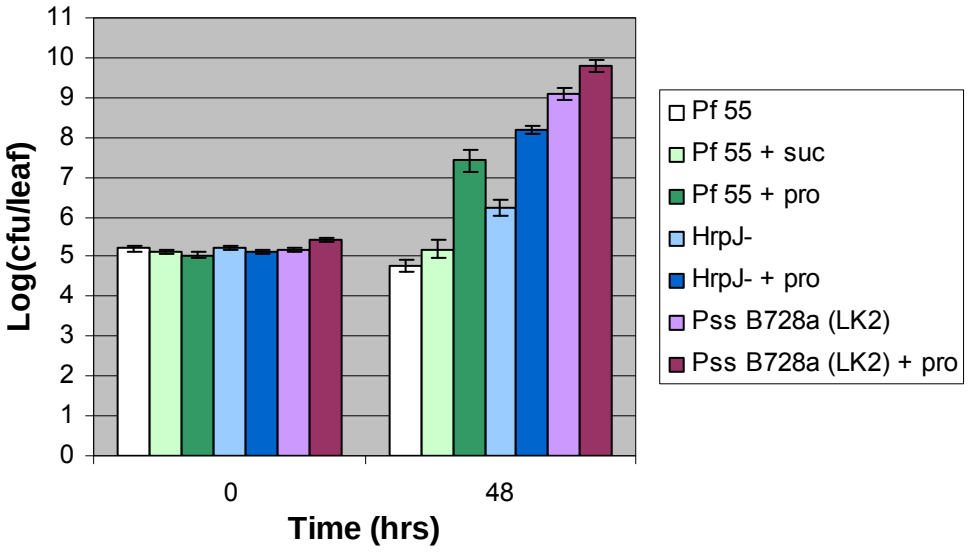


Fig. 2–7 –Addition of glucose and succinate to the inoculum of TTSS mutant of an incompatible non-host pathogen in beans (Pto DC3000 HrcC$^-$) enables endophytic growth to levels of the wild type compatible pathogen *Pss* B728a.
Pto DC3000 HrcC$^-$ alone (hrcC- DC3000) or in the presence of 5 g/L of glucose (hrcC- + glu) or succinate (hrcC- + Succinate); and *Pss* B278a (Pss B728a) were vacuum infiltrated into bean leaves. Vertical bars represent two standard errors of mean log population sizes.

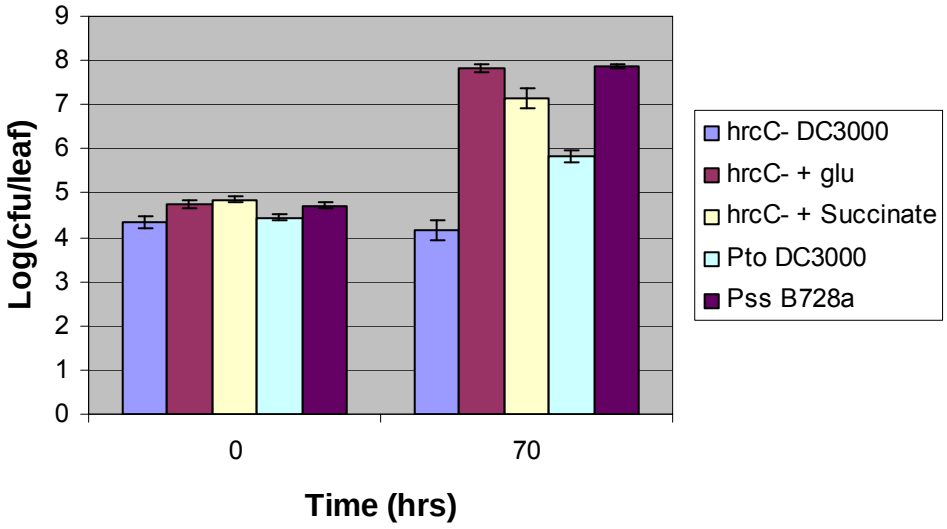
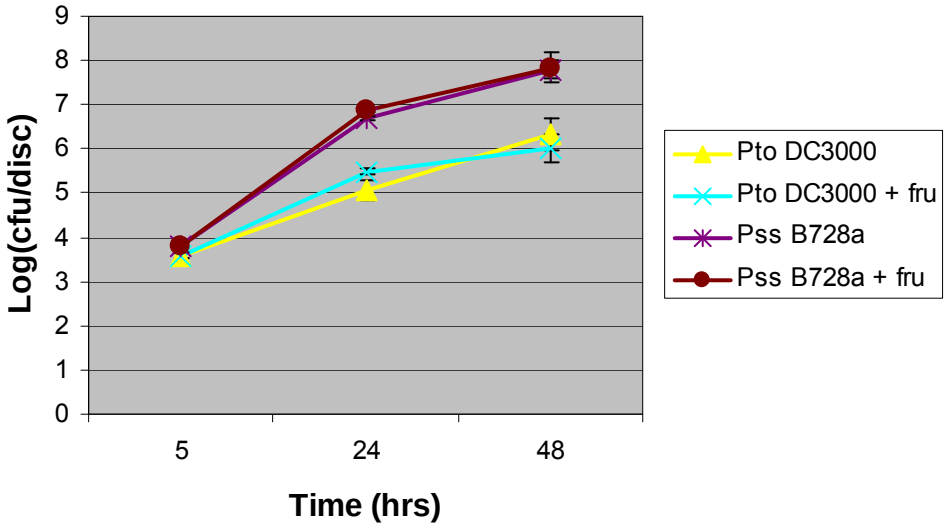


Fig. 2–8 –Addition of carbon source does not affect normal plant defense response to a non-host pathogen

a) TTSS proficient pathogens do not exhibit changes in endophytic growth upon addition of sugars to the inoculum. Endophytic population size of *Pto* DC3000 (Pto DC3000) (triangles) and *Pss* B728a (Pss B728a) (stars) alone or in the presence of 5 g/L of fructose (Pto DC3000 + fru (Xs) and Pss B728a + fru (circles)). Vertical bars represent two standard errors of mean log population sizes. **b)** HR assay of a saprophyte (*Pf*55) and host (*Pss* B728a) and non-host (*Pto* DC3000 and *Pta* 11528) pathogens in beans with and without 5 g/L of fructose; and fructose control alone.

a)



b)

B728a **B728a + Fru**

Pto DC3000

Pto + Fru

Pst11528

Pst 11528 + Fru

Pf55

Pf55 + Fru

Fru only

Fig. 2–9 –Growth of *Pss* B728a HrpJ⁻ TTSS mutant is rescued to wild type levels both upon addition of carbon sources to the inoculum and/or maintaining water-soaking conditions.

Pss B728a HrpJ⁻ alone (HrpJ-) or inoculated with 8 g/L of fructose (HrpJ- + fru), or under maintained water-soaking conditions by covering with plastic bag after infiltration (HrpJ- + water-soaking) or both (HrpJ- + fru + water-soaking); and *Pss* B728a wild type control. Vertical bars represent two standard errors of mean log population sizes.

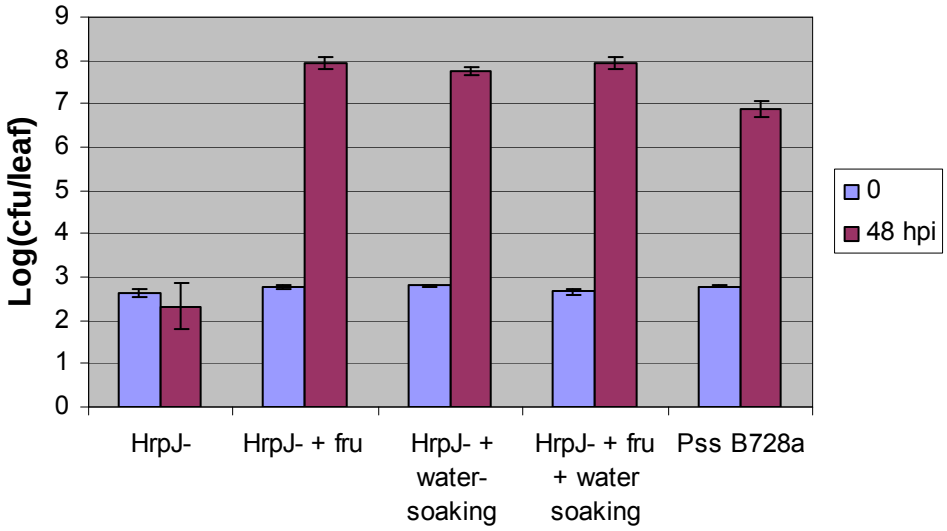
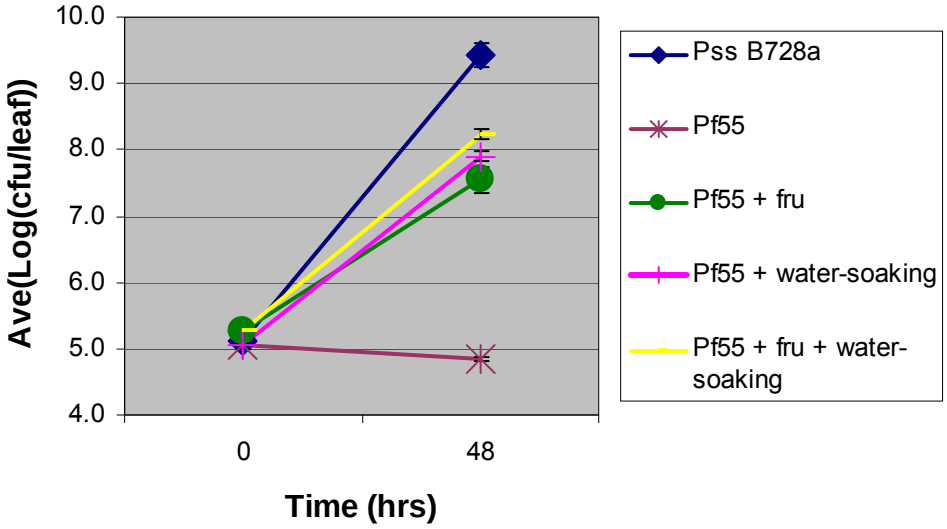
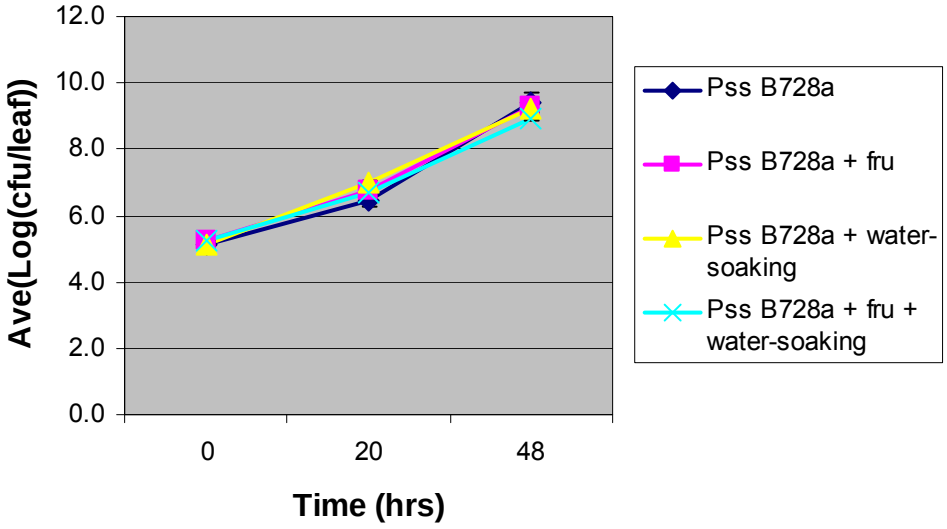


Fig. 2–10 –Vacuum infiltration of beans with various bacterial strains with added fructose, and/or maintained water-soaking after infiltration.
Strains **a)** *Pf*55, **b)** *Pss* B728a, or **c)** *Pto* DC3000 were either infiltrated alone, co-infiltrated with 5 g/L of fructose, maintained under water-soaking conditions or both. Vertical bars represent two standard errors of mean log population sizes.

a)



b)



c)

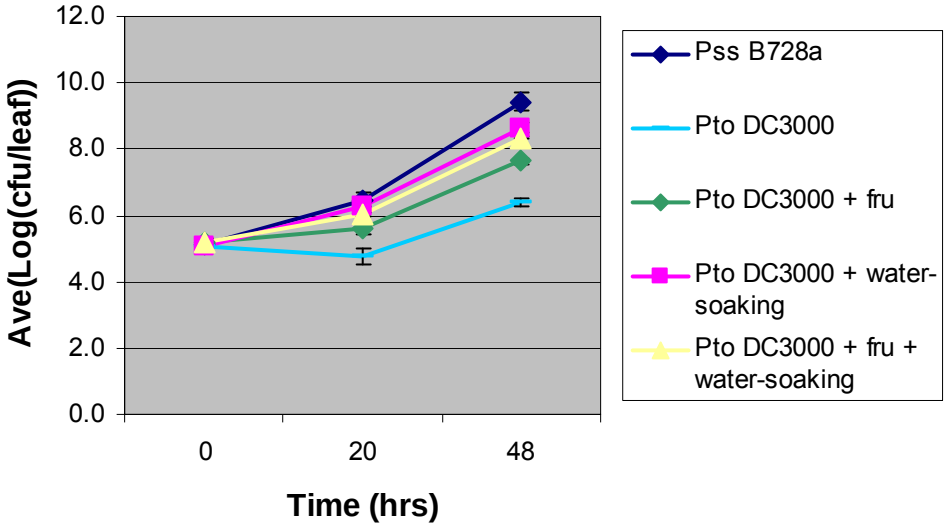
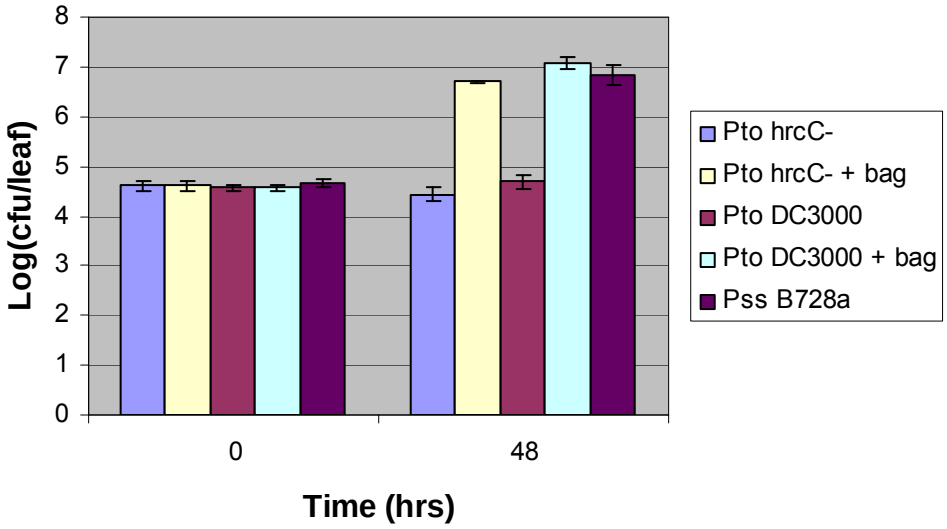

12.0
10.0
8.0
6.0
4.0
2.0
0.0
Ave(Log(cfu/leaf))
0
20
48
Time (hrs)
Pss B728a
Pto DC3000
Pto DC3000 + fru
Pto DC3000 + water-soaking
Pto DC3000 + fru + water-soaking

Fig. 2–11 –Maintenance of water-soaking of leaves after infiltration of *Pto* DC3000 HrcC$^-$ into non-host beans enables growth in the apoplast.
a) Total population size of *Pto* DC3000 and *Pto* DC3000 HrcC$^-$ alone or under maintained water-soaking conditions by covering with plastic bags after infiltration (Pto DC3000 + bag and Pto hrcC- + bag). Vertical bars represent two standard errors of mean log population sizes. Pictures of lesion-like symptoms in bagged treatments **b)** *Pto* DC3000 HrcC$^-$ after bagging **c)** *Pto* DC3000 after bagging **d)** *Pto* DC3000 or **e)** *Pss* B728a.

a)



b)

c)

d)

e)

Fig. 2–12 –Saprophyte *Pf*55 and the TTSS mutant *Pss* B728a HrpJ⁻ both exhibit extensive apoplastic growth in *N. benthamiana* leaves in which water-soaking conditions were maintained after inoculation.
*Pf*55, *Pss* B728a and *Pss* B728a HrpJ⁻ were infiltrated alone or covered with plastic bags to maintain water congestion (Pf55 + water-soaking; HrpJ- + water-soaking; and Pss B728a + water-soaking). Vertical bars represent two standard errors of mean log population sizes.

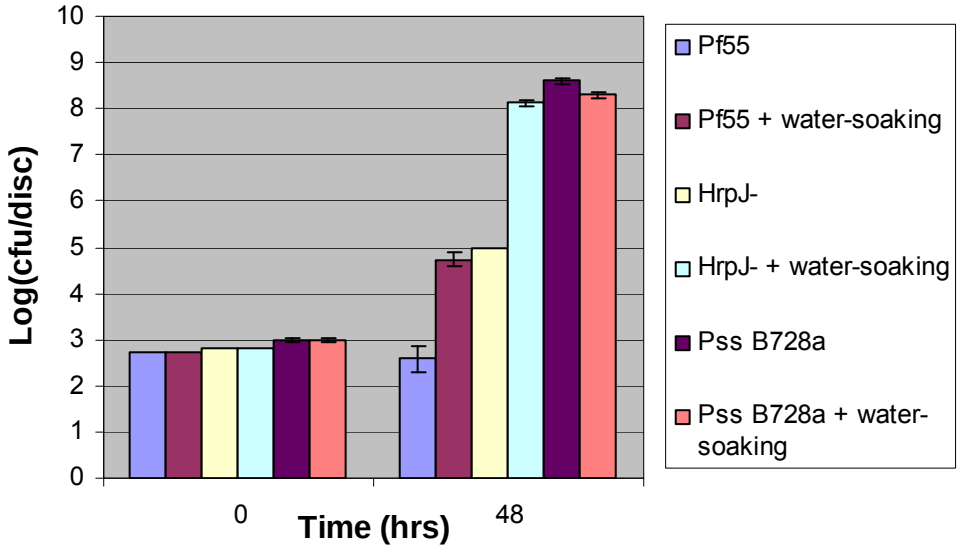
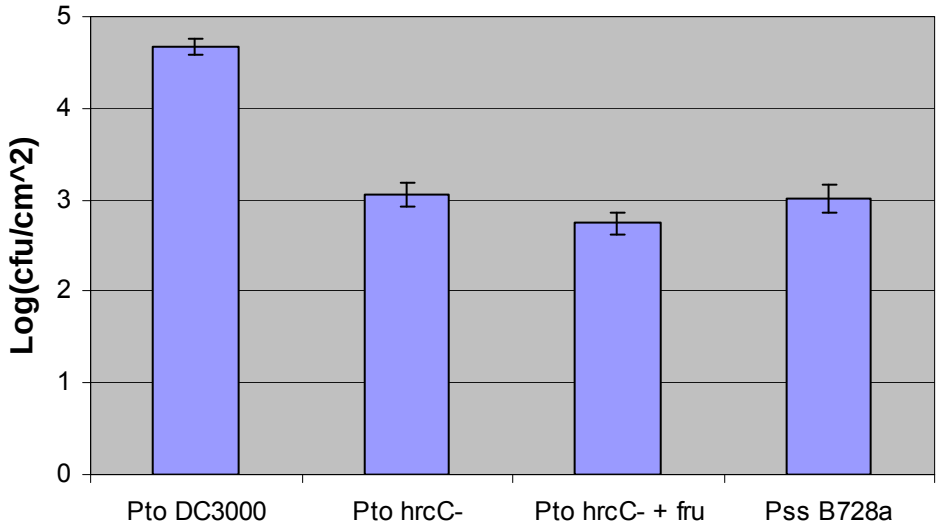


Fig. 2–13 –Non-pathogenic bacteria do not multiply endophytically in *Arabidopsis* leaves when co-inoculated with carbon sources in syringe infiltration experiments. *Arabidopsis thaliana* Col-0 were hand syringe inoculated with **a)** *Pto* DC3000 HrcC$^-$ alone (Pto hrcC-) and with 4 g/L of fructose (Pto hrcC- + fru), *Pto* DC3000 and *Pss* B728a **b)** *Pto* DC3000 HrcC$^-$ (Pto hrcC-) and *Pss* B728a HrpJ$^-$ (hrpJ-) alone or with 4 g/L of succinate (Pto hrcC- + succinate; and hrpJ- + succinate) or 4 g/L of glucose (Pto hrcC- + glu; and hrpJ- + glu). Vertical bars represent two standard errors of mean log population sizes.

a)



b)

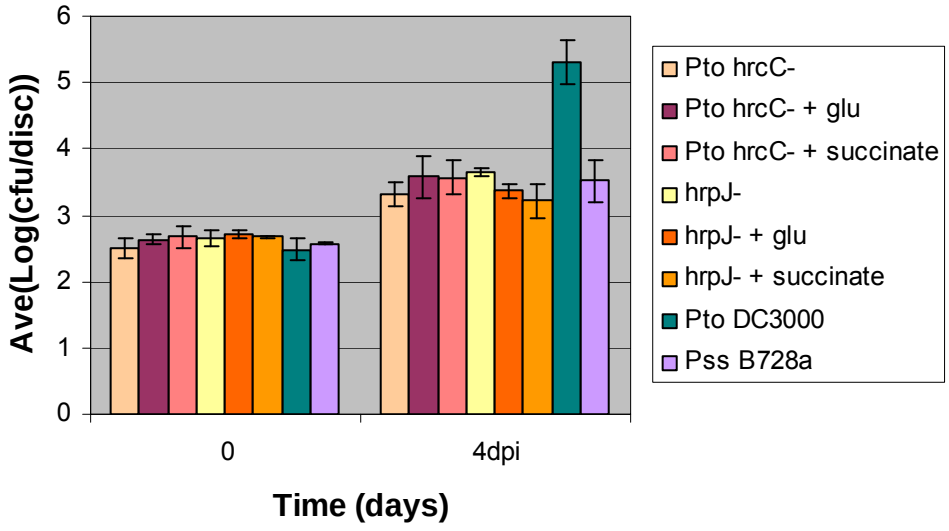


Fig. 2–14 –Multiplication of *Pf*01 and TTSS mutant *Pto* DC3000 HrcC$^-$ in *Arabidopsis thaliana* Col-0 leaves co-inoculated with high concentration of succinate as a carbon source and subsequently subjected to water-soaking conditions.
*Pf*01 alone (Pf01), in the presence of water-soaking conditions (Pf01 + B) or 8 g/L of succinate (Pf01 + Succ.), a carbon source it cannot utilize in vitro; *Pto* DC3000 HrcC$^-$ alone (Pto hrcC- DC), in the presence of water-soaking conditions (Pto hrcC + B) or 8 g/L of succinate (Pto hrcC + Succ.). Vertical bars represent two standard errors of mean log population sizes. B= bagging.

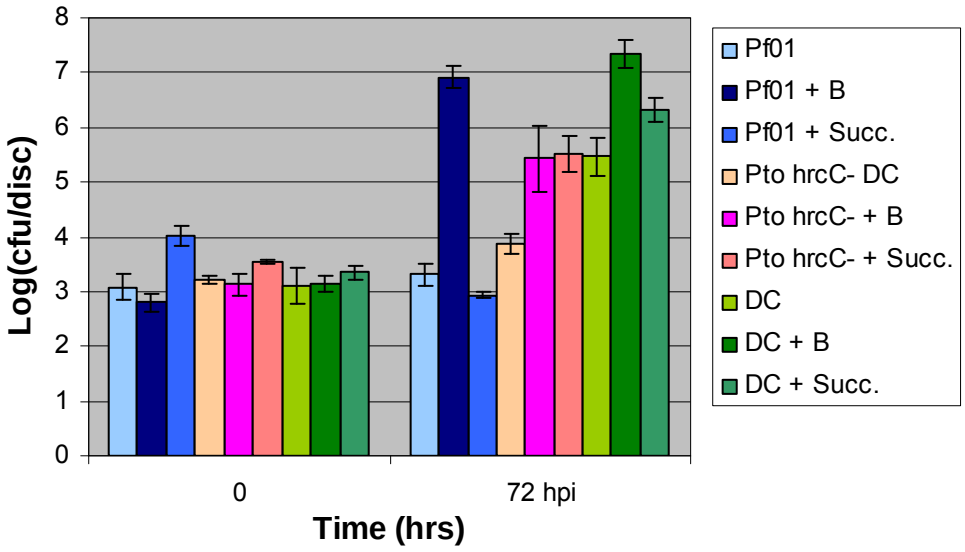
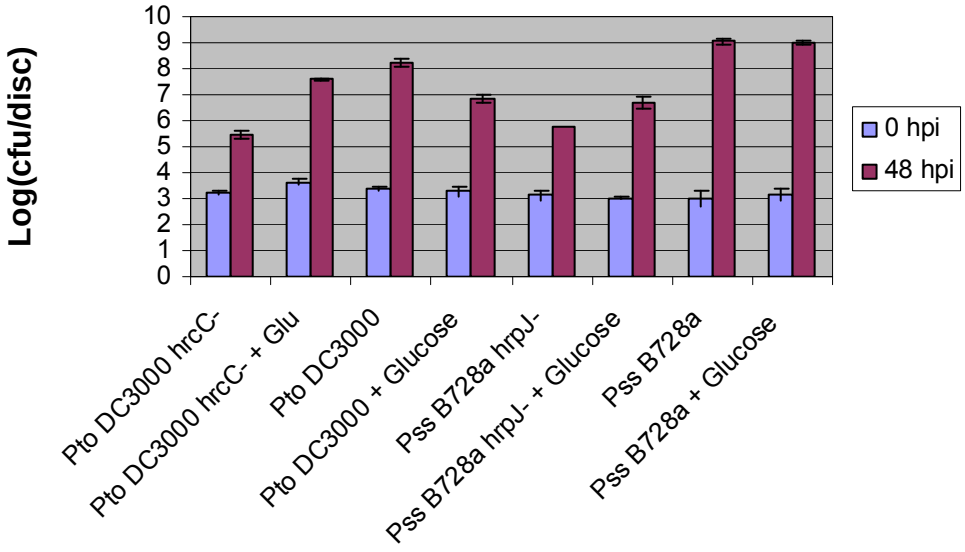


Fig. 2–15 –Endophytic growth of *hrp* mutants in of *N. benthamiana* and *Arabidopsis.* Bacterial strains were co-infiltrated into *N. benthamiana* and *Arabidopsis* by vacuum with and without added carbon sources. *Arabidopsis* is a host for *Pto* DC3000 and non-host for *Pss* B728a, while the opposite is true for *N. benthamiana*. Endophytic population growth for both types of *hrp* mutants can be achieved with 6 g/L of glucose in *N. benthamiana* but not in *Arabidopsis*. The same inoculum beaker was used for the vacuum infiltration of two sets of plants **a)** in *N. benthamiana*, and **b)** *Arabidopsis thaliana* Col-0. Vertical bars represent two standard errors of mean log population sizes.

a)



b)

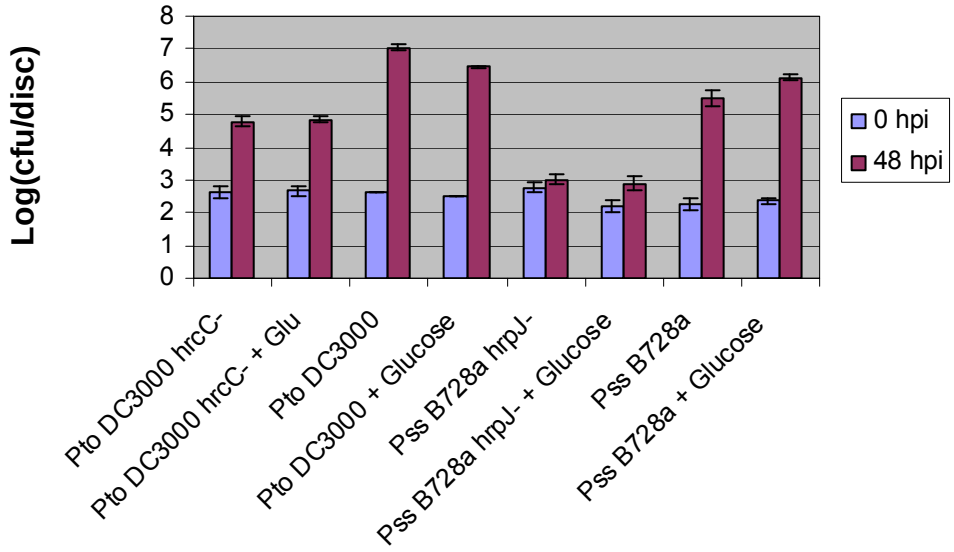
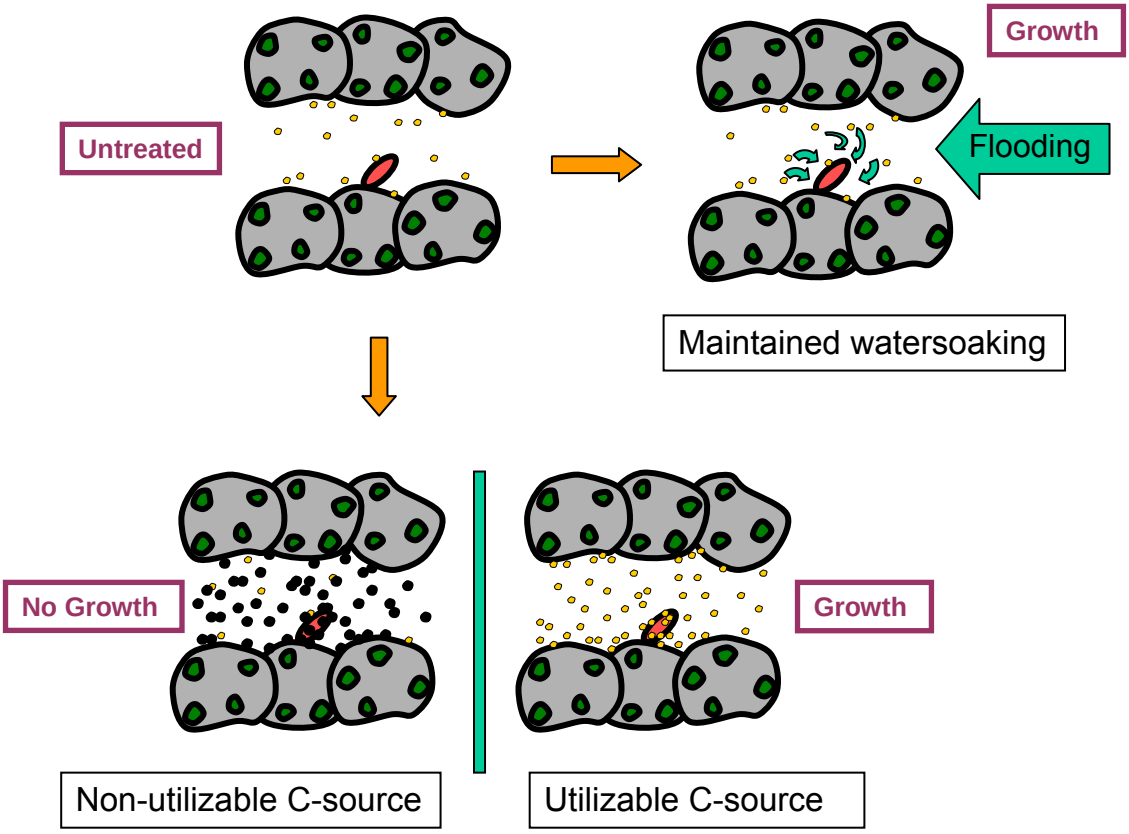


Fig. 2–16 –Model of apoplastic non-pathogenic bacterial growth under water congestion and high concentration of carbon sources.
An untreated leaf is subsequently infiltrated with buffer (right) or carbon sources (down). Flooding induces considerable water and nutrient accumulation in the apoplast and bacteria multiply. When carbon sources are infiltrated, the outcome differs when utilizable (growth) or non-utilizable (no growth) carbon sources are infiltrated once the water-congestion is dissipated.



Chapter 3
Screening for nutrient/water acquisition effector/s in *Pss* B728a

Preface

Chapter 3 describes the experiments performed with several *Pseudomonas fluorescens* plant delivery systems with the purpose of finding the type-III secretion effector/s involved in virulence mechanism/s that provide multiplying pathogens with the resources needed for growth in the apoplast of plants.

Abstract

Pathogens are able to grow endophytically in plants by expressing the type III secretion system (TTSS) and the associated TTSS effector (virulence) proteins they deliver into plants. We have explored the ability of individual TTSS effectors to induce an enhanced nutrient and/or water acquisition (ENA) effect in the apoplast inside leaves, and thus their ability to mediate the growth of saprophytic strains. In plant growth assays, we used three different *Pseudomonas fluorescens* effector delivery strains (*Pf*55, *Pf*01 EtHAn and *Pf*01 AMELIA) to express and deliver individual *Pss* B728a effectors into plants. Single effector-expressing strains are generally unable to achieve more growth than control strain, especially when they did not have a cognate chaperone to direct them to the TTSS apparatus. However, some experiments suggested that HopAH1 might be a candidate ENA effector since a *Pf*55 (pLN18) HopAH1-expressing strain could multiply inside leaves (endophytically) to higher level than controls.

AMELIA is a new single effector delivery system derived from the soil bacterium *Pf*01, and harbors the TTSS genes from a plant pathogen integrated into its genome. We showed that this strain is capable of secreting AvrRpt2 in a TTSS-dependent manner and that, unlike its predecessor EtHAn, it does not induce cell death in plants. We further show that neither AMELIA, EtHAn nor *Pf*01 multiply endophytically in plants. In spite of being a high inducer of PAMP-triggered immunity in plants (Thomas et al., 2009), AMELIA population levels could be elevated by several orders of magnitude by treatments that increased the availability of nutrients in the apoplast, such as addition of fructose or the maintenance of water-soaked conditions inside leaves. This suggests AMELIA is subject to a nutritional limitation inside the leaf that is similar to that of bacteria on the surface of leaves (Leveau, 2006). Finally we show that an infiltration strategy of grouping twelve effector-secreting AMELIA strains and co-infiltrating with saprophyte *Pantoea agglomerans* 299R (*Pa* 299R) produced a ~10-fold difference in population sizes of the *Pa* 299R in the presence of a high inoculum concentration of effector-secreting AMELIA strains as compared to the population of *Pa* 299R alone. More studies are required to further identify the effectors involved in ENA activity in co-infiltration assays.

Introduction

The intrinsic ability of pathogens to grow endophytically inside a leaf is mediated by the presence of the TTSS and the virulence effector proteins that travel this system for delivery directly into the plant cell cytosol (Alfano and Collmer, 1996). TTSS mutants (*hrp* mutants) are non-pathogenic and their growth in plants is very limited (Hirano et al., 1999; Alfano and Collmer, 1996). Thus, TTSS effector proteins mediate, to a great extent, the ability of pathogens to achieve high population densities inside the leaf (endophytic growth).

There are three main strategies by which plant pathogen effectors are thought to alter the host environment to their benefit: 1) suppressing plant defenses, 2) aiding in bacterial dispersion, and 3) providing nutrients and water to the pathogen (Chang et al., 2004). The role of effectors in modulating plant defenses has been by far the most studied. Some effectors were shown to target components of the two main branches of the plant defense response pathways: a basal level of immunity called pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and the effector-triggered immunity (ETI). For example, effectors such as AvrPto1, AvrPtoB1, AvrE1 and HopM1 have been implicated in the suppression of normal PTI responses such as papillae formation at the site of infection, callose deposition and secretion of plant defense molecules (Munkvold and Martin, 2009; Chisholm et al., 2006, Hauck et al., 2003, DebRoy et al., 2004). Other effectors seem to interfere with the production of a type of plant cell death (CD) reaction associated with ETI (the hypersensitive response or HR) by either targeting eukaryotic components of the HR response (Jamir et al., 2004) or, like AvrRpt2, by masking the recognition events triggered by another effector (Chisholm et al., 2006).

Other types of effector proteins, such as AvrB6 and PthA from *Xanthomonas* spp., induce lesion formation and cell enlargement, respectively, allowing bacteria to resurface when the leaf tissue collapses and thus aiding in dispersal to new sites of infection (Yang et al., 1994). The possibility that TTSS effectors can enhance nutrients and/or water acquisition (ENA) to plant pathogens has been suspected for a long time but there have been no reports of testing *P. syringae* TTSS effectors for their ability to induce ENA activity in the apoplast.

In a nutrient-rich environment, the inability to colonize the plant apoplast by non-pathogens and TTSS-defective mutants could be explained solely by their lack of TTSS effectors to suppress plant defense responses. However, treatments designed to alleviate nutritional-limitation (such as carbon source co-infiltration and maintenance of water-soaking conditions), enable all non-pathogenic bacteria (both saprophytes and *hrp* mutants in host and non-host interactions) to multiply in the apoplast (presented in Chapter 2). This suggests that the availability of nutrients in the leaf interior is limited and that pathogens likely overcome this limitation by secreting TTSS effectors that induce a release of resources from the host, while non-pathogens cannot grow due to their inability to actively modulate nutrients in the apoplast.

The abundance of carbon sources is the main factor that limits bacterial growth on the surface of leaves (Wilson and Lindow, 1994, Leveau and Lindow, 2001), where the number of bacteria and their distribution closely matches the availability and distribution of carbon sources (Wilson and Lindow, 1994, Leveau and Lindow, 2001; Mercier and Lindow, 2000). We hypothesize that the availability and distribution of carbon sources inside the leaf are also the main factor restricting growth of non-TTSS proficient bacteria and that a subgroup of TTSS effectors directly or indirectly alleviate the nutritional needs of actively multiplying pathogens in the apoplast.

Nothing is known about the possible mode of action of such TTSS effectors, but some studies have shown that pathogens alter the sugar content of the leaves upon infection, generally decreasing the sucrose/hexose ratio, transforming the infection site into a carbon-source consuming sink, and decreasing the levels of photosynthesis (Berger et al., 2004; Biemelt and Sonnewald, 2005; Tetlow and Farrar, 1993). The molecular mechanisms underlying such changes are largely unknown, but the targeting of the apoplastic cell wall invertase by *Xanthomonas campestris* pv. *vesicatoria* in tomato has been implicated (Kocal et al., 2008). The modification of the apoplastic compartment liquid volume and the concentration of solutes have been shown to produce dramatic changes and possible accumulation of carbon sources (Felle and Hanstein, 2002). Pathogens could also induce localized apoplastic flooding that may result in what has often been visualized as water-soaking lesions in leaves and pods of affected plants (Rudolph, 1978). Thus effectors, by inducing either a nutrient or water flow into the apoplastic compartment would be able to acquire the resources needed for bacterial multiplication. Possible subcellular targets of these effectors are many and could include the plasma membrane and cell wall (H^+/ATPase, ion channels, etc.), the chloroplasts (photosynthesis down-regulation), and the apoplast compartment, etc. We tried to avoid *a priori* assumptions about which possible effectors could have ENA activity because many could have multiple target/s and modes of action. Efectors are often complex multi-domain proteins that probably interact with many different targets and may have more than one function, as has been shown with AvrPtoB (Munkvold and Martin, 2009) and AvrRpt2 (Chisholm, 2005).

To determine which effectors induce ENA activity in the apoplast we used non-pathogenic *P. fluorescens* effector delivery systems that are capable of expressing and secreting via the TTSS individual effectors into plant hosts. Co-infiltration of non-pathogens *Pa* 299R and *Pf*55, and compatible (virulent) pathogen *Pss* B728a in beans, has revealed that effector-mediated changes can be exploited by non-pathogenic bacteria co-inhabiting the apoplast (Chapter 2). In a similar fashion the *P. fluorescens* strains expressing single TTSS effectors could increase their population size by capitalizing on the ENA activity that the effector they harbored produced in the apoplast. This gain of function approach circumvents the problem of functional redundancy of effectors that makes the study of TTSS–effector mutants not feasible. This is also likely the reason why such genes were not previously found in nutrient screens looking for traits impacting bacterial fitness in plants.

We present here the results of gain of function studies with both *Pf*55 and *Pf*01 delivery systems. The *Pf*55 delivery system developed by Alfano and co-workers exploits the foliar saprophyte *Pf*55 carrying pLN18, a cosmid construct harboring a 25-kb genomic fragment encoding the *hrp*/*hrc* pathogenicity island of *Pss*61 (a weak pathogen of beans) (Jamir et al., 2004; van Dijk et al., 2002; Huang et al., 1988). This *Pf*55 (pLN18) strain encoding the genes for regulation and assembly of the TTSS pilus was used to deliver in plant hosts individual *Pss* B728a effectors cloned in another plasmid which are constitutively expressed with a P_{nptII} promoter. We then monitored the population sizes reached by each effector-producing strain to determine which effectors could stimulate growth, thus revealing ENA activity. This effector delivery system had been shown to secrete *Pto* DC3000 effectors into tobacco and *Arabidopsis*. Strain *Pf*55 (pHIR11) carrying a cosmid with the *hrp*/*hrc* cluster as well as the *hopA*-*schA* operon produces a HopA-dependent HR in non-host tobacco and *Arabidopsis* Ws-0. Several studies by Alfano and coworkers used *Pf*55 (pLN18) to deliver individual *Pto* DC3000 effectors cloned in another plasmid to test for their ability to suppress the HopA-dependent HR generated by *Pf*55 (pHIR11) (Jamir et al., 2004; Guo et al., 2009). We decided to use non-pathogenic *Pf*55 and *Pf*01 for delivery because they use a natural route of secretion into plants and do not highly over-express effectors (as with *Agrobacterium tumefaciens*-mediated expression (Agroinfiltrations) and effector-producing transgenic plants). Also, unlike delivery using *A. tumefaciens*, *Pf*55 lacks other possible confounding virulence factors. However, the lower level of expression, acceptable in HR suppression assays where low levels of proteins are able to generate and suppress the highly amplified HR phenotype (Jamir et al., 2004), was perhaps a disadvantage for the production and/or detection of an ENA response whose detection required robust population growth in our assays.

The other delivery systems we used to test *Pss* B728a effectors were developed by J. Chang and coworkers and are based on the soil bacterium *Pf*01: *Pf*01 EtHAn and *Pf*01 AMELIA (Thomas et al., 2009; and Thomas and Chang, unpublished results). EtHAn (Effector to Host Analyzer) was created by stably integrating the ~26kb *hrp*/*hrc* cluster from *Pss* 61 spanning genes *hrpK* to *hrpH* (without effector genes) into the genome of *Pf*01 (Thomas et al., 2009). As a soil bacterium, it is thought not to be adapted for plant growth and thus likely lacking virulence factors. It also apparently elicits a PTI that is incapable of suppressing. EtHAn by itself does not grow in *Arabidopsis* but is able to initiate AvrPto-dependent callose suppression which, notwithstanding, does not lead to AvrPto-dependent increase in growth. However, because EtHAn elicits plant cell death (CD) in tomato and tobacco (Thomas et al., 2009), and also in beans and *N. benthamiana* (this study), we mostly used AMELIA, a *hrpZ* knockout derivative of EtHAn. We have shown that AMELIA does not produce a HrpZ-dependent CD in plants, and despite the loss of a TTSS helper protein for secretion, we confirmed that it is still able to translocate proteins into *Arabidopsis*. AMELIA, EtHAn and *Pf*01 all exhibit a similar lack of endophytic growth in bean, as expected of non-pathogenic strains. However, their population size can increase with treatments that increase nutrient availability in the apoplast (as shown in Chapter 2). This last question was especially relevant since AMELIA is a *Pf*01 derivative that was chosen because, as a soil bacterium, it is

non-adapted to grow well on leaves and it strongly induces PTI (Thomas et al., 2009). AMELIA has advantages over *Pf*55 since it does not carry a TTSS of its own and is probably a more efficient TTSS delivery system since it carries the *hrp*/*hrc* cluster stably inserted in its genome (Thomas et al., 2009).

Bean is the natural host of *Pss* B728a where it grows extensively. Thus, when looking for *Pss* B7828a effectors with ENA activity, we performed assays in bean plants with single-effector expressing *Pf*55, *Pf*01 EtHAn or *Pf*01 AMELIA delivery systems. Most of these studies showed that individual effector-producing strains did not exhibit much growth. However, these experiments were performed under conditions that might have been suboptimal (i.e. with a relatively low inoculum level) to yield robust apoplastic growth. We found that *Pf*55 probably has a low level of protein delivery to plants since HR-inducing effectors conferred only a weak microscopic CD in bean. However, we also found that in some cases effectors such as HopAH1, a putatively chloroplast-targeted protein stimulated some growth of the harboring saprophytic bacterium. We were, by changing environmental conditions, unable to find specific conditions that may have been required to observe induced apoplastic growth. We also tried co-inoculating several effector-producing strains to test the possibility that effectors might act cooperatively to mediate changes in the plant. Mixing of effector-expressing strains of *Pf*55 was unsuccessful in identifying effector combinations that could mediate ENA activity. However, a strategy of mixing strains with AMELIA-delivered effectors with another non-pathogenic strain revealed that this "target" bacterium benefited from this mixture of effectors. *Pa* 299R population size in bean but not in *N. benthamiana* was higher when co-infiltrated with a group of 12 effector-expressing AMELIA strains (infiltrated at high inoculum levels) than when inoculated alone. The strategy of using a nutritionally distinctive target strain to reflect changes in the plant induced by effector delivering strains may also prove more successful than direct monitoring of the individual effector-expressing strains (using high inoculum) and could facilitate further individual strain testing.

Materials and Methods

Bacterial strains

We used several bacteria as effector delivery agents to secrete and translocate effectors into plant hosts: *Pseudomonas fluorescens* 55 (pLN18), carrying a cosmid encoding all of the *hrp*/*hrc* cluster genes plus effector gene *hopA* and its chaperone *shcA*. The effector-chaperone pair was disrupted by insertion of a kanamycin resistance determinant (a gift from Jim Alfano) (Table 3-1). Antibiotics were used at the following concentrations (in μg/ml): ampicillin (Ap) 150, chloramphenicol (Cm) 20, gentamycin (Gm) 50, kanamycin (Km) 50, natamycin (Nat) 21.6, nalidixic acid (Nx) 20, nitrofurantoin (NFT) 25, Rifampicin 100, Spectinomycin (Sp) 20, tetracycline (Tc) 50.

Construction of *Pseudomonas fluorescens* strains for effector delivery into plants

The *Pss* B728a effector-carrying constructs were cloned under the control of the *nptII* promoter in plasmids pCPP5040 and pBAV226Gm conferring gentamycin resistance (Gm^R). Plasmid pBAV226Gm was generated as a Gm^R, tetracycline sensitive (Tet^S) variant of pBAV226 (Tet^R) (Heeb et al., 2000). We found *Pf*55 to have natural resistance to several antibiotics (nalidixic acid (Nx), cloramphenicol (Cm), ampicillin, spectinomycin/streptomycin and trimethoprim (T)) but sensitivity to kanamcyin (Km) and gentamycin (Gm). We decided to use Gm^R vectors for the cloning of *Pss* B728a effectors since a Km^R cassette had already been used for the marker exchange knockout of the *schA-hopA* operon to make pHIR11-derivative construct pLN18. Thus we used pML123 (Gm^R, RSF1010 replicon), pCPP5040 (Gm^R, pML123-derivative) and a Gm^R-modified version of pBAV226. pBAV226 and pBAV179 (Tet^R, derivatives of pME6010 and pME6012 with a stable pVS1 replicon) (Heeb et al., 2000) were modified to be Tet^S and Gm^R. Briefly the Gm^R cassette from pCPP5040 was PCR-amplified with *NruI*-containing specific primers. Following *NruI* digestion of the primers and the pBAV226/179 plasmids the Gm^R cassette was cloned into the single *NruI* site, interrupting the *tetA* gene in pBAV226, thus inactivating *tetA* and rendering the strains resistant to gentamycin. We used the LR clonase Gateway reaction (Invitrogen, Carlsbad CA) to clone effectors into pCPP5040 and pBAV226Gm. Briefly plasmid preparations from overnight cultures of *E. coli* (pDONR207-effector) Gateway ENTRY clones (Vinatzer et al., 2006) were linearized by restriction enzyme digestion and gel purified. The effectors under the control of the P_{nptII} promoter in pDONR were Gateway-cloned into either pCPP5040 or pBAV226Gm plasmids via an LR clonase reaction performed according to the manufacturer's instructions. The linearization step was not required but was performed to increase the efficiency of the reaction. The reaction mix was transformed into chemically competent *E. coli* TOP10 cells and plated on Gm selective plates. Transformants were analyzed by colony-PCR and were subsequently introduced into *Pf*55 or *Pf*01via *E. coli* (pRK2013)-mediated triparental mating. Transcojugants were selected in selective plates and the plasmid backbone and effector-specific amplification was confirmed by PCR. The pBAV226Gm and pCPP5040 effector-carrying constructs were introduced by triparental mating into *Pf*55 (pLN18), *Pf*55 (pCPP2089), *Pf*01 EtHAn, and into Pf01 AMELIA. Other constructs such as pLH12, pLH12-Ω and pVSP61 as well as derived constructs carrying

AvrRpt2 and AvrRpm1 (a gift of B. Staskawicz) were introduced into EtHAn and AMELIA by triparental mating.

Plant inoculations

Bacterial strains were recovered from glycerol stocks at -80C and streaked onto Luria Bertani (LB) agar amended with appropriate antibiotics. *Pseudomonas fluorescens* was incubated at 28C for 15-18 hrs, while *Pseudomonas syringae* strains were incubated at 28C for 30-35 hrs and *Pantoea agglomerans* plates were incubated overnight at 37C. Each strain was then resuspended in 10mM KPO_4 buffer (pH=7) and their concentration was estimated by turbidity (OD_{600}) and subsequently adjusted by dilution to 10^5 or 10^6 CFU/ml (for syringe inoculations) (Wilson et al., 1999).

Plant hosts used included beans (*Phaseolus vulgaris* cv. Bush Blue Lake 274), *Nicotiana benthamiana* and *Arabidopsis thaliana* ecotype Col-0. Beans were established (4-6 per pot) from seed and utilized at about 2 weeks old when trifoliate leaves were barely emerging or very small; for all other plants used one plant was established per pot. All plants were grown in a greenhouse setting.

Plant infiltrations were performed using one of the following two methods: vacuum infiltration of entire leaves as described previously (Willis et al., 1990) was used for most experiments or local infiltrations using a needleless blunt syringe. For vacuum infiltrations 2L of bacterial suspension of 10^5 or 10^6cfu/ml were added to a 2L beaker to full submergence of all the leaves in the bacterial suspensions. The plants were inverted in the beaker containing the inoculum, but the roots and soil were excluded (cotton and tape was used to prevent excessive soil from falling into the inoculum). The submerged leaves were then introduced into a chamber and a vacuum was applied for 2- 5 min, to remove air from the air-filled spaces of the plant. Upon release of the vacuum bacterial cell suspension was forced into the leaves. Only fully infiltrated leaves (evidenced by complete water-soaking) were sampled. When needed, vacuum was reapplied when too many leaves were partially infiltrated. Partially infiltrated leaves were marked or removed to avoid their sampling. Time 0 was taken after all signs of water-soaking disappeared in all plants (1.5- 2 hrs later). When maintaining water-soaking conditions, the plants were sealed in transparent plastic bags immediately after infiltration, before the water-congested leaves were allowed to dry, thus moisture in the leaf apoplast.

For hand syringe infiltrations, a small nick cut was made on either side of the midvein with a razor blade, and a needleless syringe loaded with inoculum was then applied with pressure to infiltrate at the nicked site. The water-soaked area of infiltration was then marked with a permanent marker for future sampling. After water-soaking disappeared, 0.5cm diameter discs were cut out from the infiltrated area of each leaf. Inoculation of detached bean pods was performed in a similar manner but making an initial hole with a syringe followed by the needless syringe application of the inoculum. Inoculated pods were incubated at 28C or room temperature for 3-5 days before scoring for water-soaking lesions.

Bacterial population measurement

Whole leaves (vacuum infiltration) or discs (syringe infiltration) were then sampled and macerated to determine total viable bacterial cells. Generally four biological replicates were assessed per treatment (for timepoint 0 sometimes only three replicates were obtained sampled due to the low initial variability in cell concentrations).

When estimating endophytic bacterial population sizes, sampled leaves for the same treatment were pooled together in plastic boxes and treated for 5 min. with a 15% solution (vol/vol) of H_2O_2 to achieve surface sterilization. For vacuum infiltrated plants, leaves were rinsed three times with autoclaved distilled water before being macerated with a mortar and pestle in 5 ml 10mM phosphate buffer to determine the surviving population (endophytic bacteria per leaf). For syringe-infiltrated plants, the leaves were rinsed in the same manner as described for vacuum infiltration and a single disc per leaf was cut out with an eppendorf tube cap and macerated in 0.5 ml of 10mM phosphate buffer. Further 10-fold dilutions were made of some samples and plated with a spiral plater onto appropriate selective agar plates to obtain bacterial colony-forming units (CFUs) per sample. The inoculum for co-inoculation experiments was prepared as above except *Pf*55 and *Pss* B728a were mixed in a beaker immediately before plant infiltration at 1:100 to 1:10,000 ratios with final concentrations of 10^4 to 10^6 CFU/ml *Pf*55 and 10^2 to 10^4 cfu/ml *Pss* B728a determined from the OD_{600}.

Macroscopic and microscopic hypersensitive response (HR) assays

For macroscopic visualization of plant cell death (CD) responses (HR and necrosis associated with virulent lesions) we performed syringe infiltrations of high concentration bacterial suspensions (10^8 and 10^9 CFU/ml as determined by OD_{600}). Bacterial suspensions were infiltrated on either side of a leaf midvein and the area of infiltration was delineated with a water-proof marker. CD was recorded within 24 hrs after infiltration and the quantity and quality of the HR lesion was scored.

Fluorescence and (light) microscopy were used to assess microscopic HR lesions to establish the secretion of individual *Pf*55 (pLN18 + pEffector) constructs and the increased ability of rpsL/R-carrying *Pf*55 (pHIR11) to secrete effectors.

Results

1) *Pf*55 as a TTSS effector delivery agent into plants

To deliver *Pss* B728a TTSS effectors into bean plants we used a modified *Pf*55 effector delivery system that had been previously shown to successfully secrete and translocate *Pto* DC3000 effectors into the apoplast of tobacco and *Arabidopsis* (Jamir et al., 2004). The *Pf*55-based delivery system harbors two vectors. One is cosmid pLN18 encoding the entire *hrp*/*hrc* gene cluster from the bean pathogen *Pseudomonas syringae* pv. *syringae* 61 (*Pss* 61), this gene cluster is required for TTSS-dependent delivery of effectors. The second vector is a compatible plasmid that constitutively expresses single *Pss* B728a effectors cloned under the control of the *nptII* promoter (see Fig. 3-1). We used either pBAV226Gm (low copy number), pML123 or pCPP5040 (higher copy number) as the effector-carrying plasmid (see Materials and Methods). Previous studies used this *Pf*55 delivery system to confer a $HopA_{Pss61}$-dependent HR in plants. The HR was achieved by infiltrating *Pf*55 (pHIR11) into plants, a strain that carries cosmid pHIR11 encoding $HopA_{Pss61}$ (Jamir et al., 2004; Huang et al., 1988; van Dijk et al., 2002). pHIR11 is the originally-isolated cosmid that carries both the *Pss* 61 TTSS genes and the operon *hopA*-*shcA* encoding effector $HopA_{Pss61}$ and its chaperone (van Dijk et al., 2002; Huang et al., 1988). In Jamir et al., 2004, effector delivery was performed using *Pf*55 (pHIR11) as well as with the pHIR11 derivative cosmid pLN18 (having a Km^R marker exchange mutation in the *schA*-*hopA* operon). Using *Pf*55 (pLN18) they were able to suppress the $HopA_{Pss61}$-dependent HR generated by *Pf*55 (pHIR11). They further used as a negative control strain *Pf*55 (pCPP2089) carrying a $HrcC^-$ derivative of pHIR11 which does not produce a functional TTSS apparatus (Jamir et al., 2004) (Fig. 3-1).

Our objective was to study the ability of individual *Pss* B728a effectors to trigger an ENA effect in the apoplast of beans. To achieve this goal we used *Pf*55 (pLN18) to deliver single *Pss* B728a TTSS effectors into bean plants and test the ability of each individual effector-expressing strain to induce an increased population size of the delivery strain. Our first test of the *Pf*55 delivery system was to validate our negative controls *Pf*55 (pLN18) and *Pf*55 (pCPP2089). We performed a "proof-of-concept" experiment showing that pLN18 and pCPP2089 constructs did not themselves stimulate growth of *Pf*55 (Fig. 3-2a). The growth of *Pf*55, *Pf*55 (pLN18) and *Pf*55 (pCPP2089) exhibited little growth in these 48hr-experiments while *Pss* B728a multiplied about 10,000 to 100,000-fold (Fig. 3-2a and b).

2) How much endophytic growth can a single effector produce?

We co-infiltrated different ratios of *Pf*55 and *Pss* B728a to determine the feasibility of our *Pf*55 (pLN18)–delivered single effector to stimulate its own growth by assessing how much enchanced nutrient/water acquisition (ENA) activity a given strain would need to produce in order to result in a measureable increase in population size. We also wanted to determine what would be the minimum number of bacteria that could still induce a measurable ENA effect in the apoplast. To this aim, we infiltrated into bean different ratios and concentrations of inoculum of *Pss* B728a and *Pf*55. In our co-infiltration scheme, *Pss* B728a cells would represent a strain capable of inducing nutrient/water release with a full set of effectors and *Pf*55, in turn, would constitute a non-inducing bacterial strain in which

the positive impact of higher apoplastic carbon source availability could be measured (i.e. target population for growth increase).

We tested 3 different ratios of these two strains and also varied the total number of infiltrated bacteria for one of the ratios (1:1000), in order to determine if there could be a population size threshold for detection of ENA activity (see Fig. 3-3a). Measurements of total population sizes in these syringe co-infiltration experiments at 48hpi revealed that *Pf*55 induced about 5-fold growth at a concentration of 10^6 cfu/ml in the presence of 10^3 cfu/ml *Pss* B728a, and grew 10-fold when mixed with 10^4 cfu/ml *Pss* B728a. However, coinoculation of 10^2 cfu/ml *Pss* B728a did not stimulate growth of *Pf*55 (Fig. 3-3b). Growth of *Pf*55 was not stimulated by 10^2 cfu/ml *Pss* B728a at any of the two ratios tested. This suggested that 10^2 cfu/ml *Pss* B728a were not enough cells to induce changes in the apoplast sufficient for stimulating growth of *Pf*55. Alternatively, such low population may be at the threshold measurability of our plate counting method (see Fig. 3-3c).

The co-inoculation at a ratio 1:1000 yielded ~5-fold increase in growth of *Pf*55 when 10^6cfu/ml *Pf*55 were used (10^6cfu/ml *Pf*55:10^3cfu/ml *Pss* B728a) but the ratio failed induce *Pf*55 growth when 10^7cfu/ml *Pf*55 was coinoculated with 10^4 cells/ml of *Pss* B728a, even though there had been a proportional increase in the concentration of pathogen (Fig. 3-3b). It is likely that the initial population of 10^7cfu/ml of *Pf*55 is too high to respond to the effects induced by such few pathogenic cells. A much higher concentration of *Pss* B728a (i.e. not proportional) may be needed to stimulate growth of large *Pf*55 populations. Thus the target *Pf*55 population should not be so high as to be unaffected by changes in the apoplast.

The conclusion that more apoplast-modifying strain (*Pss* B728a in this case as a full repertoire inducer) produces a greater growth of the target population (non-pathogen *Pf*55 in this case) was expected. However, because the population inducing higher ENA availability and the responsive population is the same when we use *Pf*55 (pLN18) to express effectors in the apoplast, we have two contradicting tendencies. On the one hand the *Pf*55 (pLN18) effector-expressing population should not be so high (i.e. 10^7 cfu/ml) as to make it difficult to see its increase; but at the same time should also not be so low (i.e. 10^2 cfu/ml) as to fail to produce a change in the apoplast large enough to induce growth. Thus, to test effector-expressing *Pf*55 (pLN18) strains we decided to use an inoculum level of 10^5-10^6 CFU/ml that arrived at a compromise between the two opposing requirements. By comparing the population size reached by *Pf*55 when inoculated alone at a concentration of 10^6cfu/ml with that when co-infiltrated with 10^4cfu/ml *Pss* B728a (a 1:100 ratio) (Fig. 3-3b), we further concluded that a *Pf*55 strain expressing a single nutrient release effector could be up to roughly ~100-times less effective than *Pss* B728a at inducing this multiplication effect and still result in a ~10-fold population size increase (or even up to ~1000-times less effective and still yield a measurable ~5-fold increase in growth of *Pf*55 (by comparing the growth of *Pf*55 inoculated singly at 10^6 cfu/ml with that when coinoculated with 10^3 cells/ml *Pss* B728a).

3) Secretion levels of *Pf*55 (pLN18)-effector$_{PssB728a}$ strains assayed by microscopic CD

The identification of some *Pss* B728a effectors that could cause plant CD when agroinfiltrated into beans and *N. benthamiana* plants (Vinatzer et al., 2006) was used to predict successful effector expression and translocation using the *Pf*55 delivery sytem. AvrPto1$_{PssB728a}$, HopAA1$_{PssB728a}$, HopM1$_{PssB728a}$ and HopAB1$_{PssB728a}$ are all known to produce plant CD in *N. benthamiana* while HopM1$_{PssB728a}$ and HopAB1$_{PssB728a}$ also induce CD in bean (Vinatzer et al., 2006). Thus, bean and *N. benthamiana* plants were syringe infiltrated with *Pf*55 (pLN18) strains expressing AvrPto1$_{PssB728a}$, HopAA1$_{PssB728a}$, HopM1$_{PssB728a}$ and HopAB1$_{PssB728a}$ to assess the production of macro and microscopic CD (Table 3-2). *Pf*55 (pHIR11) was able to induce a HopA-ShcA-dependent CD that was clearly visible macroscopically in beans within 17 hpi of inoculation with 10^8cfu/ml, and even when inoculated with 10^7cfu/ml at later times. This confirmed previous results by Jamir et al., 2004 in tobacco and *Arabidopsis* showing that *Pf*55 (pHIR11) was capable of delivering HopA to plants. The strain did not, however, produce CD in *N. benthamiana*, presumably because HopA is not recognized by this plant. We next used the effector delivery systems *Pf*55 (pLN18) and *Pf*55 (pCPP2089) carrying pBAV226Gm-HopM1$_{PssB728a}$, pBAV226Gm-HopAB1$_{PssB728a}$, pBAV226Gm-AvrPto1$_{PssB728a}$ and pBAV226Gm-HopAA1$_{PssB728a}$ to deliver each individual effector into bean and *N. benthamiana*. We did not observe any macroscopically evident CD produced by these strains or any other *Pf*55 (pLN18)-effector strains; even when very high levels of inoculum (>10^9cfu/ml) were infiltrated (Table 3-2). We thus decided to look for microscopic CD with these effector constructs, as evidence of their successful delivery into plants. Individual dead plant cells look very characteristically as individual shrunk and darkened cells (see *Pf*55 (pHIR11) picture in fig. 3-4). When all the cells in the tissue have died, the leaf section under the microscope has an appearance reminiscent of "burnt" tissue (see Fig. 3-4 picture of *Pto* DC3000). *Pf*55 (pLN18) expressing HopM1$_{PssB728a}$ and HopAB1$_{PssB728a}$ produced an infrequent but detectable microscopic CD in beans (data not shown) suggesting that the level of effector secretion, if proportional to the CD response, was probably low. CD in beans was effector-specific since it was absent when *Pf*55 (pLN18) expressing pBAV226Gm-AvrPto1$_{PssB728a}$ or pBAV226Gm-HopAA1$_{PssB728a}$ were used instead. When we looked for microscopic CD in *N. benthamiana*, the results were less evident and made interpretation very difficult (perhaps due to an increased thickness of the *N. benthamiana* leaf). Microscopic CD was difficult to see even in beans, since *Pf*55 and *Pf*55 (pLN18) alone (negative controls) caused some background phenotypic changes in the plant cells that made unhelpful to increase the inoculum concentration above 10^8 cfu/ml.

*Pf*55 (pCPP2089)-delivered HopM1$_{PssB728a}$ and HopAB1$_{PssB728a}$ were also able to induce microscopic CD, suggesting that either *Pf*55 (pCPP2089) strains were still capable of some protein secretion despite the mutation of *hrcC* in the cosmid carrying the TTSS cluster genes and/or that the presence of an endogenous TTSS in *Pf*55 could also be delivering small amounts of proteins. If the first scenario were expected then it was also possible that the functional *hopA-shcA* operon present in pCPP2089 might be delivered into beans and confound our results. We thus used exclusively *Pf*55 (pLN18) as negative control in subsequent microscopy and growth assay experiments. The delivery of some *Pto*

DC3000 effectors such as $HopE1_{PtoDC3000}$ and $HopG1_{PtoDC3000}$ with the *Pf*55 (pLN18) system produced a clear microscopic CD (Fig. 3-4). In summary, a subtle microscopic CD could be detected with strains expressing *Pss* B728a effectors known to generate CD in Agroinfiltration studies. CD was mild, however, and we thus hypothesized that this was due to a low level of expression/translocation of the proteins, and that we could increase expression (and thus the concomitant CD) by over-expressing TTSS regulators of the system.

Pseudomonas fluorescens SBW25 (*Pf* SBW25), a strain very closely related to *Pf*55, has a functional TTSS system of its own encoded by the *rsp*/*rsc* gene cluster (rhizosphere expressed secretion proteins) (Preston et al., 2001). In *Pf* SBW25, the overexpression of positive regulatory elements of the TTSS, RspL and RspR (homologs of HrpL and HrpR, respectively), enable this strain to generate a host-specific CD in *Nicotiana clevelandii*. Because *Pf*55 likely harbors an endogenously-coded *rsp*/*rsc* TTSS system similar to *Pf* SBW25 (G. Preston, personal communication), we over-expressed $RpsL_{PfSBW25}$ and $RpsR_{PfSBW25}$ in *Pf*55 to see if we could achieve higher levels of microscopic CD. Plasmids carrying *rspR* or *rspL* in a broad-host-range plasmid were mobilized into *Pf*55 (pHIR11) and we asked whether the trans-encoded activators could increase the expression/translocation of the HopA effector carried on pHIR11, as evidenced by a higher/faster macroscopic CD in beans (Table 3-3a). We used 10^7 and 10^9 CFU/ml because 10^7-10^8 CFU/ml is the level at which *Pf*55 (pHIR11) and *Pf* SBW25 (pHIR11) cause CD in tobacco leaves (Preston et al., 2001) (Table 3-3a). None of the control strains lacking expression of $HopA_{Pss61}$ showed any sign of CD except for a slight accentuated chlorosis of Pf (pLN18 + pRpsR) at the very high inoculum level of 10^9cfu/ml. Thus the CD induction for the HopA-expressing strains *Pf*55 (pHIR11), *Pf*55 (pHIR11 + pRpsR) and *Pf*55 (pHIR11 + pRpsL) are shown in Table 3-3b. CD ocurred faster and appeared more "confluent" in *rspL* and especially *rspR*-carrying strains (at 10^7 and 10^9 cfu/ml) as compared to *Pf*55 (pHIR11) alone (Table 3-3b). At 41 hpi, all three tested strains conferred a similar percentage of leaves with CD.

4) Non-suppressive *Pss* B728a effectors

We generated a list of candidate *Pss* B728a effectors to test, using information from the *Pseudomonas*-Plant Interaction (PPI) web site (http://pseudomonas-syringae.org/) created and maintained by Magdalen Lindeberg. The Hop Database at this website is a repository of information on hundreds of TTSS effectors and helper proteins grouped by effector family and known *Pseudomonas syringae* pathovar. The effectors on this table are categorized by several important features that are periodically updated: full sequence, Genbank accession, *hrpL* dependence, chaperone dependence, validation data with respect to secretion and translocation assays, references, etc. (Lindeberg et al., 2006). For *Pss* B728a, the current Hop database (dated 07-09-2010) included 15 effectors and 5 helper proteins on the Helpers database (dated 09-21-2009), including another 6 effectors that have been "discontinued" due to the absense of secretion or a "hrp-box" signal.

To identify TTSS effectors involved in ENA activity in plants we generated a list of candidate *Pss* B728a effectors (Table 3-4) using information from the Hop database

along with information on previously validated *Pss* B728a effector candidates (Greenberg and Vinatzer, 2003; Vinatzer et al., 2005; 2006). The small and reasonably well known effector repertoire of *Pss* B728a was an advantage for producing individual effector-carrying strains, making the *Pss* B728a repertoire more preferable than the larger, if albeit more thoroughly mined, TTSS repertoire of *Pto* DC3000.

Most of *Pss* B728a TTSS effectors were cloned, although not any of the helper proteins (harpins and the *hrpA* pilin gene). The original list of candidates contained only confirmed TTSS *Pss* B728a effectors known at the time to be lacking an established function in plant defense suppression. Thus, effectors AvrRpt2, HopX1, HopZ3, AvrE and HopM1 were not included on the list. Other effectors that were not found to be secreted or whose information as validated secreted effectors was lacking were also not included (HopAI', HopJ2, HopAH2 and HopAN1). HopAP1 was included since it is a likely candidate even though secretion could not be demonstrated in secretion assays with AvrRpt2 reporter fusions. The effectors we tested in our essays were taken from a study that confirmed secretion and translocation of several *Pss* B728a effectors (Vinatzer et al., 2006). They were cloned into *Pseudomonas syringae* expression vectors pBAV226Gm (pVS1 replicon) under the control of a constitutive *nptII* promoter and fused to a C-terminal HA tag. These single effector-carrying constructs were mobilized into the *Pseudomonas fluorescens* delivery systems and the growth of these strains in the bean apoplast was assessed.

We also tried to determine the putative subcellular localization of the effectors to ascertain their target and speculate on the mode of action they could have. We found that several effectors could putatively target the chloroplast. A cursory PSI-BLAST (Altschul et al., 1997) analysis of effectors was performed and the results obtained were very similar to that found using Phyre, an improved version of 3D-PSSM for protein function prediction based on homology to a database of proteins with known structure (Vinatzer et al., 2006).

5) Screening for ENA effectors in bean plants

A variety of *Pto* DC3000 and *Pss* B728a effectors representing different *Pseudomonas syringae* effector families were delivered into bean plants using *Pf*55 (pLN18) and tested for their ability to induce an increase in growth of this strain (Fig. 3-5). The effectors tested included plant defense suppressors (some of them even plant CD inducers). All the effectors were expressed from a second plasmid under the control of a constitutive P*nptII* promoter, with the exception of $HopA_{Pss61}$ whose operon $hopA1_{Pss61}$-$schA_{Pss61}$ was expressed *in cis* from its native promoter on pHIR11 (Fig. 3-1b). Except for $HopF1_{PtoDC3000}$ and $HopA1_{Pss61}$ constructs which carried their cognate chaperones (Shan et al., 2004; van Dijk et al., 2002) only the effectors were expressed.

While most of the *Pf*55 (pLN18)-effector constructs produced generally lower population levels than *Pf*55 (pLN18) itself at 48hpi, *Pf*55 (pLN18) expressing $HopF1_{PtoDC3000}$ and *Pf*55 (pHIR11) expressing $HopA1_{Pss61}$ had higher population sizes than the other effector strains in two independent experiments (Fig. 3-5) and were higher than

*Pf*55 (pLN18) itself in one experiment (data not shown). In vitro growth assays confirmed that *Pf*55 (pLN18)-effector strains and *Pf*55 (pLN18) itself had similar growth rates. We thus concluded that we were able to detect the growth benefit caused by individually expressed effectors in the *Pf*55 (pLN18) delivery system as an increase in apoplastic growth. We thus continued to test *Pss* B728a effectors from our list of candidates (Table 3-4). We performed several growth assays in beans with *Pf*55 (pLN18)–effectors$_{PssB728a}$ utilizing both syringe and vacuum infiltrations and by varying the conditions of the plants after inoculation, especially with respect to the level of moisture/drought stress.

HopAH$_{PssB728a}$ and HopAF1$_{PssB728a}$ were attractive candidates for ENA activity since they were predicted by bioinformatics homology-based predictors like PSORT to contain putative chloroplast transit peptides, which suggested that their function may involve the modulation of the synthesis and/or transport of sugars and starch reservoirs from plant cells. HopAH1$_{PssB728a}$ is a 427 aminoacid protein encoding a putative glycosyl-hydrolase that belongs to the three-gene HopAG1 operon *hopAG1-hopAH1-hopAI'*. *Pto* DC3000 has most of its effectors in operons (Kvitko et al., 2009) but *Pss* B728a has only the HopAG1 operon aside from the CEL and EEL effectors flanking the *hrp*/*hrc* cluster. In *Pto* DC3000, *hopAG1* is interrupted by a transposon insertion that is predicted to truncate the HopAG1$_{PtoDC3000}$ protein and may have a polar effect on the expression of downstream genes *hopAH1*$_{PtoDC3000}$ and *hopAI1*$_{PtoDC3000}$ (Greenberg and Vinatzer 2003; Schechter et al. 2004). In *P. syringae* pv. *syringae* B728a, the third gene *hopAI*$_{PssB728a}$ harbors an early STOP codon (Greenberg and Vinatzer 2003). HopAF1$_{PssB728a}$ (previously known as HopPtoJ) is a 912 amino-acid protein with homologs in *Xanthomonas* spp. and *P. agglomerans* pv. *gypsophilae*. HopAF1$_{PtoDC3000}$ was found to suppress flagellin-induced PTI in *Arabidopsis* (Li et al., 2005).

In some of our growth assay experiments, we were able to detect higher population sizes mediated by effectors HopAH1$_{PssB728a}$ and HopAF1$_{PssB728a}$ (i.e. Fig. 3-6) but not in others (data not shown). In one of the experiments, we syringe infiltrated water stressed bean plants with *Pf*55 (pLN18) expressing HopAH1$_{PssB728a}$ and HopAF1$_{PssB728a}$. We inoculated a cell concentration of 10^4 and 10^6 CFU/ml (fig. 3-6a and b). *Pf*55 (pLN18)-*hopAH1*$_{PssB728a}$ produced higher population levels than *Pf*55 (pLN18) and reached a cell density nearly as high as *Pss* B728a by 48hpi. In this experiment, the lowest initial inoculum concentration resulted in the largest differences in population sizes as compared to the negative control (Fig. 3-6a and b). Other tests of these effectors performed with plants under different drought conditions (desiccation stress before and/or during the experiment, as well as with normally watered plants) produced varying results but never as dramatic as that seen in Fig. 3-6. For example, in one such experiment we took normally-grown greenhouse bean plants with small trifoliate leaves and deffered watering for 8 days. The plants were then watered once the day before and once after infiltration with bacteria in order to maintain the health of the plants even though the soil was dry. The experiment was designed to mimic the conditions in the experiment where HopAH1$_{PssB728a}$ and HopAF1$_{PssB728a}$ were found to stimulate growth. We thus infiltrated *Pf*55 (pLN18) strains expressing those two effectors and also infiltrated HopAP1$_{PssB728a}$ and HopAM1$_{PssB728a}$ (Fig. 3-7). HopAM1$_{PtoDC3000}$ (previously known as AvrPpiB1) had been

recently shown to enhance the virulence of a weak pathogen (*Pseudomonas syringae* pv. *maculicola* M6CΔE) in *Arabidopsis* exposed to drought stress (Goel et al., 2008). However, under these stressed conditions we did not observe growth of the effector-containing *Pf*55 strain to be more than *Pf*55 (pLN18) itself (Fig. 3-7).

Because we expected effectors to perhaps induce a higher nutrient availability in the apoplast by inducing some degree of water-soaking (much in the same way water-soaked lesions are transiently seen with *Pss* B728a and *Pseudomonas syringae* pv. *phaseolicola* in leaves and pods) we decided to perform growth assays in plants under high moisture conditions. Bagging of plants had aided the formation of water-soaked lesions in previous studies (Rudolph, 1978). Our rationale was that if single effectors are somewhat inefficient in inducing water congestion, such an effect would be accentuated in plants with a high moisture content in the leaves. We thus vacuum infiltrated *Pf*55 (pLN18) expressing $HopAH1_{PssB728a}$, $HopAE1_{PssB728a}$, $HopAJ2_{PssB728a}$, and $HopAM1_{PssB728a}$ as well as *Pss* B728a to normally watered plants. After vacuum infiltration and normal drying of the infiltrated area, the pots where covered with transparent bags to maintain humid conditions. One set of infiltrated plants was covered with bags after the initial water-soaking disappeared and the other set was left uncovered and allowed to dry (Fig. 3-8). We saw no significant differences in population size at 48 hpi, between the bagged and unbagged treatments. In fact, lower numbers of cells of all strains were recovered after 48 hpi than was initially inoculated.

Because *hopAH1* had given a higher population growth increase on several occasions, we disrupted the gene in *Pss* B728a and performed several growth assays with the mutants compared to wild type *Pss* B728a and found no significant difference in growth. We also co-inoculated the mutant with wild type *Pss* B728a in beans, and observed only modestly lower growth of the mutant strain compared to the wild type strain in the mixtures (data not shown).

Reasoning that some effectors might stimulate more growth when expressed together in the apoplast we tried co-inoculating mixtures of effector-expressing *Pf*55 (pLN18) strains. This scheme would have the added benefit of letting us test groups of effectors under varying environmental conditions. We hoped to observe additive and/or synergistic effects of mixtures *Pf*55 (pLN18)-effector strains, however, we were also aware of possible negative effects (even dominant effects) that any one *Pf*55 (pLN18)-effector strain might confer to a group of strains. If positive effects would predominate, though, we could then dissect the groups and further test effectors individually or in smaller groups for their growth promoting activity. As all random combinations would be too laborious to test, we examined only some possible groupings (Fig. 3-9a) and found one of these groups to exhibit about ~10-fold more growth than *Pf*55 (pLN18) itself in one experiment (Fig. 3-9b). However, when we dissected the group and tested the *in planta* growth of individual strains, we could not reproduce the growth increase with any given effector (data not shown). It is possible that an interaction between effectors had led to the growth stimulation seen in the mixture.

6) Screening for ENA activity effectors in bean pods

Maintained watersoaking conditions in leaves is sufficient to induce growth of non-pathogenic strains (Chapter 2). *Pss* B728a produces water-soaking lesions in bean pods that is likely an ENA mechanism. We thus decided to screen individual *Pf*55 (pLN18) effector-expressing strains for their ability to induce watersoaking lesions in pods. We syringe infiltrated detached bean pods with the effector expressing strains as well as with the control strain *Pss* B728a, *Pss* B728a HrpJ$^-$ and HrcC$^-$ and *Pf*55 (pLN18) and inoculated the pods under 100% relative humidity at room temperature. The water-soaked lesions were scored 5 dpi and as expected wild type *Pss* B728a produced water-soaking zones that were ~3-4mm in diameter (when inoculated at 10^7cfu/ml but more reliably at inoculum concentration of 10^8cfu/ml and above) (Fig. 3-10). The *hrp* mutants produced smaller but detectable water-soaked zones and *Pto* DC3000 produced no water-soaking but did produce small necrotic lesions at the site of infiltration that were similar in appearance to the localized necrotic CD produced in leaves. Water-soaking was absent from *Pf*55 (pLN18) and all effector-producing *Pf*55 (pLN18) strains we tested.

7) Screening for ENA effectors with *Pf*01 (EtHAn)

We obtained another delivery system based in *Pf*01 (kindly provided by J. H. Chang). Chang and coworkers created EtHan (Effector to Host Analyzer), a new TTSS effector delivery system that harbours the *hrp*/*hrc* cluster from *Pss* 61 integrated into the genome of *Pf*01 (Thomas et al., 2009). The stable integration of the TTSS genes presumably makes this strain a more efficent and stable TTSS delivery system.

We tested this strain in several plant hosts and found that apart from its reported CD induction in tomato and tobacco (Thomas et al., 2009), it also conferred a strong macroscopic CD in beans that was evident at 20hpi as a confluent HR at high inoculum concentration (10^9 CFU/ml) but also present as a patchy CD at lower cell concentrations (10^8 cfu/ml) (Table 3-5a). In *N. benthamiana* EtHAn gave a mild but clear CD in about a quarter of the leaves and by 3 dpi EtHan had produced a delayed CD in almost two thirds of the leaves (Table 3-5b). CD was absent with *Pf*01 in this experiment, suggesting that they were caused by the TTSS cluster genes present in EtHAn. However, in a subsequent experiment, *N. benthamiana* plants gave a mild CD response to *Pf*01 itself as well (Table 3-7), making it unclear whether the CD in this particular host was EtHAn (i.e. HrpZ) dependent or *Pf*01-dependent. When we performed syringe infiltrations in *Arabidopsis* Col-0, we found that *Pf*01 by itself yielded some CD at 24hpi that was similar to that caused by EtHAn (Table 3-5c). Thus even though the TTSS cluster in EtHAn was not responsible for inducing CD in *Arabidopsis* (as reported by Thomas et al., 2009), in our hands *Pf*01 was nevertheless inducing some CD in this plant species. In another experiment in *Arabidopsis* in which CD was scored earlier (17 hpi), we found that while less CD was obtained, it was conferred by all *Pf*01 strains including a HrcC$^-$ EtHAn derivative. However a strain of EtHAn expressing the effector AvrRpt2 (EtHAn (pVSP61-AvrRpt2)) conferred, as expected, a much higher CD in *Arabidopsis* that was well above that conferred by *Pf*01 (Table 3-6). We thus confirmed that EtHAn is able to secrete effectors into plants and that the strain induces a CD in several plant hosts including bean, tobacco, tomato and possibly also *N. benthamiana*.

Because of its propensity to induce CD, the utility of EtHAn as a delivery system for detection of nutrient/water acquisition in the apoplast was of limited use. However, we decided to mobilize the pBAV226Gm-effector$_{PssB728a}$ constructs into EtHAn and measure the growth of some EtHAn (effector) strains in *N. benthamiana*. This plant host was utilized since the CD we had observed was always mild and generally occurred only in plants inoculated with very high inoculum concentrations (Table 3-5b). We thus measured growth of EtHAn carrying effectors that had previously stimulated population growth of *Pf*55 (pLN18) strains that delivered effectors such as HopAH1$_{PssB728a}$ and HopAF1$_{PssB728a}$ (Fig. 3-6). However, we could detect no growth of these effector-containing strains (Fig. 3-11), confirming previous results obtained with EtHAn. EtHAn (AvrPto1) (Thomas et al., 2009) and other individual *Pto* DC3000 effector-expressing EtHan strains were also unable to multiply more than EtHAn itself in *Arabidopsis* (J.H. Chang, personal communication).

In our experiment, the population size of EtHAn-harboring AvrPto1$_{Pss\ B728a}$ at 9 dpi was higher than that of EtHAn itself as well as that of the other EtHAn-effector expressing strains, but we believe this to be the result of the method of infiltration we utilized in this experiment (Fig. 3-11). Different strains were infiltrated individually on opposite sides of the midvein of *N. benthamiana* leaves. EtHAn (AvrPto1) was infiltrated opposite to *Pss* B728a. The apoplast-modification changes that the pathogen likely introduced were probably enough to benefit EtHAn (AvrPto1) after 9 days even though it was on the other side of the leaf.

8) Screening for ENA activity effectors with *Pf*01 (AMELIA)

The CD induced by EtHAn strains was suspected to be caused by the helper protein HrpZ that is encoded by the *Pss* 61 TTSS gene cluster. TTSS helper proteins have been shown to be secreted by the TTSS themselves and aid in the delivery of effectors into plants (Petnicki-Ocwieja et al., 2005). We obtained an EtHAn-derived strain named AMELIA (A Modified EtHAn-like Injection Apparatus) where the *hrpZ* gene had been disrupted (a kanamycin resistance cassette (Km^R) was added to a deletion-containing variant and subsequently removed by flp-dependent excision of the Km^R marker) (a gift by W. Thomas and J.H. Chang). We performed several studies to determine whether this AMELIA strain no longer produced a CD phenotype and if it was still capable of delivering effectors into plants since the TTSS helper HrpZ was no longer present. The absence of HrpZ and the presence of a functional TTSS cluster in the vector was confirmed by PCR, we therefore tested for its ability to induce CD in bean as evidence of secretion competency. AMELIA produced no CD in beans just like *Pf*01 itself (and unlike EtHAn that produced a strong CD in beans) (Table 3-7). In *Arabidopsis* and *N. benthamiana* AMELIA also behaved in a similar manner to *Pf*01, producing a lesser incidence of CD (about 50% in *Arabidopsis* and 30% in *N. benthamiana*).

Next we wanted to determine that the TTSS cluster in AMELIA would not itself stimulate endophytic growth and second that the strain would respond to higher apoplastic nutrient availability by growth. This last question was especially relevant since AMELIA is a *Pf*01 derivative; as a soil bacterium it is not adapted for growth on leaves and it strongly induces PTI (Thomas et al., 2009). We thus determined the apoplastic growth of

AMELIA with two treatments that should have simulated high apoplastic nutrient concentrations in bean (Fig. 3-12). *Pf*01, EtHAn as well as AMELIA all multiplied similarly in bean when vacuum infiltrated and did not multiply significantly unless fructose was added to the inoculum or apoplastic water-congestion was maintained. This confirms that the TTSS present in AMELIA does not stimulate growth by itself and, importantly that the growth of this PTI-inducing strain could increase with higher apoplastic nutrient/water-availability.

We next asked whether AMELIA could secrete and translocate effectors into plants. We thus syringe infiltrated high concentrations of AMELIA, AMELIA (AvrRpt2) and AMELIA (AvrRrpm1) carrying pVSP61-derived constructs with effectors AvrRpt2 and AvrRpm1 (that are recognized in a gene-for gene manner) into *Arabidopsis* Col-0 and subsequently scored for CD results. We also infiltrated AMELIA (pLH12) carrying AvrRpt2 in a different plasmid vector and pLH12-Ω with a Sp^R insertion inactivating AvrRpt2 as a negative control (Whalen et al., 1991) as well as *Pss* B728a and *Pto* DC3000 controls. The CD assessed at 16 hpi induced by AMELIA strains differed in *Arabidopsis* and bean (Table 3-8). While AvrRpt2 and AvrRpm1-expressing AMELIA caused effector-dependent CD, they did not incite any macroscopic symptoms in bean leaves. In contrast, *Pto* DC3000 caused a strong CD in non-host bean as noted before while *Pss* B728a caused no virulence symptoms at this early stage. However, the high inoculum level (10^9 cfu/ml) used did not allow us to differentiate CD caused by virulent versus avirulent strains in *Arabidopsis*. Nevertheless, we could confirm that AMELIA can translocate effectors via its TTSS by the results we obtained in *Arabidopsis*, even though AMELIA (AvrRpt2) and (AvrRpm1)-generated CD was not evident macroscopically in bean.

Pss B728a effector constructs in plasmid pBAV226Gm were next mobilized into AMELIA and growth of these strains was assessed in bean (Fig. 3-13). By 24 hpi the population size of all AMELIA strains had dropped. The strain AMELIA (pVSP61) was used as a negative control since it carries the same origin of replication (pVS1) as effector-carrying plasmid pBAV226Gm. AMELIA (pVSP-AvrRpt2) and AMELIA (pVSP61-AvrRpm1) were also infiltrated. None of the effectors tested including even AvrRpt2 and AvrRpm1-expressing strains (capable of inducing a clear CD in *Arabidopsis*), stimulated measureable growth. Thus the growth stimulation conferred by single effectors is apparently very limited and could not be detected under our experimental conditions. We therefore decided to assess growth that might be stimulated by grouping all of the effector-expressing AMELIA strains together. However, instead of assessing stimulation of the collection of AMELIA strains, we instead monitored growth of a different species (*Pa* 299R) which we had added to the mixture. We distinguished the effector-expressing strains (grouped AMELIA (effector)) and the target populations (*Pa* 299R) so we could better assess the effector-producing and nutrient-responsing functions of the two different bacterial populations (Fig. 3-14). Thus, as suggested by earlier experiments with co-infiltration of *Pss* B728a (as effector-expressing) with *Pf*55 (responsive population) (See Fig. 3-3), we could infiltrate a very high concentration of the group of AMELIA (effector) (each effector-producing strain was present at 10^7 cfu/ml), with a low number (10^5 cfu/ml) of the responsive *Pa* 299R strain so as to be able to assess

the population growth. Twelve effector-expressing AMELIA strains were grouped to yield aprox. 1.2 x 10^8 cfu/ml that were vacuum infiltrated alone (AMELIA (12 effectors)) or co-infiltrated with 10^5 cfu/ml of *Pa* 299R (AMELIA (12 effectors) + *Pa* 299R). The co-infiltrated population was plated on two different selective media to distinguish AMELIA and *Pa* 299R populations. In two out of three similar experiments on beans we obtained a modest overall increase in growth for *Pa* 299R in the presence of the mixture of AMELIA strains, but at 48 hpi there was also a 10-fold higher population size of *Pa* 299R in the presence of the mixture of effector-producing strains than in their absence (one such experiment is shown in Fig. 3-14). No difference in growth of *Pa* 299R in the presence and absence of the mixtured AMELIA strains was observed in *N. benthamiana*. This experiment was performed only once in *Arabidopsis,* revealing modest increases in growth of *Pa* 299R. This study should be repeated to verify this phenomenon in *Arabidopsis*.

Discussion

We previously found that the availability of carbon sources play a central role in the ability of bacteria to grow in the apoplast of leaves. Those experiments showed that TTSS effectors are involved in processes that make nutrients more available to pathogens in the apoplast. Addition of exogenous carbon source, maintaining of apoplastic water congestion, and co-inoculation with a TTSS-proficient pathogen all can enable extensive apoplastic multiplication of non-pathogens. Thus, there are likely some TTSS effectors in each pathogen's repertoire whose presence contributes to their endophytic growth in plants by enhancing nutrient and/or water acquisition (ENA). Moreover, ENA activity should enable growth of bacteria in both host and non-host plants unless their multiplication is arrested by, for example, an ETI-associated HR. Conversely, non-pathogenic strains would be unable to multiply endophytically due to an absence of TTSS effectors conferring ENA activity.

Unrelated effector proteins often redundantly target similar plant pathways to achieve the same function (Kvitko et al., 2009). Similarly we believe that there are redundant TTSS effectors inducing ENA. That is, there must be more than one functionally redundant ENA effector in each pathogen's genome as otherwise their presence would have been discovered in earlier transposon mutagenesis studies of bacterial fitness. Also, it is entirely possible that different unrelated ENA effectors might be present in the TTSS repertoire of a given pathogen.

TTSS effectors are in many cases large multi-domain proteins that can target multiple host proteins (Chisholm et al., 2005; Munkvold and Martin, 2009). The possibility that even suppressors of plant defenses might have ENA activity prompted us to revise our original decision to exclude effectors that target the plant defense system. We thus developed a full repertoire list, including "helper" proteins that are thought to function in the apoplast, as well as $AvrE1_{PssB728a}$ and $HopM1_{PssB728a}$, well conserved effectors residing in the CEL near the *hrp*/*hrc* cluster. *Pto* DC3000 CEL mutants were shown to be impaired in growth *in planta* and expression of either $AvrE1_{PtoDC3000}$ or $HopM1_{PtoDC3000}$ alone could restore the ability of the CEL mutant to multiply (Badel et al., 2006; Kvitko et al., 2009).

In the present work we sought to find ENA effectors in the genome of *Pss* B728a, a well characterized pathogen with a small and reasonably well established TTSS repertoire (Feil et al., 2005). We used a gain of function strategy utilized before to study individual effectors where the TTSS cluster genes are expressed in a non-pathogenic strain such as *Pf*55 or *Pf*01 enabling the delivery of effectors (Jamir et al., 2004, Guo et al., 2009, Thomas et al., 2009). Unlike previous studies that focused on observing a phenotype of suppression of plant defenses in high inoculum HR assays, we looked for a phenotype of increased population growth of the delivery agent itself when inoculated at low inoculum concentration. Evidence of growth of the strain might be more difficult to obtain than phenotypes that are based on amplified responses such as the HR. However, growth assays are ultimately a measure that unequivocally shows the relevance of any factor positively impacting bacterial fitness. For example, EtHAn (AvrPto1) successfully induces a PTI-dependent reduction in callose formation, a factor that should positively impact the

growth of this strain if PTI negatively affected apoplastic growth. However, EtHAn (AvrPto1$_{PtoDC3000}$) failed to grow more than EtHAn itself (Thomas et al., 2009), suggesting that PTI is not a major factor limiting growth in the apoplast.

Our choice of a non-pathogenic strain as an effector delivery agent was designed to avoid other virulence factors that are likely present in other delivery systems such as *Agrobacterium tumefaciens*. Unlike *A. tumefaciens*, the *P. fluorescens* delivery systems use a route and a lower level of protein secretion that more closely resembles the conditions during natural infection in contrast to the great overexpression of effectors that takes place in transgenic plants (Guo et al., 2009). However the *P. fluorescens* delivery systems also probably have lower delivery efficiency than that of the pathogen themselves.

Unexpected variability in induction of CD by effectors delivered by *Pf*55 (pHIR11) raised questions of inefficiency in delivering effectors. HopA1$_{Pss61}$ as expressed from *Pf*55 (pHIR11) triggers a macroscopic HR in tobacco and *Arabidopsis* (Jamir et al., 2004). In our experiments, we also found *Pf*55 (pHIR11) to produce a macroscopic *hopA1$_{Pss61}$*-dependent HR in bean but not in *N. benthamiana*. However, no other *Pto* DC3000 and *Pss* B728a effectors tested were able to produce macroscopic CD in beans or *N. benthamiana* even when very high numbers of bacteria were infiltrated (Table 3-2). This was puzzling because some *Pss* B728a effectors had been shown in previous studies to induce CD when transiently expressed in plants using Agroinfiltrations. Our results with macroscopic CD show that the effectors are indeed secreted but hint at a low level of expression/translocation as compared to Agroinfiltrations. When we examined microscopic CD induced by HopM1$_{PssB728a}$ and HopAB1$_{PssB728a}$-expressing *Pf*55 (pLN18) strains we found they were only able to induce a subtle but detectable microscopic CD in bean when very high inoculum densities were infiltrated. This microscopic CD was induced by HopM1$_{PssB728a}$ and HopAB1$_{PssB728a}$ but absent in plants infiltrated with *Pf*55 (pLN18) expressing AvrPto1$_{PssB728a}$ and HopAA1$_{PssB728a}$ in bean, as expected; however the CD induced by *Pf*55 itself when infiltrated at 10^9cfu/ml often made it very difficult to observe.

We thus tried to determine how efficient our effector delivery system would need to be to produce a detectable amount of growth (in other words whether our strategy could work) (Fig. 3-3abc). Co-infiltration *Pf*55/*Pss*B728a enabled us to determine the concentration and ratio of both the ENA-producing strain (*Pss* B728a) and nutrient-responsive (measured) bacterial population to use. Originally we utilized the *Pf*55 delivery strains as both effector delivery agent and nutrient-responsive population. This forced us to reach a compromise between the need for high numbers of effector secretion and a sufficiently low number of responsive bacteria so that growth could be detected. Later, with AMELIA (effector)-producing strains we differentiated the high concentration of effector producers (AMELIA (effector)) from low concentration of nutrient responsive *Pa* 299R. This later strategy appeared to be more successful, probably because *Pa* 299R can consume sucrose, a nutrient we would expect to be made available in the ENA response. Since *P. fluorescens* cannot consume sucrose, *Pa* 299R would selectively benefit from sucrose release.

The growth benefit caused by individually expressed effectors in the *Pf*55 (pLN18) delivery system was sufficient to produce a measureable population growth in the apoplast. However, in most cases the *Pf*55-delivered effectors produced neither a growth increase nor a macroscopic CD, suggesting that it achieved only a low level of protein delivery to the plant. The level of CD is not an exact reflection of the level of protein delivered to the plant because effector function is not solely dependent on its level of expression, secretion and translocation but also on its stability in both the bacteria and the plant, as well as the level of recognition by the plant (which in turn is influenced by environmental factors) (Vinatzer et al., 2006). However, several lines of evidence suggest that a low level of protein was delivered via TTSS pilus with the *Pf*55 (pLN18) delivery system. The fact that no microscopic lesions were conferred by the system except for the only effector expressed *in cis* from pHIR11 itself ($HopA_{Pss61}$) suggests that effectors expressed *in trans* in the Gm^R plasmids are being lost, or not efficiently expressed or translocated. The first hypothesis is not likely since pVS1-based plasmids are considered stable (Joyner and Lindow, 2000). Thus, the effectors expressed from the pVS1-based plasmids under the control of the *nptII* promoter are likely not being effectively targeted to the TTSS pilus. The presence of a chaperone encoded on pHIR11 could have helped direct $HopA_{Pss61}$ to the TTSS apparatus and thus enabled efficient secretion and the macroscopic CD formation observed here. The other effector that was expressed with its chaperone, $HopF1_{PtoDC3000}$, was interestingly one of the two (along with $HopA_{Pss61}$) effectors that induced higher population growth of *Pf*55 (pLN18). This made us wonder if the reason for its higher growth was mainly due to its more efficient targeting to the TTSS needle. The fact that the co-expression of the *rpsL*/*R* SBW25 positive TTSS regulators facilitated microscopic CD detection of *Pf*55 (pHIR11)-dependent CD in beans also suggests that low level of expression and/or targeting of effectors to the TTSS needle occurred in an *Pf*55 (pHIR11) system. Alfano and coworkers found that over-expression of the effector itself could partially overcome the lack of a chaperone (van Dijk et al., 2002). In a similar way, up-regulation of TTSS cluster genes may partially overcome poor targeting of the proteins to the TTSS apparatus. It is important to point out that we do not know whether *rpsL*/*R* over-expression modulates $hrpL_{Pss61}$ and *hrp*/*hrc* gene expression and/or endogenous expression of *Pf*55 $rspL_{Pf55}$ *rsp*/*rsc* genes. Thus we cannot determine if such changes were through increased expression of the TTSS machinery genes or increased effector expression itself.

Considering the possibility that effectors could act in a coordinated fashion to target specific functions and/or have small additive effects, we grouped some effector-expressing strains and tested their growth *in planta* where we varied the environmental conditions (moist/dry) before and during experiments (fig. 3-6, 3-7, 3-8). In experiments with the *Pf*55 delivery system, we observed a limited effect of this strategy. For example, in some experiments, the population sizes recovered of *Pf*55 (pLN18 + pHopAH) were higher than that of *Pf*55 (pLN18) itself. However, a *Pss* B728a *hopAH* mutant was not significantly impaired in its growth in bean. We observed a small reduction in fitness when the mutant was co-inoculated with *Pss* B728a (data not shown). It is likely, however, that the wild type pathogen would have complemented the impaired growth of the mutant in such mixed inoculum competition assays, thus obscuring small differences in endophytic multiplication. A more successful strategy could be the knock out of the whole

HopAG1 operon followed by a test for decreased fitness of the mutant in growth assays in host and non-host plants.

The fact that none of the effector-secreting *Pf*55 strains produced macroscopic watersoaked lesions was not surprising. Experiments in leaves showed that even under moist conditions we could not induce higher growth of *Pf*55 (pLN18) effector-expressing strains. If water-soaking was sufficient to induce growth then the lack of growth would suggest that water-soaking had not occurred. It is also important to note that *Pss* B728a HrpJ$^-$ can produce water-soaking lesions, if albeit reduced, suggesting that its ability to induce water-soaking is probably due to some factor/s that are not TTSS dependent. However, if TTSS effectors confer water-soaking ability we could discover it with a sensitive assay that could monitor local changes in water potential in the immediate vicinity of bacterial cells. For this purpose, a new biosensing strain that responds to increasing water availability in the apoplast could be made and either co-infiltrated with *Pf* (effector)-producing strains or the reporter gene construct responsive to high water availability could be introduced into the effector delivery strain itself.

The possibility that EtHAn (and later AMELIA) was a more stable effector delivery system prompted us to test it. The most promising results were obtained with the AMELIA delivery system. Unlike EtHAn, we found evidence that AMELIA produces no HrpZ-dependent CD in plants but is still able to deliver effector proteins. However single effector delivery by either EtHAn or its derivative AMELIA proved to be just as unsuccessful as the *Pf*55 effector-delivery strains at inducing growth of the delivery strains in the apoplast. Either the amount of change in the apoplast that a single effector can produce is too small to measure in our growth assays or effectors cooperate to mediate plant changes. However, it could also be that, as shown in our early experiments with *Pf*55-*Pss*B728a co-infiltrations, single effector-delivery strains inoculated at 10^5-10^6 cfu/ml might not be achieving sufficiently high density to produce a measurable effect in the apoplast (see Fig. 3-3). Thus we decided to increase the inoculum size of AMELIA-effector expressing strains, and separately measure a different target population (the non-pathogenic strain *Pa* 299R) that was co-inoculated at a lower concentration. We only performed one growth assay with this scheme of dual species. In this experiment we grouped together AMELIA strains expressing individual effectors and asked whether this group could generate measureable ENA activity in the apoplast. Calculations of the number of bacteria and plant cells in an infiltrated area indicated that infiltration of 10^7cfu/ml bacterial cells would establish an effector-producing AMELIA cell for each plant cell. In the presence of a mixture of twelve effector-expressing AMELIA the growth size of *Pa* 299R in bean was higher than in the absence of it. It might be possible that some effectors function additively and/or cooperatively when delivered into the same plant cell by different bacteria. It remains to be seen, however, whether this growth increase is due to a nutrient/water acquisition effect. We will then next perform in planta growth assays with individual AMELIA (effector)-expressing strains at this high inoculum level and will co-infiltrate them with *Pa* 299R (whose population we will monitor). We should perform such assays in both host and non-host plants since it has been suggested that different effectors in a given pathogen's repertoire are important for the growth in different hosts

and even non-host plants (Vinatzer et al., 2006), and thus the molecular targets for effector-mediated nutrient and/or water acquisition are likely conserved in different plant species.

Table 3-1 - List of bacterial strains and plasmids used in this work.

Strain or plasmid	Characteristics*	Reference or source
Escherichia coli DB3.1	*F⁻ gyrA462 endA1 Δ(sr1-recA) mcrB mrr hsdS20 (r_B^-, m_B^-) supE44 ara-14 galK2 lacY1 proA2 rpsL20* (Sm^r) *xyl-5 λ⁻ leu mtl-1*	Invitrogen, Carlsbad, CA, U.S.A.
Escherichia coli TOP10	*Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu)*	Invitrogen, Carlsbad, CA, U.S.A.
P. syringae pv. *syringae* B728a	Wild type isolated from Phaseolus vulgaris (snap bean) Rif^r	Loper and Lindow, 1987
*Pss*B728a HrpJ-	hrpJ::ΩSpec; T3SS-deficient	Hirano *et al.,* 1999
P. syringae pv. *tomato* DC3000	Wild type; spontaneous Rif^r	Cuppels 1986
P. syringae pv. *tomato* DC3000 HrcC$^-$	*hrcC* mutant defective in Type III secretion, Cm^r	A. Collmer / J. Alfano
Pantoea agglomerans 299R	Rif^r	Brandl et al., 1996
Pseudomonas fluorescens 55	Nal^r	M. Sasser
Pseudomonas fluorescens SBW25	Wild type	G. Preston
Pseudomonas syringae pv. *tabaci* 11528	Isolated from *N. tabacum*	Brian Staskawicz
Pseudomonas syringae pv. *maculicola* ES4326	Wild-type pathovar *maculicola*	F. M. Ausubel
Pf01	Cm^r	J. H. Chang
Pf01(EtHAn)	Genomic integration of *hrp/hrc* cluster of *Pss* 61 Cm^r	Thomas et al., 2009
Pf01(AMELIA)	*hrpZ*- derivative of EtHAn, Cm^r	W. Thomas and J.H. Chang
pBluescript-II KS+	Cloning vector, Ap^r	Stratagene, La Jolla, CA, U.S.A.
pENTR/D-TOPO	Gateway system entry vector, Km^r	Invitrogen. Carlsbad, CA, U.S.A.
pRK2013	IncP Tra RK2+ ArepRK2 repEI, 7, Km^r	Ruvkin and Ausubel (1981)
pDONR207	Gateway^{TM} cloning vector	Invitrogen (Carlsbad, CA)
pHIR11	Cosmid pLAFR3 derivative carring *hrp/hrc* cluster of *P. syringae* pv. *syringae* 61, Tc^r	Huang et al., 1988

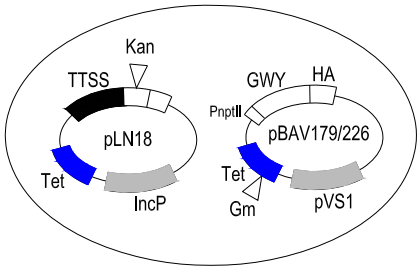
pLN18	pLAFR3 derivative containing 25-kb P. syringae pv. syringae 61 hrc-hrp cluster with shcA and hopPsyA replaced by an nptII cassette, Tcr Kmr	Jamir et al., 2004
pCPP2089	pHIR11 derivative containing TnphoA insert into hrcC, Tcr, Km^r	Huang et al., 1991
pML123	Broad-host-range cloning vector, Gm^r Km^r	Labes et al., 1990
pML123-*rspR*	pML123 carrying *rspR*	Preston et al., 2001
pML123-*rspL*	pML123 carrying *rspL*	Preston et al., 2001
pCPP5040	pML123-hemagglutinin (HA) derivative gateway destination vector, Gm^r Cm^r	J. Alfano
pBAV179	Gateway™ *P. syringae* expression vector (*nptII* promoter and C-terminal HA-tag)	J. Greenberg
pBAV226	Gateway™ *P. syringae* expression vector (*nptII* promoter and C-terminal HA-tag)	J. Greenberg
pCPP5040-*hopF1*/*F2*	pML123 derivative containing *hopF1-shcF2-ha*, Gm^r	Jamir et al. 2004
pCPP5040-*hopE1*	pML123 derivative containing *hopE1-ha*, Gm^r	Jamir et al. 2004
pCPP5040-*hopG1*	pML123 derivative containing *hopG1-ha*, Gm^r	Jamir et al. 2004
pCPP5040-*hopAM1*	pML123 derivative containing *hopAM1-ha*, Gm^r	Jamir et al. 2004
pCPP5040-*avrPtoB1*	pML123 derivative containing *avrPtoB1-ha*, Gm^r	Jamir et al. 2004
pBAV226Gm	pBAV226 derivative with GmR cassette insertion in *tetA* gene	This work
pBAV226Gm-*hopAA1*	pBAV226Gm derivative with *hopAA1-HA*	This work
pBAV226Gm-*hopAB1*	pBAV226Gm derivative with *hopAB1-HA*	This work
pBAV226Gm-*hopAE1*	pBAV226Gm derivative with *hopAE1-HA*	This work
pBAV226Gm-*hopAF1*	pBAV226Gm derivative with *hopAF1-HA*	This work
pBAV226Gm-*hopAH1*	pBAV226Gm derivative with *hopAH1-HA*	This work
pBAV226Gm-*hopAJ2*	pBAV226Gm derivative with *hopAJ2-HA*	This work
pBAV226Gm-*hopAK1*	pBAV226Gm derivative with *hopAK1-HA*	This work

pBAV226Gm-*hopAM1*	pBAV226Gm derivative with *hopAM1-HA*	This work
pBAV226Gm-*hopAP1*	pBAV226Gm derivative with *hopAP1-HA*	This work
pBAV226Gm-*hopH1*	pBAV226Gm derivative with *hopAA1-HA*	This work
pBAV226Gm-*hopI1*	pBAV226Gm derivative with *hopI1-HA*	This work
pBAV226Gm-*hopM1*	pBAV226Gm derivative with *hopM1-HA*	This work
pBAV226Gm-*avrPto1*	pBAV226Gm derivative with *avrPto1-HA*	This work
pBAV226Gm-*avrE1* (N−terminal)	pBAV226Gm derivative with *avrE1-HA* (1 kb N-terminal region)	This work
pVSP61	pVS1 derivative, Km^r	B. Staskawicz
pVSP61::*avrRpm1*	pVSP61 derivative carrying *avrRpm1*, Km^r	B. Staskawicz
pVSP61::*avrRpt2*	pVSP61 derivative carrying *avrRpt2*, Km^r	B. Staskawicz
pLH12	pLAFR3 derivative with 1.4 kb insertion with *avrRpt2* gene	B. Staskawicz / Whalen et al., 1991
pLH12-Ω	pLH12::Ω with disrupted *avrRpt2*.	B. Staskawicz / Whalen et al., 1991

* Nal^r, Rif^r, Cm^r, Sp^r, Gm^r, Km^r, and Tc^r = resistant to nalidixic acid, rifampicin, chloramphenicol, spectinomycin, gentamycin, kanamycin, and tetracycline.

Fig. 3-1 –Schematic diagram of the *Pf*55 effector delivery system.
a) *Pseudomonas fluorescens* 55 (pLN18, pBAV179/226Gm). This strain carries a pLAFR3 derivative (pLN18, pHIR11 or pCPP2089) and a compatible pVS1 replicon plasmid, generally pBAV226Gm (low copy number) or pCPP5040 (pML123-derived). Only pLN18 and pBAV226Gm are depicted here. **b)** pLAFR3-derived cosmids pHIR11 (the originally isolated cosmid carrying the *hrp*/*hrc* (TTSS) cluster from *Pss* 61 as well as the HopA operon *hopA-schA*; pLN18 (a pHIR11 derivative a in which the *hopA-schA* operon is disrupted. Only pHIR11 and pLN18 carry the functional TTSS cluster genes. pCPP2089 is a pHIR11 derivative with a transposon insertion in the *hrcC* gene that renders it TTSS-deficient. Note the strain carries a functional *hopA-schA* operon.

a)



b)

- pHIR11 – pLAFR3 derivative.
- pLN18 – pHIR11 derivative, hopPsyA-shcA::KmR
- pCPP2089 – pHIR11 derivative, hrcC::TnPhoA

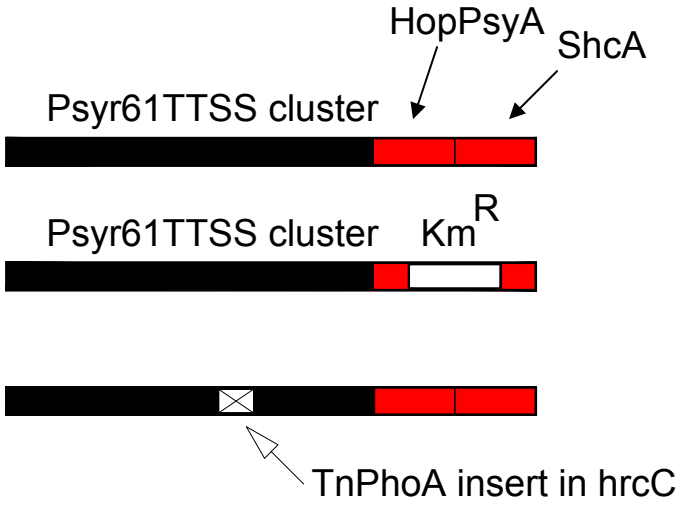
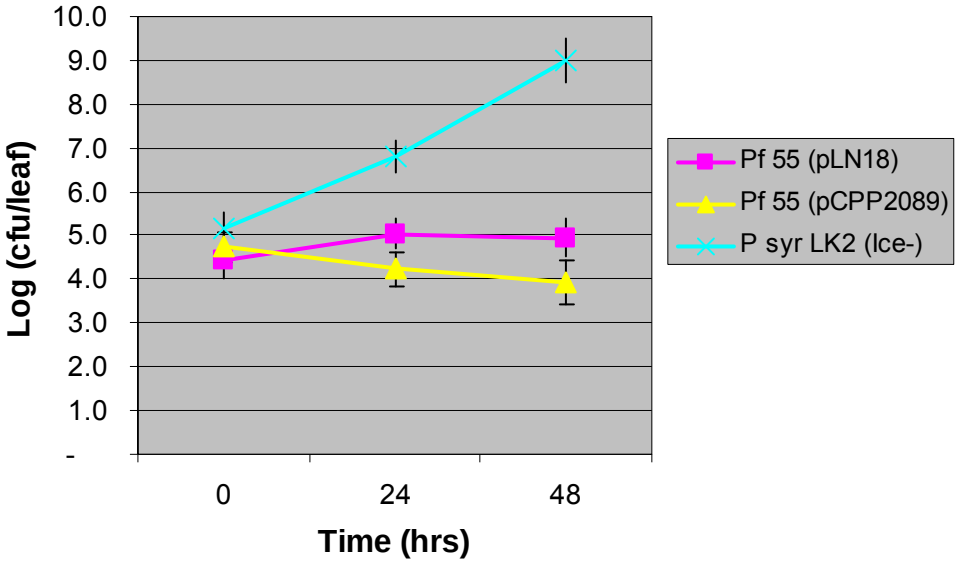


Fig. 3-2 –*Pseudomonas fluorescens* 55 (*Pf*55), *Pf*55 (pLN18) and *Pf*55 (pCPP2089) do not grow endophytically in bean plants.
a) Total bacterial population size of *Pf*55 (pLN18) expressing the *hrp*/*hrc* cluster from *Pss* 61 (squares), *Pf*55 (pCPP2089), carrying a HrcC- derivative of pHIR11 (triangles), and *Pss* B728a (LK2), an isogenic Ice- derivative of *Pss* B728a (Xs). **b)** Population sizes of *Pf*55, *Pf*55 (pLN18), and *Pf*55 (pHIR11) carrying the original *hrp*/*hrc* cluster and the *hopA-schA* operon from *Pss* 61. Vertical bars represent two standard errors of mean log population sizes.

a)



b)

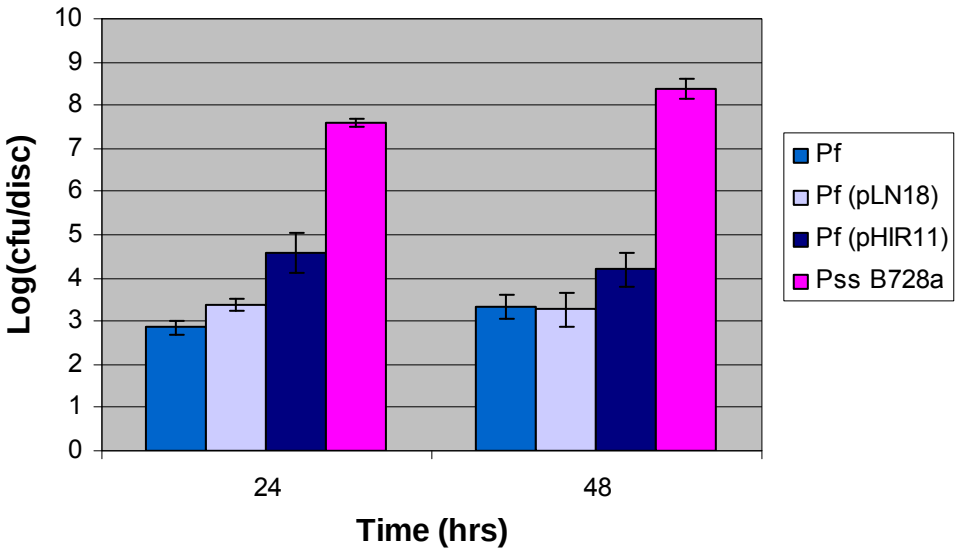
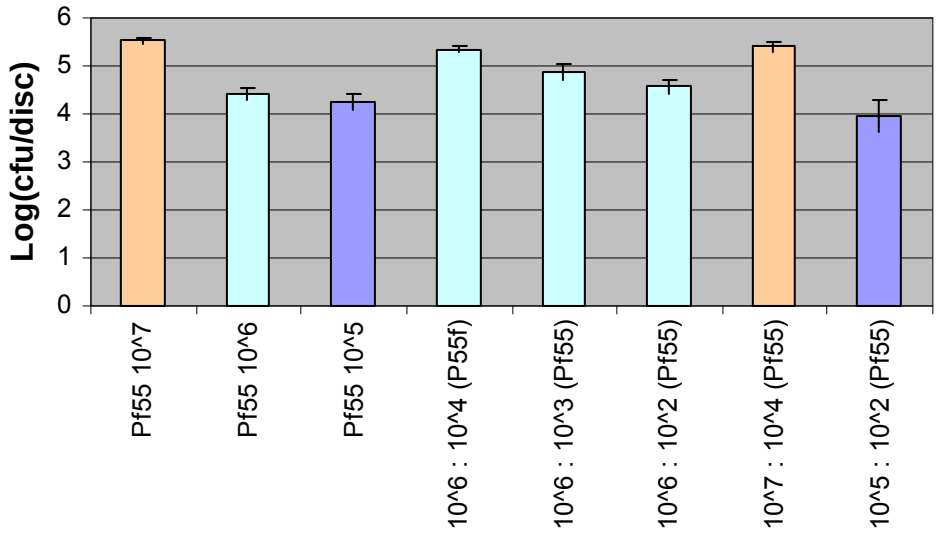


Fig. 3-3 –Mixtures of *Pf*55 and *Pss* B728a reveal the ability of effector-secreting strains to rescue growth of saprophytes in the apoplast.
a) Ratios and concentration of bacteria infiltrated with a syringe into bean plants. *Pss* B728a served as a bacterium with enhanced nutrient and/or water acquisition (ENA) activity (full effector repertoire). *Pf*55 served as a target bacterium whose growth was indication of the ENA activity caused by pathogen. **b)** Population sizes of *Pf*55 at 48hpi at each ratio with and without *Pss* B728a co-infiltration. **c)** Population sizes of *Pss* B728a at 48hpi at each ratio with and without *Pf*55 co-infiltration. Vertical bars represent two standard errors of mean log population sizes.

a)

Measured population at 48hpi with inoculum levels:
1:100 ratio – 10^6 *Pf*55 : 10^4 *Pss* B728a
1:1,000 ratio – 10^6 *Pf*55 : 10^3 *Pss* B728a
1:10,000 ratio – 10^6 *Pf*55 : 10^2 *Pss* B728a
1:1,000 – 10^7 *Pf*55 : 10^3 *Pss* B728a
1:1,000 – 10^5 *Pf*55 : 10^2 *Pss* B728a

b)



c)

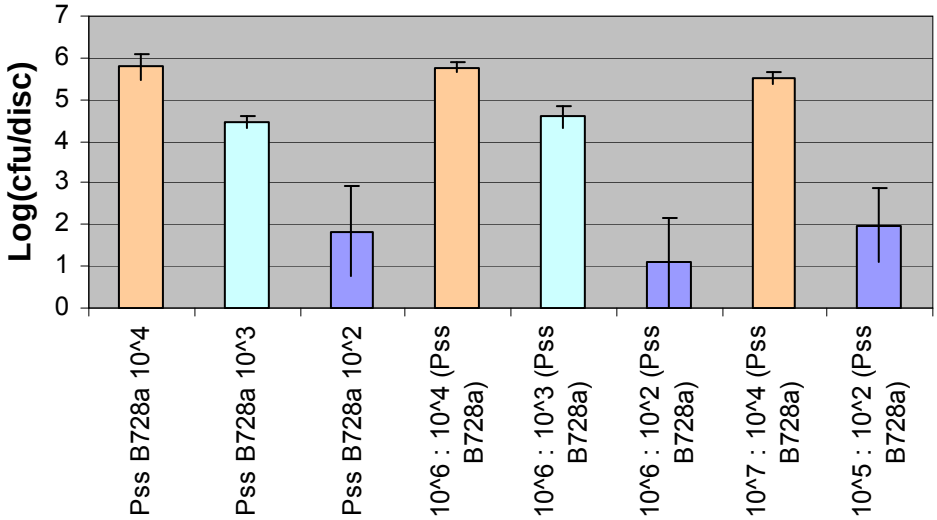
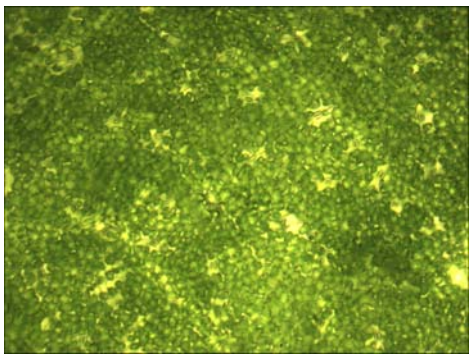

Log(cfu/disc)
7
6
5
4
3
2
1
0
Pss B728a 10^4
Pss B728a 10^3
Pss B728a 10^2
10^6 : 10^4 (Pss B728a)
10^6 : 10^3 (Pss B728a)
10^6 : 10^2 (Pss B728a)
10^7 : 10^4 (Pss B728a)
10^5 : 10^2 (Pss B728a)

Table 3-2 –Macroscopic and microscopic cell death (CD) elicitation by different strains in bean plants.

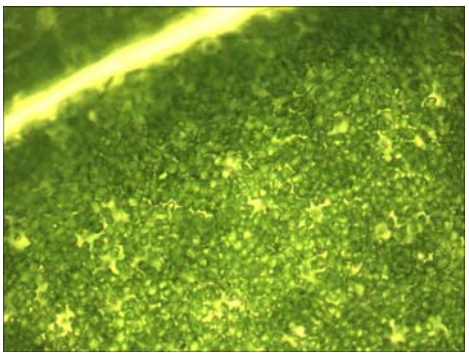
CD reflects either HR necrosis or virulent necrosis (lesions). No symptoms= no visible necrosis and/or chlorosis present. The results presented here were taken from four different experiments and the plants were scored within 24 hpi in all cases. *Pf*55, *Pf*55 (pLN18), *Pf*55 (pCPP2089), *Pf*55 (pHIR11), *Pss* B728a, *Pss* B728a HrpJ-, *Pto* DC3000, *Pseudomonas syringae* pv. *maculicola* ES4326, *Pf*55 (pLN18) or *Pf*55 (pCPP2089) strains expressing *Pto* DC3000 effectors under constitutive expression (P_{nptII} promoter) in pCPP5040 (HopF1-ShcF, AvrPtoB, and AvrPpiB1) or *Pss* B728a effectors under constitutive expression (P_{nptII} promoter) in pBAV226Gm (HopM1, HopAB1, HopAA1, AvrPto1). 1-Experiment #1 - scored at 24 hpi; 2-Experiment #2 - scored at 14hpi, 20 hpi, and 24 hpi; 3-Experiment #3 - scored at 16 hpi; 4-Experiment #4.

	MACRO	MICRO
*Pf*55, *Pf*55 (pLN18), *Pf*55 (pCPP2089)	No symptoms[1,2]	No CD
Pss B728a HrpJ-	No symptoms[1]	No CD
Pto DC3000, *Pma* ES4326	CD[1]	CD
*Pf*55 (pHIR11)	CD by 16hpi[1,2,3]	CD
Pss B728a	Lesions[1,2,3]	CD
*Pf*55 (pLN18/pCPP2089 + pCPP5040/pBAV226Gm):		
HopF1-ShcF, AvrPtoB, and AvrPpiB1	No symptoms[2]	No CD
HopM1, HopAB1	No symptoms[4]	CD
HopAA1, AvrPto1	No symptoms[4]	No CD

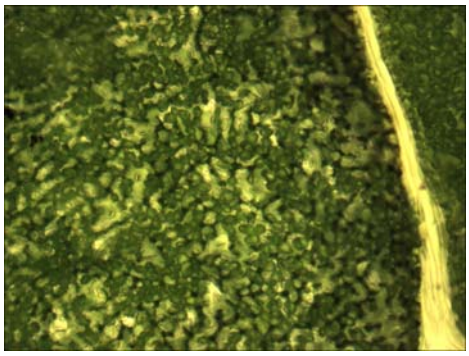
Fig. 3-4 –Microscopic cell death (CD) of *Pf*55 (pLN18) strains expressing HopE1$_{PtoDC3000}$, HopG1$_{PtoDC3000}$ or HopAM1$_{PtoDC3000}$ (formerly known as AvrPpiB1).



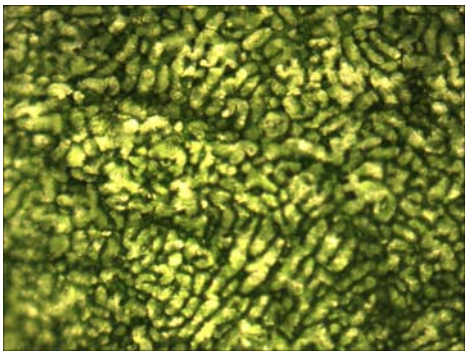
*Pf*55 (pLN18)



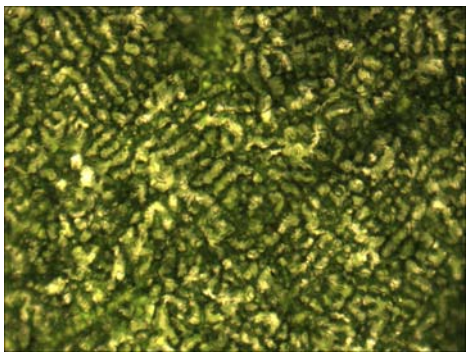
*Pf*55 (pCPP2089)



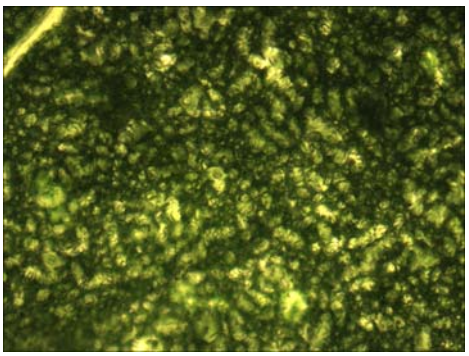
*Pf*55 (pHIR11)



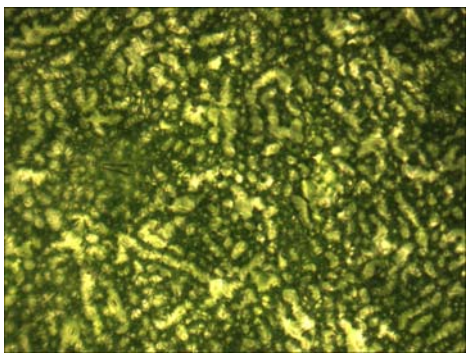
*Pf*55 (pHIR11)



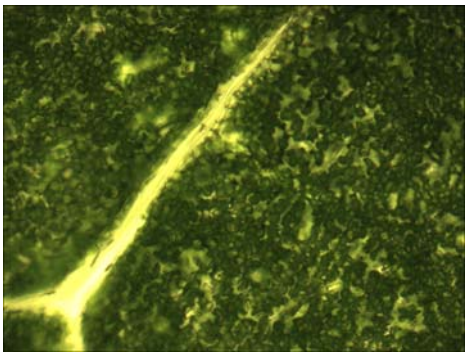
Pto DC3000



*Pf*55 (pLN18 + pHopE1$_{PtoDC3000}$)



*Pf*55 (pLN18 + pHopG1$_{PtoDC3000}$)



*Pf*55 (pLN18 + pHopAM1$_{PtoDC3000}$)

Table 3-3 –Increased efficiency of *Pf*55 effector delivery with co-expression of *rps*L/R.
a) *Pf*55 strains carrying *Pf*55 (pHIR11) alone, or carrying pML123-*rsp*R or –*rsp*L constructs (Pf55 (pHIR11 + pRspL/pRspR)), *Pf*55 (pLN18) alone or carrying pML123-*rsp*R or –*rsp*L constructs (Pf55 (pLN18 + pRspL/pRspR)), and *Pf*55 carrying *rsp*R/L constructs (Pf55 (pRspR/pRspL)) were infiltrated into beans at a concentration of 10^7 and 10^9 CFU/ml. **b)** Microscopic CD (number of leaves with microscopic CD/ total number of infiltrated leaves) was analyzed at the time points presented.

a)

- Pf55 (pHIR11) – known to give HR on beans.
- Pf55 (pHIR11 + pRspR)
- Pf55 (pHIR11 + pRspL)
- Pf 55 (pLN18)
- Pf55 (pRspR)
- Pf55 (pRspL)
- Pf55 (pLN18 + pRspR)
- Pf55 (pLN18 + pRspL)

b)

	Cell death (Number of leaves with CD/ total number of infiltrated leaves)					
	10^7 cfu/ml	10^9 cfu/ml	10^7 cfu/ml	10^9 cfu/ml	10^7 cfu/ml	10^9 cfu/ml
Hpi	Pf55 (pHIR11)		Pf55 (pHIR11 + **pRspR**)		Pf (pHIR11+ **pRspL**)	
17	0 / 11	0 / 11	0 / 10	0 / 10	0 / 10	0 / 10
20.5	0 / 11	3 / 11	2 / 10	10 / 10	0 / 10	8 / 10
21.5	0 / 11	5 / 11	----	-----	-----	------
23	1 / 11	7 / 11	2 / 10	10 / 10	0 / 10	9 / 10
41*	3 / 11	11 / 11	3 / 10	10 / 10	1 / 10	10 / 10

* The # of CD leaves did not change after 41hpi.

Table 3-4 –List of effector candidates from *Pss* B728a.

Name/ Original name	Characteristics	Length (aa)	Putative localization	Expressed
hopAK1 */ hopPmaH*	C-terminal Pectin lyase domain; homologs in many bacterial species including *P. agglomerans* pv. *gypsophilae*.	543	Not pred./Cytoplasmic	**YES**
hopAF1 */ holPtoN, hopPtoJ*	Homolog in *P. agglomerans* pv. *gypsophilae* and Xanthomonas sp.	912	Chloroplast	**YES**
hopAH1*/ holPsyAH*	Operon hopAG1, AH and AI'. Glycosil hydrolase. Paralog $HopAH2_{PssB728a}$.	427	Chloroplast	**YES**
hopAG1*/ holPsyAG*	Operon hopAG1, AH and AI'. C-terminal NUDIX / Hydrolase domain.	716	Chloroplast	**NO**
hopAJ2 */ hopPmaG*	*hopPmaG* (*hopAJ1*) - putative transglycosilase	445	Mitochondria	**NO Hrp box**
hopI1 */ hopPmaI*	DNAJ domain. In *Pma* Tn insertion causes reduced growth (Guttman et al., 2002). Confirmed chloroplast localization (induces tylakoid disorganization in *N. benthamiana*, tobacco and *Arabidopsis*. DnaJ domain not needed for biological activity in chloroplast.	361	Chloroplast	**YES**
hopAE1*/ holPsyAE*	Chimera HopPmaA-like with a novel C-terminal region (Greenberg and Vinatzer, 2003), similar C-terminal with HopPmaA, HopPtoD2, HopPtoP. Induces CD on *N. benthamiana*.	914	Chloroplast	**YES**
hopH1 */ hopPtoH*	*Pss* has hopH1 and hopH2 *(hopAP1*, a chimera of *hopPtoH*). Distant homology with *C. botulinum* neurotoxin (PSI-BLAST).	218	Not pred./Cytoplasmic	**YES#**
hopAP1*/ hopPtoH2*, chimeric with *hopH1 (HopH2)*	A chimera of *hopH1.*	176	Not pred./Cytoplasmic	**YES**
hopAA1 */ hopPtoA1, CEL ORF5, hopPtoA2*	Produces CD in *N. benthamiana*. Paralog HopPtoA2 is ipx in *Pto*. Localized to mitochondria and kills yeast (targets respiration).	554	Mitochondria - Chloroplast	**YES**
hopM1*/ holPtoX (Pss), hopPtoM, CEL ORF3 en Pto*	Involvement in virulence (causes lesions). ipx12 in Pto IVET. Causes CD on bean, *N. benthamiana* and tobacco. Restores virulence to CEL mutant in *Pto* DC3000.	718	Chloroplast	**YES**
avrPto1*/ avrPto, avrP*	Homolog in *Pto* is suppressor of PTI.	163	Chloropl/ Cytopasmic?	**YES**
avrE1 */ avrE (N-terminal region) CEL*	In *P. fluorescens* SBW25, similar and function is interchangeable with DspA/E E amylovora. Also present in *P. agglomerans* pathogens. Restores virulence to CEL mutant in *Pto*.	1821	Not pred./Cytoplasmic	**YES**
HopAB1 *(AB1, AB2, AB3, AB3-1 and -2) / virPphA, AvrPtoB, virPtoA, hopPmaL, holPmaN*	Homolog in *Pph* is a suppressor of plant defenses. Can interfere with CD.	516	Not pred	**YES**
avrRpm1*/ avrRpm1, avrPmaA, avrPpiA2.R2, avrPpiA1*	Binds RIN4, a potential regulator of basal plant defenses (Mackey et al., 2002)	228		**YES**
avrPto1*/ avrPto, avrP*	Homolog in *Pto* is suppressor of PTI.	163	Chloropl/ Cytopasmic?	**YES**
avrB3*/ hopPsyC (AvrPphC)* EEL ORF1	AvrPphC (homolog of AvrB3 in *P. phaseolicola*) is a suppressor of plant defenses.	323		**YES**
hopX1 */ hopPsyE*, AvrPphE, EEL ORF2	AvrPphE (homolog in *Pto*) is a suppressor of plant defenses (Jamir et al., 2004)	383		**YES**
hopZ3 */ hopPsyV, HopPmaD*, EEL ORF5	Family of YopJ/P/AvrBsT cysteine proteases are suppressors of defenses in animal pathogens. *Pss* B728a deletion produces an increase in growth of mutant.	411		**YES**
hopAN1 */ ipx53*	*Pto* IVET ipx; conserved in Pf-5, *R. solanacearum*, *B. cepacia*	429	Not pred./Cytoplasmic	**NO**
hopJ1 */ hopPmaJ*	Secretion could not be detected with AvrRpt2 reporter fusion.	150	Mitochondria - Chloroplast	**Not secreted**
hopL1 */ hopPtoL, ORF29*	Homolog to SPI-2 regulated SrfC.	899	Not pred./Cytoplasmic	**NO Hrp box**

Name/ Original name	Characteristics	Length (aa)	Putative localization	Expressed
hopAI1'/ *holPsyAI*	Operon *hopAG*, *hopAH* and *hopAI'*. Early stop in $hopAI_{PssB728a}$, functional in *Pto*; homology to OspF effector from Shigella.	20	Chloroplast / Truncated gene	**Truncated**
hopAC1*/ holPsyAC, holPtoAC*	Originally did not met criteria for effector; in *Pto* with a Tn insertion; C-terminal region is HopPtoB-like in both (HopPtoB in *Pto*, *Pph* and Pss DH105 not in B728a)		Not pred./Cytoplasmic	**Not secreted**
hopAH2*/ holPsyAH2*	Secretion could not be detected with AvrRpt2 reporter fusion.	380	Not pred./Cytoplasmic	**Not secreted**

IVET – in vivo expression technology; ipx – in planta expressed gene; *Pss* – *P. syringae* pv. *syringae* B728a; *Pma- P. syringae* pv. *maculicola* ES4326; *Pto* – *P. syringae* pv. *tomato* DC3000.

Fig. 3-5 –TTSS effectors from *Pto* DC3000 and *Pss* B728a known for their plant defense suppression role were tested for increased growth of *Pf*55 in bean.
*Pf*55 (pHIR11) carries *hrp*/*hrc* cluster and *hopA*/*shcA* operon from *Pss* 61; *Pf*55 (pLN18) carries the *hrp*/*hrc* cluster from *Pss* 61; *Pf*55 (pCPP2089) carries a *hrcC* deletion derivative of pHIR11. *Pf*55 (pLN18) carry *Pto* DC3000 effector genes in pCPP5040 plasmid (*hopF1*/*shcF* (HopF1/F2), and *hopAM1* (AvrPpiB1) and *Pss* B728a effector genes in pBAV226Gm (HopAB1, and AvrPto1). All strains were infiltrated at a concentration of 10^7 CFU/ml. Vertical bars represent two standard errors of mean log population sizes.

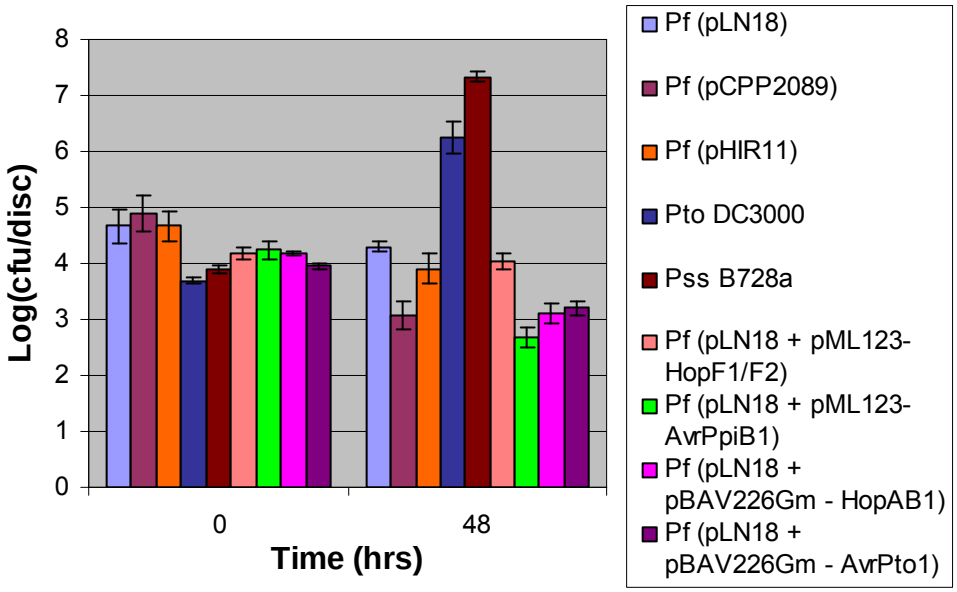
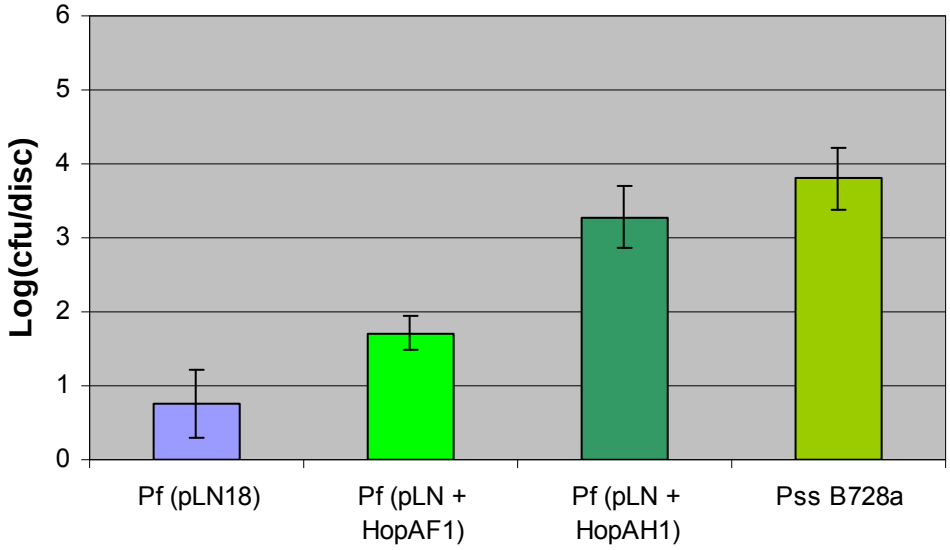


Fig. 3-6 –Population size of *Pseudomonas fluorescens* 55 (*Pf*55) harboring TTSS effectors putatively-targeted to the chloroplast.
a) *Pf*55 (pLN18), *Pf*55 (pLN18 + pHopAF1), *Pf*55 (pLN18 + pHopAH1) and *Pss* B728a were inoculated at 10^4cfu/ml. **b)** *Pf*55 (pLN18), *Pf*55 (pLN18 + pHopAF1), *Pf*55 (pLN18 + pHopAH1) and *Pss* B728a were inoculated at 10^6cfu/ml. Plants were water-stressed at the beginning of the experiment. Vertical bars represent two standard errors of mean log population sizes.

a)



b)

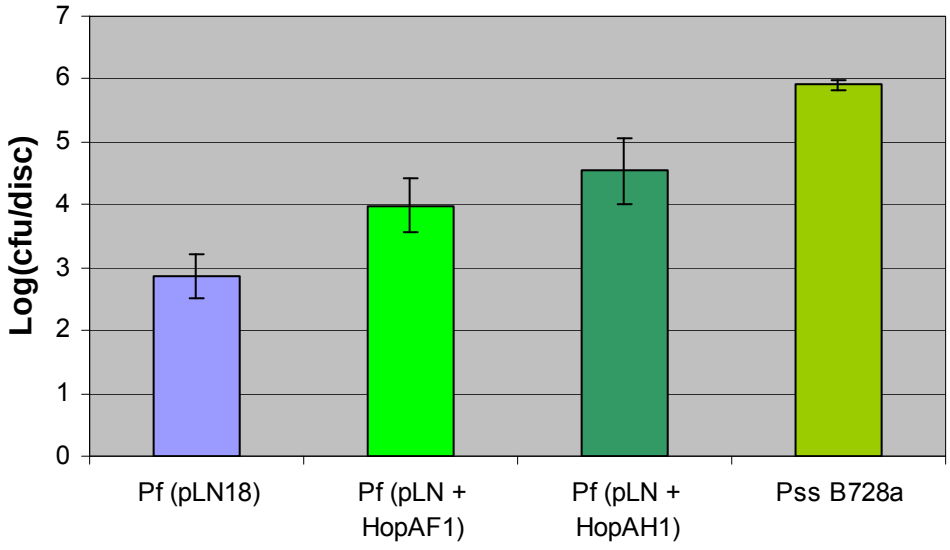


Fig. 3-7 –Growth of *Pf*55 (pLN18) harboring *Pss* B728a effectors in water-stressed bean.
*Pf*55 (pLN18) alone or expressing *Pss* B728a effectors under constitutive *nptII* promoter in pBAV226Gm (*Pf*55 (pLN18 + pBAV226Gm-effector)) and *Pss* B728a were infiltrated into water-stressed beans. Effectors: HopAH1 (AH1); HopAE1 (AE1); HopAJ2 (AJ2); and HopAM1 (AM1). Greenhouse beans were exposed to water stress before experiment followed by normal dry growth conditions during bacterial growth. Vertical bars represent two standard errors of mean log population sizes.

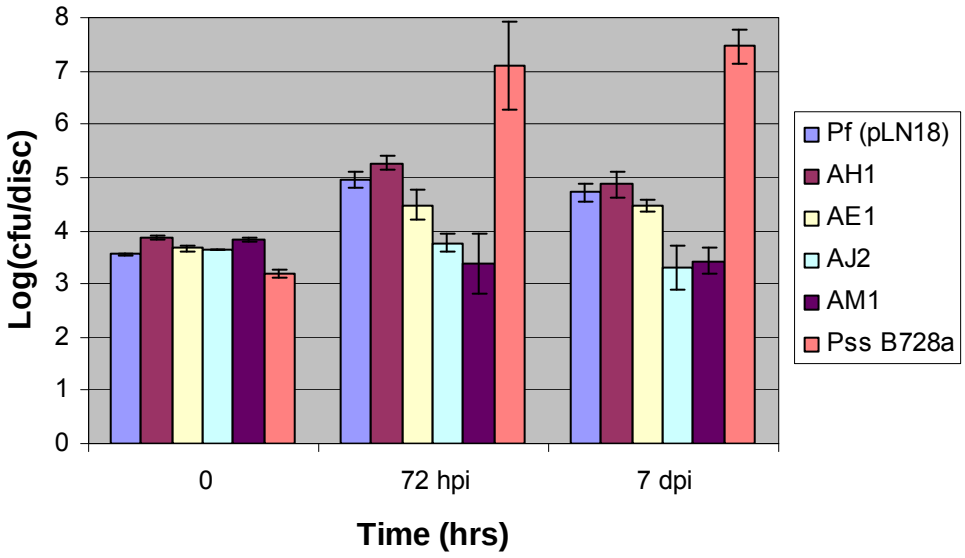


Fig. 3-8 –Growth of *Pf*55 (pLN18) harboring *Pss* B728a effectors in plants with and without maintenance of humidity conditions.
Bean plants infiltrated with *Pf*55 (pLN18), *Pf*55 (pLN18 + pBAV226Gm-AvrPto1) (AvrPto1), *Pf*55 (pLN18 + pBAV226Gm-N-terminal portion of AvrE1) (N-terminal AvrE1), *Pf*55 (pLN18 + pBAV226Gm-HopAK1) (HopAK1), *Pf*55 (pLN18 + pBAV226Gm-HopAP1) (HopAP1), and *Pss* B728a (Pss B728a) were infiltrated alone or were covered with plastic bags after water congestion from infiltration had dried (no water congestion was visible) (b). Vertical bars represent two standard errors of mean log population sizes. b= bagged after water congestion was no longer visible.

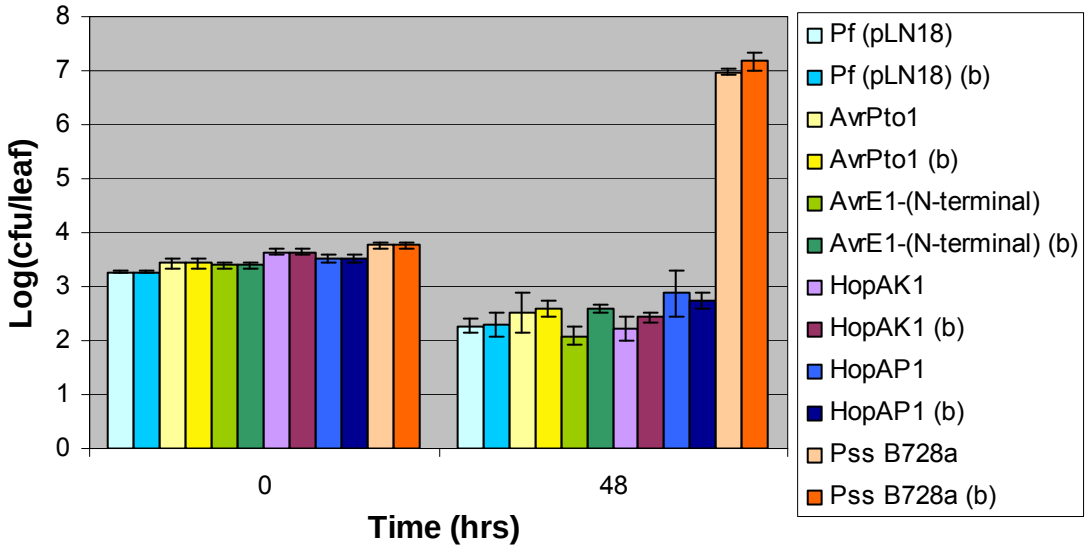


Fig. 3-9 –Growth of mixed groups of *Pf*55 (pLN18) strains expressing effectors from different sources in bean.
a) Effector containing groups. **b)** Bacterial population growth levels for the three-grouped strains, *Pf*55 (pLN18), *Pf*55 (pHIR11) and *Pss* B728a in greenhouse grown plants that were not watered during the experiment. Vertical bars represent two standard errors of mean log population sizes.

a)

Group	Each strain 1x10^5 CFU/ml inoculum	
I	*Pf*55 (pBAV226Gm -*hopAH1*, -*AF1*, -*I1*, -*AJ2*, -*H1*, -*AP1*)	*Pss* B728a effectors
II	*Pf*55 (pCPP5040 -*avrPtoB*, -*hopE1*, *hopF1*/*shcF*, *hopG1*, -*hopAM1*)	*Pto* DC3000 effectors (known suppressors of plant defenses)
III	*Pf*55 (pBAV226Gm -*avrPto1*, -*hopM1*, -AA1, -*AB1*, -*AK1*, -*avrE1*(N-terminal))	*Pss* B728a (plant defense suppressing) effectors AvrPto1, HopM1, HopAA1, HopAB1

b)

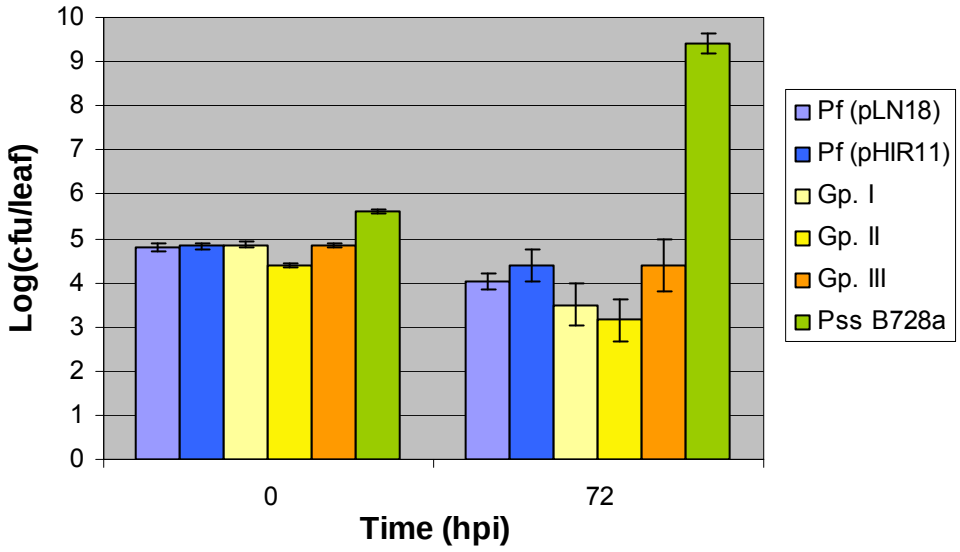
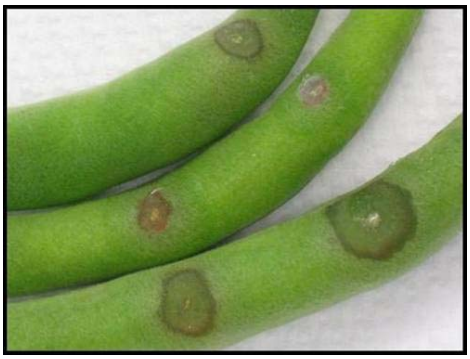


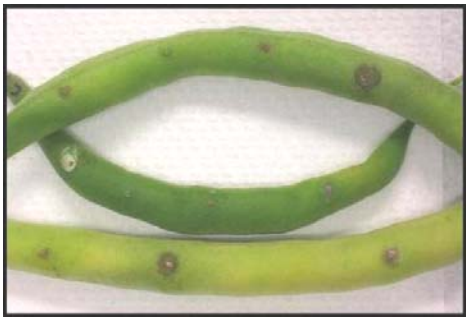
Fig. 3-10 –Screening for ENA activity effectors in bean pods
Detached pods were syringe infiltrated with concentrated bacterial suspensions (pictures show 10^8 and 10^9 CFU/ml zones), incubated covered under humid conditions. Water-soaking lesion zones were scored 5dpi.



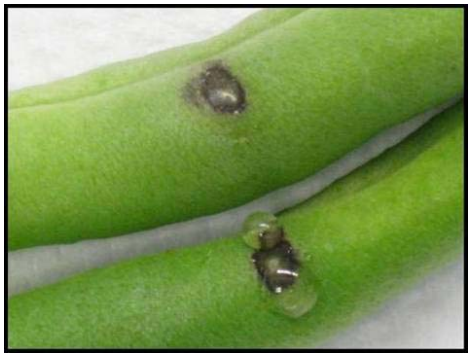
Pss B728a

Buffer

*Pf*55 (pLN18) control



Pss B728a HrpJ-



Pto DC30000

Table 3-5 –*Pf*01 (EtHAn) but not *Pf*01 cause CD in bean and *N. benthamiana*
Syringe inoculation of *Pf*01 (EtHAn) and *Pf*01 in **a)** bean, **b)** *N. benthamiana*, or **c)** *Arabidopsis* leaves in opposite sides of leaves with cell suspensions of 10^8 and 10^9 cfu/ml. CD (number of necrotic leaves/total number of infiltrated leaves) was scored within 24 hpi.

a)

	Beans before trifoliate emergence			
	*Pf*01 (EtHAn)		*Pf*01	
	10^9 cfu/ml	10^8 CFU/ml	10^9 cfu/ml	10^8 cfu/ml
20 hrs	All confluent CD	Patchy CD	No CD	No CD

b)

	N. benthamiana - before flowering			
	Pf0-1 (EtHAn)		*Pf*01	
	10^9 cfu/ml	10^8 cfu/ml	10^9 cfu/ml	10^8 cfu/ml
20 hrs	4 / 16 (mild)	No CD	No CD	No CD
44 hrs	4 / 16 CD	No CD	No CD	No CD
72 hrs	10 / 16 CD	3 / 16	No CD	No CD

c)

	Arabidopsis - Col-0 (small)			
	Pf0-1 (EtHAn)		*Pf*01	
	10^9 cfu/ml	10^8 cfu/ml	10^9 cfu/ml	10^8 cfu/ml
24 hpi	6 / 12 (CD)	6 / 12	4 / 12 (CD)	5 / 12
44 hpi	11 / 12	7 / 12	7 / 12	9 / 12

Table 3-6 –*Pf*01 (EtHAn) can secrete AvrRpt2 in a TTSS-dependent manner in *Arabidopsis* Col-0

*Pf*01 (EtHAn), *Pf*01 (EtHAn) HrcC- and *Pf*01 (EtHAn) expressing effector AvrRpt2 or vector control pVSP61 alone were infiltrated into *Arabidopsis* Col-0 and scored for CD (number of necrotic leaves/total number of infiltrated leaves) induction at 17 hpi.

Strain	Time	CD
*Pf*01 (Ethan)	17hpi	2/15
*Pf*01 (Ethan hrcC::Tn5)	17hpi	3/14
*Pf*01 (Ethan) + pVSP61	17hpi	5/15
*Pf*01 (Ethan) + pVSP-AvrRpt2	17hpi	12/16

Fig. 3-11 –Growth of *Pf*01 (EtHAn)-delivered effector strains in *N. benthamiana*
*Pf*01 (EtHAn), *Pf*01 (EtHAn) HrcC- (PfO1 (-)) and *Pf*01 (EtHAn) carrying pBAV226Gm-HopAF1 (AF1), pBAV226Gm-HopAH1 (AH1) and pBAV226Gm-AvrPto1 (AvrPto1) and Pss B728a were syringe infiltrated into *N. benthamiana* plants. Vertical bars represent two standard errors of mean log population sizes.

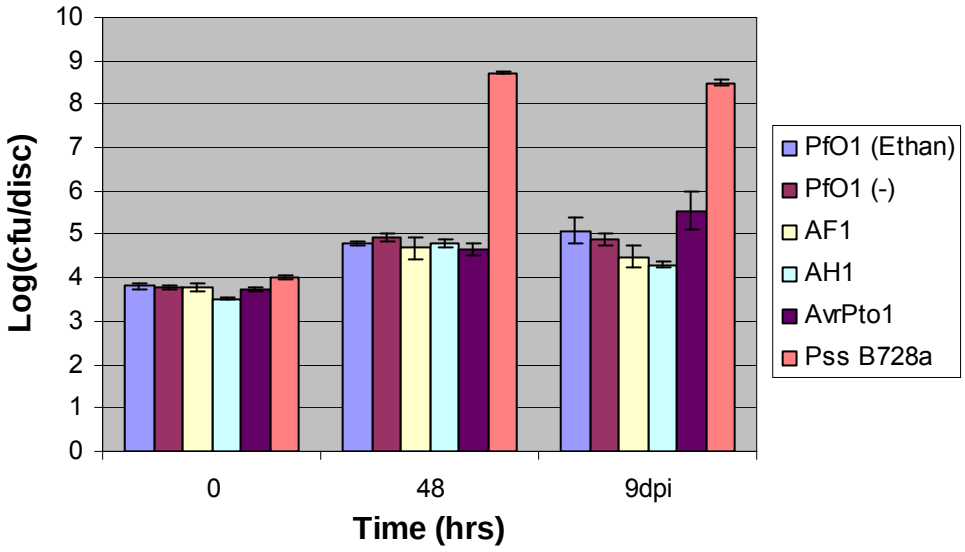


Table 3-7 –AMELIA does not induce CD on bean unlike EtHAn.
Infiltration of *Arabidopsis*, bean and *N. benthamiana* plants with *Pf*01, *Pf*01 (AMELIA), *Pf*01 (EtHAn), *Pf*01 (EtHAn) with a genomic insertion of AvrRpt2 (*Pf*01 (EtHAn) + AvrRpt2), AvrRpm1 (*Pf*01 (EtHAn) + AvrRpm1) or vector control (*Pf*01 (EtHAn) + E.V.). CD (number of necrotic leaves/total number of infiltrated leaves) was scored at 18-20 hpi.

		***Arabidopsis* Col-0**		**Bean**		***N. benthamiana***	
Strain	**Time**	**CD**	**%**	**CD**	**%**	**CD**	**%**
Buffer	18-20hpi	0/18	0	ND	ND	ND	ND
PfO1	18-20hpi	8/16	50	0/13	0	4/14	29
PfO1 (Amelia)	18-20hpi	10/18	56	0/13	0	5/15	33
PfO1 (Ethan)	18-20hpi	7/16	45	11/11	100	5/16	31
PfO1 (Ethan) + E.V.	18-20hpi	12/17	71	16/18	89	7/17	41
PfO1 (Ethan) + AvrRpt2	18-20hpi	16/17	94	16/16	100	8/13	62
PfO1 (Ethan) + AvrRpm1	18-20hpi	16/16	100	16/16	100	5/13	39

Fig. 3-12 –AMELIA population levels rise with ENA treatments (maintained water-soaking and addition of exogenous fructose to the apoplast).
Bean plants were vacuum infiltrated with, *Pf*01 (Pf01), *Pf*01 (EtHAn) (EtHAn), *Pss* B728a (Pss B728a) and *Pf*01 (AMELIA) alone (Amelia) or with 5 g/L of fructose added to the inoculum (Amelia + 5g/L Fructose) or maintained water-congested conditions after infiltration (Amelia Bagged). Vertical bars represent two standard errors of mean log population sizes.

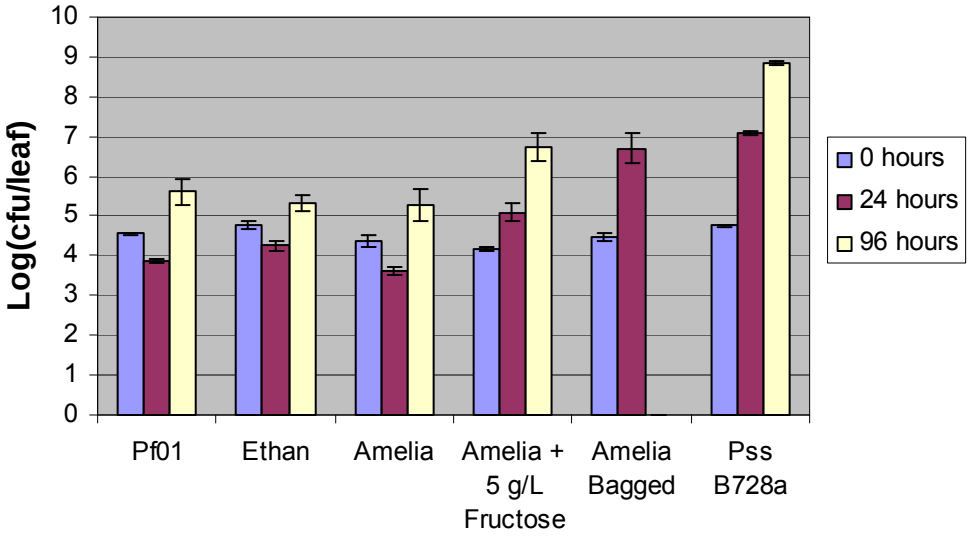


Table 3-8 –AMELIA can secrete effector AvrRpt2 in a TTSS-dependent manner and induce macroscopic CD in *Arabidopsis* but not bean.

AMELIA-delivered AvrRpt2 and AvrRpm1 produce CD in *Arabidopsis*. AMELIA alone (Amelia) or carrying pVSP61-based constructs with AvrRpt2 (Am (pAvrRpt2)), AvrRpm1 (Am (pAvrRpm1)) or vector control (Am (pVSP61)) and AvrRpt2-expressing plasmid pLH12 (Am (pLH12)) or an AvrRpt2-deletion mutant (Am (pLH12-Ω)); *Pss* B728a, *Pto* DC3000 or buffer control were syringe infiltrated into *Arabidopsis* plants. CD (number of necrotic leaves/total number of infiltrated leaves) was scored at 16 hpi. The % incidence as well as a score value (total scores of individual leaves/ maximum score (4) x total number of leaves infiltrated) are presented.

	Time	*Arabidopsis* (~16 hpi)				bean (~16 hpi)		
	Plant/Pot	1	2	3	% / score	1	2	%
Amelia	Incidence	3/7	0/6	1/5	22%	0/7	0/9	0%
	Score	2,2,4		1	0.13			
Am (pVSP61)	Incidence	1/6	1/4	0/5	13%	0/8	0/8	0%
	Score	1	1		0.03			
Am (pAvrRpt2)	Incidence	5/6	3/5	2/5	63%	0/8	0/9	0%
	Score	1,2,3,3,1	1,1,2	2,1	0.27			
Am (pAvrRpm1)	Incidence	3/7	3/5	3/5	53%	0/8	0/8	0%
	Score	1,2,2	1,2,3	1,4,4	0.29			
Am (pLH12)	Incidence	2/7	5/8	6/9	54%	0/9	0/8	0%
	Score	1,2	3,1,1,1,3	1,1,2,3,1,1	0.22			
Am (pLH12-Ω)	Incidence	3/6	0/6	4/6	39%	0/10	0/6	0%
	Score	1,2,3		1,1,1,4	0.18			
Pto DC3000	Incidence	3/7	5/7	2/6	50%	7/7	8/8	100%
	Score	1,1,2	1,1,2,3,3	1,1	0.20			
Pss B728a	Incidence	1/6	0/6	1/7	11%	0/8	0/8	0%
	Score	1		1	0.03			
Buffer	Incidence	0/7			0%			
	Score				0			

Fig. 3-13 –AMELIA strains harboring a range of effectors do not multiply in bean.
Pss B728a, AMELIA strains carrying Pss B728a effectors cloned in pBAV226Gm: *hopAH1*, *hopAK1*, *hopAJ2*, *hopAA1*, *hopAP1*, *hopAE1* and *hopH1* (Am (HopAH), Am (HopAJ2), Am (HopAA1), Am (HopAP1), Am (HopAE1) and Am (HopH1), respectively); and carrying pVSP61 alone or pVSP61-*avrRpt2*, or -*avrRpm1* (Am (pVSP61), Am (AvrRpt2) and Am (AvrRpm1), respectively) were vacuum infiltrated into bean. Vertical bars represent two standard errors of mean log population sizes.

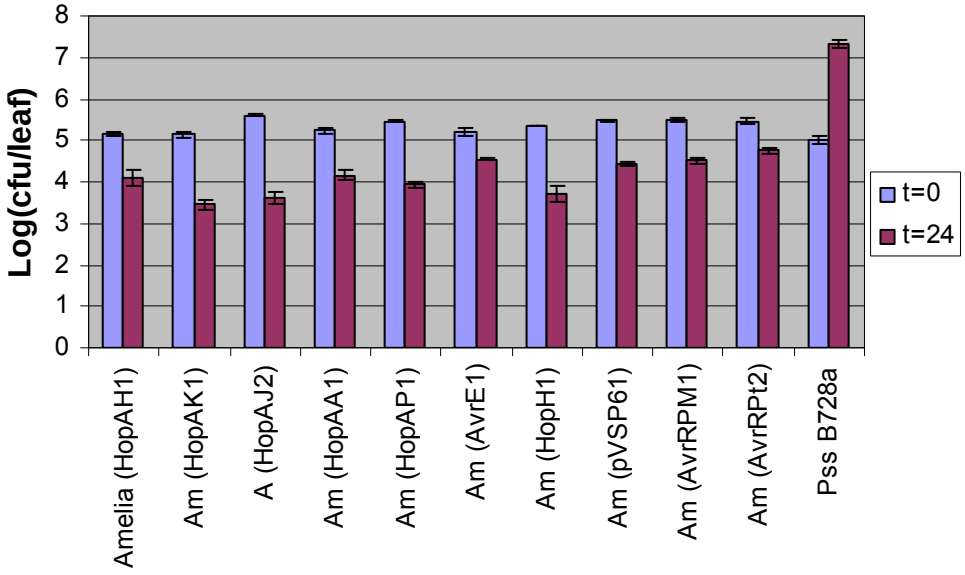
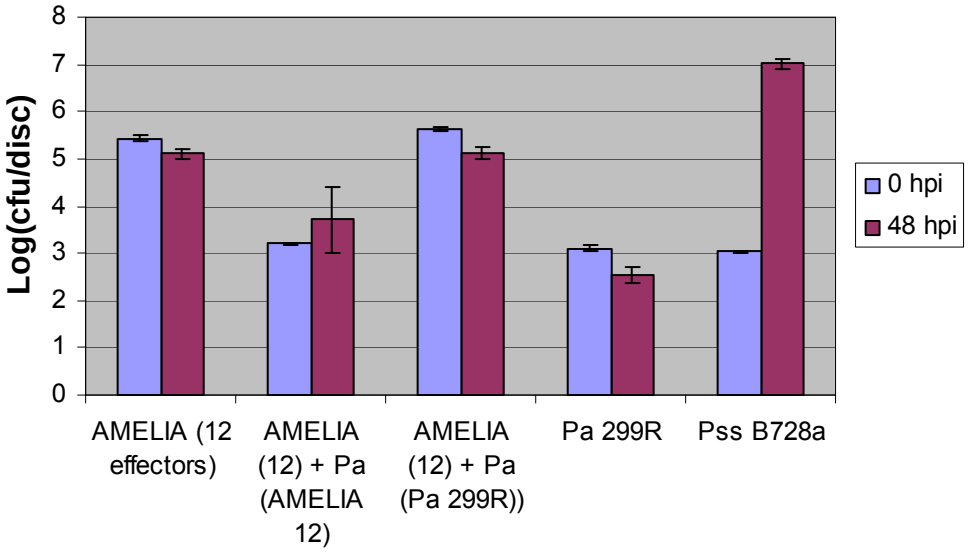


Fig. 3-14 –Twelve combined single effector-producing AMELIA co-infiltrated with *Pa* 299R.

Combined effector-producing AMELIA strains (tested for ENA activity) were vacuum co-infiltrated with *Pa* 299R as target population to measure growth increase. AMELIA strains harbored pBAV226Gm-effector constructs with the following TTSS effectors: *hopH1*, *hopAF1*, *hopAH1*, *hopAB1*, *hopAA1*, *avrPto1*, *hopAK1*, *hopAJ2*, *hopAE1*, *hopI1*, *hopAP1*, and *hopM1* (without *shcM*). AMELIA strains each expressing one of the 12 effectors were infiltrated at 10^7 CFU/ml inoculum (Amelia (12 effectors)); co-infiltration mixture of AMELIA strains (infiltrated at a 10^7 CFU/ml inoculum concentration) and *Pa* 299R (infiltrated at 10^5 CFU/ml inoculum concentration) were plated in Rifampicin selective media for *Pa* 299R bacterial counts (AMELIA (12 effectors) + *Pa* 299R (*Pa* 299R)) or plated on Gm selective media for grouped AMELIA counts (AMELIA (12 effectors) + *Pa* 299R (Amelia (12)); *Pa* 299R alone at 10^5 CFU/ml inoculum concentration (Pa 299R); and *Pss* B728a infiltrated alone at 10^5 CFU/ml inoculum concentration (Pss B728a). Vertical bars represent standard error of mean log population sizes. Vertical bars represent two standard errors of mean log population sizes.



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