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Wensheng Wei

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Wensheng Wei

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ABSTRACT

Type III Protein Secretion in Plant-Bacterial Pathogen Interactions

By

Wensheng Wei

Hypersensitive response and pathogenicity (*hrp*) genes control the ability of major groups of plant pathogenic bacteria to elicit the defense response in resistant plants and to cause disease in susceptible plants. A number of Hrp proteins share significant sequence similarities with components of type III secretion systems and flagellar assembly machineries in animal pathogenic bacteria. The *hrp* gene-encoded type III secretion system (the Hrp system) is believed to deliver bacterial virulence or avirulence (Avr) proteins into host cells upon infection. However, the molecular mechanism of type III protein secretion is poorly understood in both animal and plant pathogenic bacteria. My doctoral dissertation research is mainly focused on studying the mechanism of type III protein secretion and the function of secreted proteins in a model plant pathogenic bacterium, *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000.

I began by examining the function of the *hrpA* gene, which encodes an extracellular protein secreted via the Hrp system of *Pst* DC3000. A non-polar *hrpA* mutant was constructed by replacing the *hrpA* gene open reading frame (ORF) with the *aph* ORF. In collaboration with Dr. Martin Romantschuk's group I demonstrated that HrpA is a major structural protein of a novel filamentous appendage on the bacterial surface, called the Hrp pilus. The *hrpA* mutant could no longer cause disease in *Arabidopsis thaliana* or elicit a resistance response in the non-host tobacco or resistant

tomato plants, providing genetic evidence for a critical role of the Hrp pilus in plant-*Pst* DC3000 interactions.

In the functional study of the Hrp pilus, I found that the *hrpA* gene was required for expression of all examined *Pst* DC3000 genes (*hrc*, *hrp* and *avr*) that are associated with the type III secretion. HrpA-mediated gene regulation was achieved through activation of two previously characterized regulatory genes (*hrpR* and *hrpS*) at the RNA level. Ectopic expression of the *hrpRS* operon restored gene expression in the *hrpA* mutant, but did not restore type III protein secretion. These results define a dual role of the Hrp pilus structural protein in global regulation of the Hrp system and control of type III protein secretion in *Pst* DC3000.

I also explored the possibility of using an Avr protein as a bait to identify host interacting proteins. Specifically, there is one unique Avr family of proteins, known as Avr/Pth proteins, that are presumably secreted via the Hrp system of the cotton pathogen *Xanthomonas campestris* pv. *malvacearum* (*Xcm*). Some members (e.g., Avrb6) of this family are also required for bacterial virulence in susceptible cotton cultivars. A yeast two-hybrid system was employed to identify cotton receptors of Avrb6. After screening over 1.8×10^8 cotton cDNA clones, I confirmed that over 152 clones encode proteins that specifically bind to the bait, the central repeat region of Avrb6. Sequencing analysis revealed three clones, a serine/threonine kinase homologous to the resistance protein Pto in tomato, a calcineurin B-like protein, and a putative transcription factor with a C_2H_2-type zinc-finger motif. The functional relevance of these cotton proteins to *Xcm* avirulence and virulence in plants remains to be determined.

To my parents

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Chapter 1

LITERATURE REVIEW AND INTRODUCTION

Plant disease has had disastrous effects on crop production and the well-being of human populations. The range of phytopathogenic organisms that attack plants is diverse and includes viruses, mycoplasmas, bacteria, fungi, nematodes, protozoa, and parasites. Each has a unique mode of pathogenicity (Staskawicz *et al.*, 1995). Plant disease can result in enormous economic loss. For example, the worldwide losses of major crops, including rice, maize, potatoes, soybeans, cotton and coffee, were estimated to be 13.3% ($76.9 billion) from 1988-1990 (Baker *et al.*, 1997). Protection of crops from disease can substantially improve agricultural production. Thoughtful application of new technologies based on molecular understanding of plant defense mechanisms, combined with understanding of the complex ecology of the disease process, can lead to more effective protection strategies against plant pathogens.

1.1. Plant Responses to Pathogen Attack

Microbial pathogens are responsible for numerous diseases in higher plants, animals, and humans worldwide, including such common ones as rice leaf blight, apple fire blight, and plague (He, 1998). Plants are sessile organisms that lack a circulating, somatically adaptive immune system to protect themselves against pathogens. They instead have evolved other mechanisms for defense against a spectrum of pathogens. Plants are, in fact, resistant to most microorganisms by means of constitutive chemical or

physical barriers such as the plant cuticle which consists of layers of wax that a potential pathogen must penetrate or bypass to incite disease. Disease is therefore the exception rather than the rule when microbes and plants meet. Yet, yield loss due to plant disease remains an important component of modern agriculture, as many pathogens are evolutionarily specialized to overcome preformed defense barriers (Dangl and Holub, 1997).

1.1.1. Hypersensitive Response (HR)

Plant strategies used to inhibit the growth of pathogens include the activation of localized cell death (classically referred to as the hypersensitive response, HR) (Greenberg, 1997), the release of reactive oxygen intermediates (Lamb and Dixon, 1997) and nitric oxide (Delledonne *et al.*, 1998; Durner *et al.*, 1998), the fortification of plant cell walls, and the production of numerous antimicrobial secondary metabolites and defense-related proteins (Hammond-Kosack and Jones, 1997). Physiological features associated with the HR include a rapid oxidative burst, ion fluxes characterized by K^+-H^+ exchange, cellular decompartmentalization, cross-linking and strengthening of the plant cell wall, production of antimicrobial compounds (phytoalexins), and induction of pathogenesis-related (PR) proteins such as chitinases and glucanases (van Kan *et al.*, 1992). Although the molecular mechanism is obscure, the HR is likely to be important for limiting a pathogen's nutrient supply, since the dying tissue rapidly becomes dehydrated (Staskawicz *et al.*, 1995).

Programmed cell death (PCD) is the active process of cell death that occurs during development and in response to environmental cues (Greenberg *et al.*, 1994). The

HR appears to be a form of PCD in plants. The appearance of HR is genetically controlled and purified HR-inducing factors from bacteria, called harpins, will not induce the HR unless the plant tissue is transcriptionally active (He *et al.*, 1993). In addition, HR-inducing bacteria will not cause the HR if protein synthesis is blocked in the plant. In the search for a signal for HR induction, several groups have determined that H_2O_2 is rapidly produced by plant cells in culture during the HR in a phenomenon termed the oxidative burst. Levine *et al.* (1994) showed that enhancing H_2O_2 production during the HR leads to dramatic increases in the amount of cell death observed in a soybean cell culture system. Alvarez *et al.* (1998) showed that inoculation of *Arabidopsis* leaves with avirulent *Pseudomonas syringae* induced secondary oxidative bursts in discrete cells in distant tissues, leading to low-frequency systemic micro-HRs. The primary oxidative burst induces these systemic responses, and both the primary burst and the secondary microbursts are required for systemic immunity. Nitric oxide, which acts as a signal in the immune, nervous and vascular systems, was found to potentiate the induction of hypersensitive cell death in soybean cells by reactive oxygen intermediates and function independently of such intermediates to induce genes for the synthesis of protective natural products. Moreover, inhibitors of nitric oxide synthesis compromise the hypersensitive disease-resistance response of *Arabidopsis* leaves to *Pseudomonas syringae*, promoting disease and bacterial growth (Delledonne, 1998).

In addition, the induction of the HR by some pathogens and elicitors may be mechanistically similar to apoptosis in animals, since apoptotic features such as DNA breaks with 3'OH ends, blebbing of the plasma membrane, as well as nuclear and cytoplasmic condensation are present in some cells undergoing the HR. In some cases

the HR is also accompanied by internucleosomic DNA cleavage, another apoptosis-associated event (Mittler *et al.*, 1995; Ryerson and Heath, 1996). In addition, some caspase inhibitors specifically inhibit *Pseudomonas syringae* pv. *tomato* DC3000-induced HR on tobacco (unpublished data from our laboratory). Programmed cell death, therefore, is likely involved in plant defense against pathogenic attack.

1.1.2. The Gene-for-Gene Hypothesis

Many instances of plant disease resistance follow the gene-for-gene model. It was first proposed by Flor, who demonstrated that resistance of flax to the fungal pathogen *Melampsora lini* was a consequence of the interaction of paired cognate genes in the host and the pathogen (Flor, 1971). Genetic analyses in many systems have demonstrated that recognition functions are provided by dominant alleles of genes in the plant (Resistance, or *R*-genes) that interact, either directly or indirectly, with either the direct or indirect product of a pathogen gene (avirulence, or *avr* gene). The gene-for-gene hypothesis is a genetic explanation for the interaction between plants and representatives of all classes of pathogens: fungi, bacteria, viruses, and insects.

The biochemical explanation of gene-for-gene resistance is not known for most cases. However, evidence suggests that molecular recognition of some bacterial avirulence determinants occurs inside plant cells by resistance proteins, either directly or indirectly. This hypothesis is based primarily on research in bacteria showing that several avirulence proteins function inside the plant to induce resistance-gene-dependent cell death (Gopalan *et al.*, 1996; Leister *et al.*, 1996; Scofield *et al.*, 1996; Tang *et al.*,

1996; Van den Ackerveken *et al.*, 1996). More details will be discussed later in this chapter.

1.1.3. Disease Resistance (*R*) Genes

Plant disease resistance (*R*) genes confer an ability to resist infection by pathogens expressing specific corresponding avirulence genes. Intense effort in the past decade has led to successes in cloning *R* genes conferring resistance to a wide spectrum of plant pathogens including viruses, bacteria, fungi, and nematodes. A number of *R* genes have been isolated from several plant species (Hammond-Kosack and Jones, 1997). Many of them encode transmembrane or cytoplasmic proteins containing leucine-rich repeats (LRRs), which suggests protein-protein interactions.

R genes can be grouped into five classes (Baker *et al.*, 1997). The structural feature shared by their products is a leucine-rich repeat (LRR) motif and/or a serine-threonine kinase domain. The first class encodes cytoplasmic receptor-like proteins that contain an LRR domain and a nucleotide binding site (NBS). The family of genes encoding proteins with the LRR-NBS motif includes *RPS2*, *RPM1* and *RPS5* from *Arabidopsis* (Grant *et al.*, 1995; Mindrinos *et al.*, 1994; Warren *et al.*, 1998); *Prf* from tomato (Salmeron *et al.*, 1996); *N* from tobacco (Whitham *et al.*, 1994); L^6 and *M* from flax (Lawrence *et al.*, 1995); *RPP5* from *Arabidopsis* (Parker *et al.*, 1997); I_2 from tomato (Ori *et al.*, 1997); *Cre3* from wheat (Lagudah *et al.*, 1997); *Mi-1* from *Lycopersicon peruvianum* (Vos *et al.*, 1998); and *Dm3* from lettuce (Meyers *et al.*, 1998). The second class of genes includes *Pto*, which confers resistance to strains of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* that carry the *avrPto* gene (Martin *et al.*, 1993). *Pto* is

the first 'gene-for-gene' *R* gene to be cloned from tomato. Unlike either of the LRR classes of *R* genes, Pto is a member of the Raf family of serine-threonine protein kinases. The third class includes *Cf-2* and *Cf-9* from tomato and $HS1^{pro-1}$ from sugar beet. *Cf-2* (Dixon *et al.*, 1996) and *Cf-9* (Jones *et al.*, 1994) encode putative transmembrane receptors with large extracytoplasmic LRR domains and confer resistance to different races of *Cladosporium fulvum*. $HS1^{pro-1}$ (Cai *et al.*, 1997) encodes a transmembrane LRR protein that confers resistance to the beet cyst nematode *Heterodera schachtii*. The fourth class is represented by the rice gene *Xa21*, which confers resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Song *et al.*, 1995). *Xa21* encodes a putative transmembrane receptor with an extracellular LRR domain and an intracellular serine-threonine kinase domain. The Xa21 structure suggests an evolutionary link between LRR proteins (Cf) and kinases (Pto). The fifth class includes the *HM1* gene, which confers resistance to the fungal pathogen *Cochliobolus carbonum* race 1 (Johal and Briggs, 1992). *HM1* encodes a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductase that inactivates the host-selective toxin produced by *C. carbonum* race 1. *HM1* is distinct from other *R* genes because an Avr component is not involved in toxin degradation by the *HM1* product. The common features among different *R* gene products suggest that certain signaling events are held in common in plant defense.

1.2. Bacterial Pathogenicity

The common Gram-negative phytopathogenic bacteria include *Agrobacterium*, *Erwinia*, *Pseudomonas*, *Xanthomonas*, and *Ralstonia*. Any given type of symptom can be caused by bacterial pathogens belonging to several genera, and each genus may

contain pathogens capable of causing different types of diseases, such as leaf spots and blights; soft rots of fruits, roots, and storage organs; wilts; overgrowths; scabs; and cankers. Bacterial pathogens have evolved a variety of strategies to subvert host cellular functions in order to secure their multiplication and survival. These strategies are often very complex and fine-tuned, particularly in the case of pathogens that have co-evolved or have had a long-standing association with their hosts (Galan and Bliska, 1996).

1.2.1. Pathogenicity Islands

Since the early 1980s, independent molecular characterization of genes controlling bacterial pathogenicity of animals and plants have led to the discovery of large clusters of pathogenicity genes. Such gene clusters are either in extrachromosomal elements, such as large plasmids in *Ralstonia solanacearum* (Arlat *et al.*, 1996), *Yersinia* (Goguen *et al.*, 1984), and *Shigella* (Sansonetti *et al.*, 1982), or in specific chromosomal regions called pathogenicity islands (Hacker *et al.*, 1997), such as those in *Salmonella* (Mills *et al.*, 1995), *Erwinia amylovora* (Barny *et al.*, 1990), *Pseudomonas syringae* (Lindgren *et al.*, 1986), *Xanthomonas campestris* (Bonas *et al.*, 1991), and enteropathogenic *Escherichia coli* (EPEC) (Jarvis *et al.*, 1995).

A pathogenicity island can be defined according to the following criteria (Hacker *et al.*, 1997):

i) Presence of (often many) virulence genes

ii) Presence in pathogenic strains and absence or sporadic distribution in less-pathogenic strains of one species or a related species

iii) Different G-C content in comparison to DNA of host bacterium

iv) Occupation of large chromosomal regions (often 30 kb)

v) Represent compact, distinct genetic units, often flanked by direct repeats

vi) Association with tRNA genes and/or insertion sequence (IS) elements at their boundaries

vii) Presence of (often cryptic) 'mobility' genes (IS elements, integrases, transposases, origins of plasmid replication)

viii) Instability

The G-C content of pathogenicity islands usually does not match that of other bacterial DNA. For example, the human intestinal bacterium *Escherichia coli* has an average G-C content of 51%. But the genes in the diarrhea-causing enteropathogenic strain of *E. coli* are on a pathogenicity island with a G-C content of 38% (Barinaga, 1996). It is important to point out that the clustering of genes and the sometimes unusual G-C content of these gene clusters are often taken as evidence for horizontal transfer of an entire block of genes with related function. Acquisition of an entire virulence system in a single step would potentially enable an otherwise nonpathogenic or weakly pathogenic bacterium to become highly virulent on a new host. Acquiring a pathogenicity island would therefore be a major evolutionary step in the life of a pathogen.

1.2.2. The Type III Secretion System in Bacteria

In the past decade, research into the molecular basis of bacterial pathogenesis has led to the conclusion that although different bacteria may use unique mechanisms to subvert hosts, a few strategies are common. A recent development is the identification of

a highly specialized protein secretion system in several Gram-negative bacteria. This system, which is termed type III, has evolved to deliver proteins from the bacterial cytoplasm to the host cytosol (Salmond and Reeves, 1993). These bacterial proteins can then stimulate or interfere with host cellular processes, thereby dictating the terms of the bacterial-host cell interaction. Type III secretion systems are present in both animal and plant pathogenic bacteria, which indicate that they are capable of operating not only across bacterial genera but also across host kingdoms (Galan and Collmer, 1999).

1.2.2.1. The Type III Protein Secretion System

Pathogenicity of Gram-negative bacteria is commonly found to require the type III secretion system. Type III secretion systems have three distinguishing features: (i) the absence in the secreted proteins of a cleavable signal peptide that is characteristic of proteins secreted via the *sec*-mediated general secretion pathway; (ii) the requirement for customized accessory proteins (chaperones) for many of the secreted proteins; and (iii) a widespread requirement of host cell contact for full activation of the secretion pathway (Galan and Collmer, 1999).

The architecture of the cell envelope of Gram-negative bacteria dictates that proteins destined to be delivered to the outside must traverse several barriers: the inner membrane, the periplasmic space, the peptidoglycan layer, and finally the outer membrane (Agrios, 1997). Consequently, these bacteria have evolved a variety of mechanisms to transfer proteins from the cytoplasm to the extracellular environment (Pugsley, 1993). Type III secretion systems are distinct from those of Types I and II (Salmond, 1994). Type I systems are *sec*-independent, involving secretion via a one-step

mechanism through a channel or gated pore formed between the inner and outer membranes (Pugsley, 1993; Salmond, 1994). Type II pathways (also known as the *sec* or general secretion pathways) involve a two-step mechanism. The first step involves export from the cytoplasm to the periplasm via a *sec*-dependent mechanism requiring cleavage of a NH_2-signal sequence, and the second step involves transport across the outer membrane (Pugsley, 1993; Salmond, 1994). It is believed that all enzymes secreted via this pathway are synthesized with a classical N-terminal signal sequence and are processed by signal peptidase en route to the periplasm (Salmond, 1994).

All known type III secretion systems of animal and plant pathogenic bacteria share a number of core structural components that are highly conserved (Hueck, 1998; Lee, 1997). These components can be divided into at least two groups. One group consists of predicted outer membrane proteins, including a protein with sequence similarity to the secretin family of protein transporters, as well as several less conserved lipoproteins. The other group consists of several integral membrane proteins with close similarity to components of the flagellar export apparatus.

Type III secretion systems are used for different purposes in different bacteria, reflecting the distinct habits of these pathogens (He, 1998). Plant pathogenic bacteria are extracellular pathogens. The type III secretion system is believed to be used by these bacteria to secrete virulence proteins that ultimately cause leakage of plant nutrients to the extracellular space (apoplast) of infected tissues. In contrast, intracellular pathogens such as *Salmonella* spp. and *Shigella* spp. use type III secretion systems for invasion of host cells (Galan and Bliska, 1996; Menard *et al.*, 1996). *Yersinia* spp. also invades host cells, but the type III secretion system is not used for invasion. Rather, it is used to resist

the uptake of bacteria by both phagocytes and non-phagocytic cells in the later stage of pathogenesis, when bacteria are located extracellularly.

For historical reasons, different names have been assigned in different bacteria to many apparently functional homologues of the same gene. Recently, efforts have been made to streamline the nomenclature of type III secretion genes in plant pathogenic bacteria so that at least nine conserved *hrp* genes have now been re-named *hrc* (for *hrp* conserved) genes and given the last-letter designation of their *Yersinia ysc* homologues (Bogdanove *et al.*, 1996).

1.2.2.2. *hrp* Genes

Components of the type III secretion systems of plant pathogenic bacteria are encoded by *hrp* (for hypersensitive response and pathogenicity) genes (Lindgren *et al.*, 1986). *hrp* genes are so named because they are required for the bacterium to cause disease in susceptible plants and to elicit the hypersensitive response (HR) in resistant plants.

hrp mutant bacteria behave very much like saprophytic bacteria that do not have any apparent interactions with plants. Since the first publications on *hrp* genes in the plant pathogenic bacterium *P. syringae* (Lindgren *et al.*, 1986), three additional bacterial species have been used as model systems for the study of the organization and function of *hrp* genes: *E. amylovora*, *Ralstonia* (formerly *Pseudomonas*) *solanacearum*, and *X. campestris*.

Analysis of *hrp* gene organization, sequence relatedness, and regulatory systems indicates that *hrp* genes in *P. syringae* and *E. amylovora* belong to one group (group I),

whereas *hrp* genes in *X. campestris* and *R. solanacearum* form another group (group II) (Alfano *et al.*, 1997). *hrp* genes in *P. syringae* pv. *syringae* (*Pss*) strain 61 and *E. amylovora* Ea321 are clustered in a single 25 to 30-kb chromosomal region to form a pathogenicity island. A cosmid pHIR11, which contains the whole *hrp* gene cluster of *Pss* 61, enabled *Escherichia coli* TB1 to elicit an HR-like reaction when infiltrated into tobacco leaves, thus defining the minimum number of genes required for HR elicitation by these bacteria (Huang *et al.*, 1988).

There are 27 genes in the *P. syringae hrp* gene cluster (Fig. 1.1). Three (*hrpL*, *hrpS*, and *hrpR*) are involved in the up-regulation of many pathogenicity-related genes (e.g. *hrp*, *avr*) in response to the nutrient-poor condition in the plant apoplast, where bacteria proliferate (Xiao *et al.*, 1994). Three (*hrpA*, *hrpZ*, and *hrpW*) encode proteins that are secreted outside bacteria in *hrp*-inducing conditions (Charkowski *et al.*, 1998; He *et al.*, 1993; Yuan and He, 1996). Most of the other *hrp* genes are believed to be involved in protein secretion.

Experiments using reporter genes have demonstrated that *hrp* genes are actively transcribed in plants and in minimal media, but are repressed in complex media (Arlat *et al.*, 1992; Lindgren *et al.*, 1989; Rahme *et al.*, 1992; Schulte and Bonas, 1992; Wei *et al.*, 1992; Xiao *et al.*, 1992). Expression of *hrp* genes is affected by various carbon and nitrogen sources, pH, osmolarity, temperature, and possibly plant signal molecules (Arlat *et al.*, 1992; Rahme *et al.*, 1992; Schulte and Bonas, 1992; Wei *et al.*, 1992; Xiao *et al.*, 1992). It is widely believed that the minimal media mimic the apoplastic environment of plants (Rahme *et al.*, 1992). In contrast to *in planta* growth, *hrp* mutants are not impaired in their ability to grow in culture, since they are able to grow in minimal media (Lindgren

et al., 1986). Thus, *hrp* genes do not encode products required for general growth or metabolism.

hrp gene clusters were categorized as pathogenicity islands (Alan Collmer, personal communication). Other genes whose products affect plant-reaction phenotypes are sometimes physically linked to *hrp* gene clusters as part of pathogenicity islands. For example, some *avr* genes are found linked to *hrp* clusters in strains of *P. syringae* (Lorang and Keen, 1995; Mansfield *et al.*, 1994). The *hrmA* locus is adjacent to the *P. s.* pv. *syringae* 61 *hrp* cluster (Heu and Hutcheson, 1993). *dsp* (for disease-specific) genes are also linked to *hrp* regions of *E. amylovora* (Barny *et al.*, 1990) and *R. solanacearum* (Arlat *et al.*, 1992); inactivation of *dsp* genes results in mutants that are nonpathogenic or reduced in virulence but still retain the ability to elicit the HR.

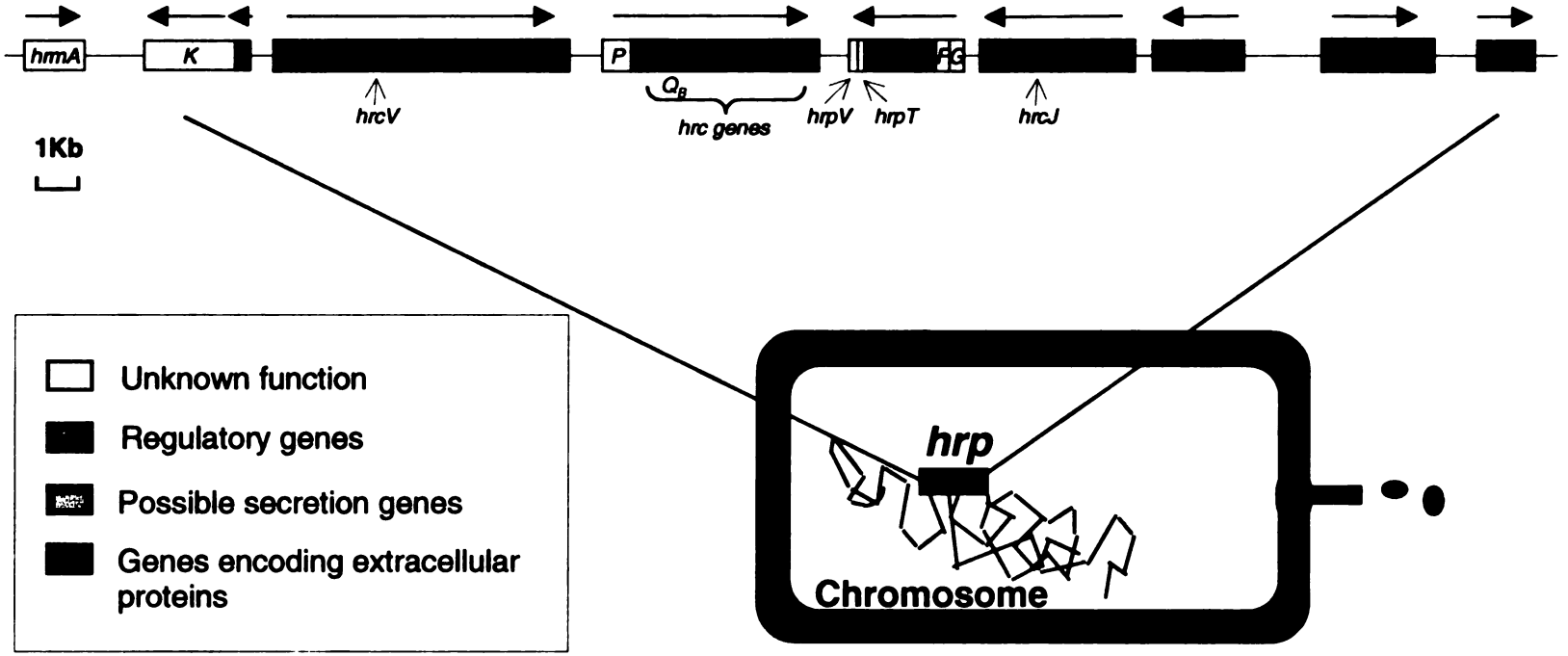

Figure 1.1. The *hrp* gene cluster of *Pseudomonas syringae*. Arrows indicate the direction of transcription for each operon. Regulatory and secretion genes are indicated by blue and red boxes, respectively. Genes encoding extracellular proteins are indicated by green boxes. Genes of unknown biochemical functions are indicated by yellow boxes. *hrmA* has recently shown to be an *avr*-like gene.

1.2.2.3. The Regulation Cascade Controlling the Hrp System

In *Pseudomonas syringae* pv. *syringae* 61 (*Pss* 61), *hrpR*, *hrpS*, and *hrpL* have been shown to be part of a multi-component regulatory system for the expression of *hrp* and *avr* genes. HrpR and HrpS show high sequence similarities to each other and are related to the NtrC class of response regulators (Grimm *et al.*, 1995; Grimm and Panopoulos, 1989; Xiao *et al.*, 1994). Proteins of the NtrC class are members of two-component regulatory systems, consisting of an environmental sensor and a response regulator, involved with the transcriptional activation of genes required for a wide variety of metabolic processes (Albright *et al.*, 1989). HrpR and HrpS differ from most members of the NtrC family in lacking the amino-terminal domain that modulates the regulatory activity; however, they do contain the conserved carboxy-terminal DNA-binding domain (Grimm *et al.*, 1995; Grimm and Panopoulos, 1989; Xiao *et al.*, 1994).

Studies have demonstrated that HrpL shares homology with the ECF (extra cytoplasmic functions) family of response regulators (Lonetto *et al.*, 1994; Xiao *et al.*, 1994). *hrpL* contains a conserved σ^{54} motif in its promoter region, which is consistent with a requirement for *rpoN* for its activation (Morett and Segovia, 1993; Xiao *et al.*, 1994). A consensus sequence has also been found in the promoter region of a number of *Pss hrp* genes; this region functions as a HrpL-dependent promoter (Xiao and Hutcheson, 1994). Significantly, this sequence, GGAACCNA-N14-CCACNNA, was originally identified in the promoter regions of *Pss avr* genes (Innes *et al.*, 1993) and has been referred to as the *hrp*- or *avr*-box (Innes *et al.*, 1993; Lorang and Keen, 1995).

It is believed that the regulatory cascade for the transcription of *P. syringae hrp* genes involves the activation of the *hrpL* promoter by the HrpS and HrpR proteins in

conjunction with RpoN (Xiao *et al.*, 1994). The HrpL protein then acts as an alternative sigma factor for the expression of HrpL-responsive *avr* and *hrp* genes (Xiao *et al.*, 1994), which usually have "*hrp*-box" *cis*-element in their promoters. A slightly different regulatory cascade may be operative in *P. s.* pv. *phaseolicola*, as genetic analysis indicated that HrpR functions as a transcriptional activator controlling *hrpS* expression (Grimm *et al.*, 1995).

Erwinia amylovora contains genes sharing homology with *P. syringae hrpL*, suggesting that these bacteria contain certain common regulatory elements (Wei and Beer, 1995).

1.2.2.4. Secreted Proteins and Secretion Signals of the Hrp Secretion System

There appear to be two classes of proteins that traverse the Hrp secretion system. The first class of proteins, exemplified by harpin proteins, is secreted in *hrp*-inducing minimal medium. The other class of proteins, typified by avirulence (Avr) proteins, appear to be secreted through the Hrp system directly into the plant cell (He, 1998).

HrpN of *E. amylovora* was the first Hrp protein shown to elicit an HR in tobacco (Wei *et al.*, 1992), and it was subsequently shown to be secreted via the Hrp system (Wei and Beer, 1993). The properties of HrpN define the characteristics of the class of HR elicitors known as harpins: They are hydrophilic, rich in glycine, heat stable, lack cysteine, and elicit HR when injected into the apoplast of certain plants. In *P. syringae* pv. *syringae*, the *hrpZ* gene product, HrpZ (or $harpin_{Pss}$), was found to exhibit HR-eliciting activity in the tobacco leaf apoplast. HrpZ shares many properties with HrpN and therefore is also a member of the Harpin family of proteins. HrpZ was also shown to

traverse the Hrp secretion pathway (He *et al.*, 1993). Homologues of HrpZ are present in all *P. syringae* pathovars surveyed (He *et al.*, 1993). HrpZ was found to be essential but not sufficient for HR elicitation by saprophytic bacteria carrying pHIR11 (Alfano *et al.*, 1996).

In addition to secreting HrpZ, *P. syringae* pv. *tomato* (*Pst*) strain DC3000 also secretes at least three other proteins (EXP-60, EXP-22, and EXP-10) via the Hrp system in culture (Yuan and He, 1996). The EXP-60 is HrpW, whose gene is immediately adjacent to the right border of the *hrp* gene cluster in *Pst* DC3000 (Charkowski *et al.*, 1998). *hrpW* encodes a 42.9-kDa protein with domains resembling harpins and pectate lyases (Pels). HrpW indeed has HR-eliciting activity, but it does not have measurable pectate lyase activity. EXP-10 is encoded by the *hrpA* gene situated adjacent to the *hrpZ* gene in the same operon. The primary sequence of HrpA, a small, hydrophilic protein, shares no homology with any other proteins (Preston *et al.*, 1995). The exact function of the *hrpA* gene was unknown.

avr genes mediate the elicitation of hypersensitive disease resistance in plants that contain the corresponding disease resistance genes (Leach and White, 1996). However, the phenotypic expression of *avr* genes in bacteria requires *hrp* genes (Huynh *et al.*, 1989). In fact, a 25-kb *hrp* gene cluster of *P. syringae* pv. *syringae* strain 61 appears sufficient to enable *E. coli*, which otherwise has no apparent interaction with plants, to deliver Avr proteins to the plant (Gopalan *et al.*, 1996; Pirhonen *et al.*, 1996).

Type III-dependent secretion of some effector proteins can be detected in cultures of induced mammalian bacterial pathogens in the absence of the host (Hueck, 1998). However, secretion of avirulence proteins from plant bacterial pathogens has been

difficult to detect. Only *E. amylovora* has been shown to secrete an avirulence protein, DspE (DspA), into bacterial cultures in a *hrp*-dependent manner (Bogdanove *et al.*, 1998; Gaudriault *et al.*, 1997).

The recent development of a heterologous *E. coli hrp* secretion system capable of secreting copious amounts of avirulence proteins into bacterial cultures has provided an alternative method for studying the *hrp* secretion pathway (Ham *et al.*, 1998). Using this system, the secretion of AvrB, AvrPto, and AvrRpt2 was efficiently detected (Ham *et al.*, 1998; Mudgett and Staskawicz, 1999).

It is apparently disadvantageous for bacteria to send their avirulence proteins into the plant cell because recognition of Avr proteins by the corresponding plant receptors in resistant plants triggers rapid plant cell death and other defense response, which lead to plant resistance to bacteria. This puzzle can be explained that bacterial proteins that traverse the Hrp secretion system originally serve virulence functions. However, during host-bacterium coevolution, plants have recognized some of these virulence proteins as elicitors of their defense response. The virulence factor therefore becomes avirulence factor (He, 1998). As a matter of fact, it's already been found some *avr* proteins are actually virulence factors when they are in different plant-bacterial interactions, such as *avrBs2* of *X. campestris* pv. *vesicatoria* (Kearney and Staskawicz, 1990), *avr/pth* genes of *X. campestris* pv. *malvacearum* (Swarup *et al.*, 1992; Yang *et al.*, 1994), *avrA* and *avrE* of *P. syringae* pv. *tomato* (Lorang *et al.*, 1994), and *avrRpm1* of *P. syringae* pv. *maculicola* (Ritter and Dangl, 1995).

Proteins traversing the type III pathway carry multiple signals that route them to the secretion pathway. No common amino acid or peptide sequence has been identified

among the secretion signals. Experiments carried out with YopE and YopN, two type III secreted proteins of the enteropathogen *Yersinia*, revealed that the first ~15 amino acids of these proteins were sufficient to direct the secretion of a heterologous protein (Cornelis *et al.*, 1998; Sory *et al.*, 1995). However, frameshift mutations that completely altered the peptide sequences of these signals failed to prevent secretion. Thus, the signal that leads to the type III secretion of Yop proteins appears to be encoded in their messenger RNA rather than in the peptide sequence (Anderson and Schneewind, 1997). The 5' mRNA regions of some of the *yop* messages are predicted to have stem-loop structures that bury AUG translational signals, which suggests that the translation of these proteins may be arrested until the 5' mRNA interacts with a component of the secretion apparatus. The secretion signals of two proteins from the plant pathogen *P. syringae*, AvrB and AvrPto, also appear to reside in their 5' mRNA, which suggests that this type of substrate recognition may be a general feature of type III secretion systems (Galan and Collmer, 1999).

Some type III secreted proteins can be secreted posttranslationally through an alternative secretion signal (Anderson and Schneewind, 1997; Cheng *et al.*, 1997). This alternative mechanism involves the function of specific chaperones that bind the cognate secreted proteins on discrete domains located within the first 100 amino acids of many proteins that travel the type III pathway (Cornelis *et al.*, 1998; Sory *et al.*, 1995; Wattiau *et al.*, 1994; Wattiau and Cornelis, 1993). DspF of *Erwinia amylovora* and AvrF of *Pseudomonas syringae* are small (16 kDa and 14 kDa) and acidic with predicted amphipathic alpha helices in their C termini; they resemble chaperones for virulence

factors secreted by type III secretion systems of animal pathogens (Bogdanove *et al.*, 1998).

1.2.3. Similarity between Type III Secretion System and Flagellar Assembly

At least eight type-III secretion proteins share sequence similarities with flagellar assembly components (Bogdanove *et al.*, 1996). Intriguingly, type-III secretion components of plant pathogenic bacteria are more closely related to components of the flagellar machinery than are their counterparts in animal pathogenic bacteria. Thus, it is possible that type III secretion systems first emerged in plant pathogenic bacteria as an evolutionary adaptation of the flagellar export apparatus in order to secrete proteins other than flagellin, thereby assisting bacteria to form a close association with plant cells (Galan and Collmer, 1999). Given the similarity of the type III proteins to those that assemble flagella, it is tempting to hypothesize that they form a flagellum-like structure that secretes and translocates proteins into the host cells (Barinaga, 1996).

1.2.4. Interkingdom Transport Hypothesis

The *avr* genes interact genetically with their corresponding disease resistance genes in plants to trigger resistance responses in a gene-for-gene manner (Leach and White, 1996). However, purified bacterial Avr proteins elicit no response when added to the plant apoplast, where bacteria proliferate (Gopalan *et al.*, 1996; Leach and White, 1996), and Avr proteins have not been detected outside bacteria *in planta* (Gopalan *et al.*, 1996; Leach and White, 1996).

Amino acid sequence analysis predicts that cloned plant disease resistance genes corresponding to known bacterial *avr* genes encode intracellular proteins instead of cell surface receptors (Bent, 1996). With the exception of AvrD of *Pseudomonas syringae* pv. *tomato* (Kobayashi *et al.*, 1990), none of the bacterial Avr proteins has been shown to possess any catalytic activity that could generate diffusible, low-molecular-weight elicitors of the HR (Leach and White, 1996). Thus, the biochemical basis of the activity of bacterial *avr* gene products has remained elusive.

The type III secretion systems in animal pathogenic bacteria translocate effector proteins from the bacteria to their host cells (Gauthier and Finlay, 1999). The functional dependence of Avr proteins on *hrp* genes suggests possible transport of Avr proteins into the interior of the plant cell via the Hrp secretion system. Indeed, using transgenic and transient expression of AvrB of *P. syringae* in leaves of *Arabidopsis thaliana*, Gopalan *et al.* (1996) showed that the putative delivery of AvrB into plant cells required the Hrp secretion system.

Similar evidence supporting interkingdom transport of Avr proteins has been obtained independently for *P. syringae* pv. *tomato* AvrPto (Scofield *et al.*, 1996; Tang *et al.*, 1996), AvrRpt2 (Leister *et al.*, 1996) and *X. campestris* pv. *vesicatoria* AvrBs3 (Van den Ackerveken *et al.*, 1996). In the case of AvrPto, the tomato disease resistance gene *Pto* product has been shown to bind specifically to AvrPto in the yeast two-hybrid system (Scofield *et al.*, 1996; Tang *et al.*, 1996).

The AvrBs3 family of Avr proteins was found to contain functional plant nuclear localization signals (NLSs) that target the reporter protein β-glucuronidase (GUS) to the plant nucleus (Yang and Gabriel, 1995). It has now been shown that these NLSs are

required for the AvrBs3 family of proteins to trigger disease resistance-associated HR, suggesting that some members of the AvrBs3 family enter not only the plant cell but also the plant nucleus (Van den Ackerveken *et al.*, 1996).

1.3. Project Summary

Plant pathogenic bacteria are extracellular pathogens, and transport of bacterial proteins requires passage of the effector proteins across not only the bacterial envelope but also the plant cell wall (>100 nm thick) and plasmalemma. This is in contrast to animal-bacterium interactions, in which protein transfer can be accomplished by direct contact between the membranes of bacteria and host cells (Cornelis and Wolf-Watz, 1997). How do bacteria manage to overcome the plant cell wall and send their proteins into host cells? How do bacteria sense host contact? Are supramolecular appendages assembled by the Hrp system? Is there a host receptor protein capable of physically binding to virulence or avirulence proteins? As the key to plant bacterial pathogenicity, the *hrp*-encoded type III protein secretion system has remained mysterious.

In these thesis projects, I addressed some of these questions. *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) was used as a model pathogenic bacterium. The species *P. syringae* is divided into pathovars largely on the basis of host specificity, i.e., *P. syringae* pathovars *syringae* (brown spot of beans), *tomato* (bacterial speck of tomatoes), and *glycinea* (bacterial blight of soybeans) (Alfano and Collmer, 1997). *Pst* DC3000 is a pathogen of both tomato and *Arabidopsis*, and it contains a functional *hrp* gene cluster as well as several avirulence genes, e.g., *avrPto*. All these features make it a good pathogenic bacterium to study the type III secretion system.

Chapter 2 describes my efforts to identify and characterize a novel bacterial surface appendage, called the Hrp pilus. This project was initiated by the finding that the HrpA protein is secreted outside bacteria in *hrp*-inducing conditions (Yuan and He, 1996). By constructing a non-polar *hrpA* mutant, I was able to demonstrate that the *hrpA* encodes the major structural protein of a pilus-like structure. I further demonstrated that *hrpA* is essential for *Pst* DC3000 to cause disease in the host plants and to elicit an HR in a resistant host or nonhost plant. This project was carried out in collaboration with Dr. Martin Romantschuk's group at the University of Helsinki, Finland. They independently made the observation that the HrpA protein is associated with a pilus-like structure. Results from chapter 2 suggest that the Hrp pilus plays a critical role in the pathogenicity of *Pst* DC3000.

Chapter 3 reports my research on determining the function of the Hrp pilus. Two models were proposed to describe the potential role of Hrp pilus in type III protein secretion: contact model and conduit model. Contact model suggests that the role of Hrp pilus is to physically attach the bacterium to plant cells. While the conduit model suggests that the Hrp pilus is directly involved in the protein delivery from bacterium to plant cell. I was especially interested in knowing whether the Hrp pilus is required for protein delivery. To test this model, I examined the secretion of HrpW in the *hrpA* mutant. I found that HrpW protein secretion was indeed blocked in the *hrpA* mutant in which the Hrp pilus is not produced. However, further study revealed that HrpW production was significantly suppressed in the *hrpA* mutant. This rather surprising finding prompted us to consider a possible involvement of the HrpA protein in *hrp* gene regulation. Subsequent western and northern blot analyses confirmed that the *hrpA*

mutation down-regulated expression of all examined *hrc/hrp* and *avr* genes at the RNA level. Furthermore, I demonstrated that *hrpA* acts through two previously known positive regulatory genes, *hrpR* and *hrpS*. Ectopic expression of *hrpR/S* genes restored gene expression in the *hrpA* mutant, but the secretion of HrpW and AvrPto was still blocked. These results suggest that the Hrp pilus plays a dual role in protein secretion and global regulation of the Hrp system.

Chapter 4 describes my efforts to identify cotton proteins that interact with Avrb6. Avrb6 belongs to the Avr/Pth family of proteins in *Xanthomonas campestris* pv. *malvacearum* (*Xcm*) and is believed to be targeted into the plant nucleus via the Hrp system. By employing a yeast two-hybrid system, I have isolated several cotton proteins that specifically interact with the bait protein, Avrb6ΔNC — the central repeat region of Avrb6. These proteins include a serine/threonine kinase highly homologous to Pto, a disease resistance gene product identified from tomato; calcineurin B; and a putative transcription factor containing a zinc-finger motif. The functional significance of these proteins in *Xcm* avirulence and virulence remains to be determined.

1.4. REFERENCES

Agrios, G. N. (1997). *Plant Pathology*, 4th ed. Edition (San Diego: Academic Press).

Albright, L. M., Huala, E., and Ausubel, F. M. (1989). Prokaryotic signal transduction mediated by sensor and regulator protein pairs. *Annu Rev Genet* **23**, 311-36.

Alfano, J. R., Bauer, D. W., Milos, T. M., and Collmer, A. (1996). Analysis of the role of the *Pseudomonas syringae* pv. *syringae* HrpZ harpin in elicitation of the hypersensitive response in tobacco using functionally non-polar *hrpZ* deletion mutations, truncated HrpZ fragments, and *hrmA* mutations. *Mol Microbiol* **19**, 715-28.

Alfano, J. R., and Collmer, A. (1997). The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. *J Bacteriol* **179**, 5655-62.

Alfano, J. R., Klm, H. S., Delaney, T. P., and Collmer, A. (1997). Evidence that the *Pseudomonas syringae* pv. *syringae hrp*-linked *hrmA* gene encodes an Avr-like protein that acts in an *hrp*-dependent manner within tobacco cells. *Mol Plant Microbe Interact* **10**, 580-8.

Alvarez, M. E., Pennell, R. I., Meijer, P. J., Ishikawa, A., Dixon, R. A., and Lamb, C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* **92**, 773-84.

Anderson, D. M., and Schneewind, O. (1997). A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. *Science* **278**, 1140-1143.

Arlat, M., Gough, C. L., Zischek, C., Barberis, P. A., Trigalet, A., and Boucher, C. A. (1992). Transcriptional organization and expression of the large *hrp* gene cluster of *Pseudomonas solanacearum*. *Mol Plant Microbe Interact* **5**, 187-93.

Arlat, M., Van Gijsegem, F., Huet, J. C., Pernollet, J. C., and Boucher, C. A. (1996). PopA1, a protein which induces a hypersensitivity-like response on specific Petunia genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. *Emerg Infect Dis* **2**, 270-88.

Baker, B., Zambryski, P., Staskawicz, B., and Dinesh-Kumar, S. P. (1997). Signaling in plant-microbe interactions. *Science* **276**, 726-33.

Barinaga, M. (1996). A shared strategy for virulence. *Science* **272**, 1261-3.

Barny, M. A., Guinebretiere, M. H., Marcais, B., Coissac, E., Paulin, J. P., and Laurent, J. (1990). Cloning of a large gene cluster involved in *Erwinia amylovora* CFBP1430 virulence. *Mol Microbiol* **4**, 777-86.

Bent, A. (1996). Plant disease resistance genes: function meets structure. *Plant cell* **8**, 1757-1771.

Bogdanove, A. J., Bauer, D. W., and Beer, S. V. (1998). *Erwinia amylovora* secretes DspE, a pathogenicity factor and functional AvrE homolog, through the Hrp (type III secretion) pathway. *J Bacteriol* **180**, 2244-7.

Bogdanove, A. J., Beer, S. V., Bonas, U., Boucher, C. A., Collmer, A., Coplin, D. L., Cornelis, G. R., Huang, H. C., Hutcheson, S. W., Panopoulos, N. J., and Van Gijsegem, F. (1996). Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. *Mol Microbiol* **20**, 681-3.

Bogdanove, A. J., Kim, J. F., Wei, Z., Kolchinsky, P., Charkowski, A. O., Conlin, A. K., Collmer, A., and Beer, S. V. (1998). Homology and functional similarity of an *hrp*-linked pathogenicity locus, *dspEF*, of *Erwinia amylovora* and the avirulence locus *avrE* of *Pseudomonas syringae* pathovar tomato. *Proc Natl Acad Sci U S A* **95**, 1325-30.

Bonas, U. S., R. Fenselau, S. Minsavage, G. V., Staskawicz, B. J., and Stall, R. E. (1991). Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. *Mol Plant Microbe Interact* **4**, 81-88.

Cai, D., Kleine, M., Kifle, S., Harloff, H. J., Sandal, N. N., Marcker, K. A., Klein-Lankhorst, R. M., Salentijn, E. M. J., Lange, W., Stiekema, W. J., Wyss, U., Grundler, F. M. W., and Jung, C. (1997). Positional cloning of a gene for nematode resistance in sugar beet. *Science* **275**, 832-4.

Charkowski, A. O., Alfano, J. R., Preston, G., Yuan, J., He, S. Y., and Collmer, A. (1998). The *Pseudomonas syringae* pv. *tomato* HrpW protein has domains similar to harpins and pectate lyases and can elicit the plant hypersensitive response and bind to pectate. *J Bacteriol* **180**, 5211-7.

Cheng, L. W., Anderson, D. M., and Schneewind, O. (1997). Two independent type III secretion mechanisms for YopE in *Yersinia enterocolitica*. *Mol Microbiol* **24**, 757-65.

Cornelis, G. R., Boland, A., Boyd, A. P., Geuijen, C., Iriarte, M., Neyt, C., Sory, M. P., and Stainier, I. (1998). The virulence plasmid of *Yersinia*, an antihost genome. *Microbiol Mol Biol Rev* **62**, 1315-52.

Cornelis, G. R., and Wolf-Watz, H. (1997). The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. *Mol Microbiol* **23**, 861-7.

Dangl, J., and Holub, E. (1997). La dolce vita: a molecular feast in plant-pathogen interactions. *Cell* **91**, 17-24.

Delledonne, M., Xia, Y., Dixon, R. A., and Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. *Nature* **394**, 585-8.

Dixon, M. S., Jones, D. A., Keddie, J. S., Thomas, C. M., Harrison, K., and Jones, J. D. (1996). The tomato *Cf-2* disease resistance locus comprises two functional encoding leucine-rich repeat proteins. *Cell* **84**, 451-9.

Durner, J., Wendehenne, D., and Klessig, D. F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc Natl Acad Sci U S A* **95**, 10328-33.

Flor, H. H. (1971). Current status of the gene-for-gene concept. *Annu Rev Phytopathol* **9**, 275-296.

Galan, J. E., and Bliska, J. B. (1996). Cross-talk between bacterial pathogens and their host cells. *Annu Rev Cell Dev Biol* **12**, 221-55.

Galan, J. E., and Collmer, A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**, 1322-8.

Gaudriault, S., Malandrin, L., Paulin, J. P., and Barny, M. A. (1997). DspA, an essential pathogenicity factor of *Erwinia amylovora* showing homology with AvrE of *Pseudomonas syringae*, is secreted via the Hrp secretion pathway in a DspB-dependent way. *Mol Microbiol* **26**, 1057-69.

Gauthier, A., and Finlay, B. B. (1999). Protein translocation: delivering virulence into the host cell. *Curr Opin Microbiol* **2**, 94-8.

Goguen, J. D., Yother, J., and Straley, S. C. (1984). Genetic analysis of the low calcium response in *Yersinia pestis* mu d1(Ap lac) insertion mutants. *J Bacteriol* **160**, 842-8.

Gopalan, S., Bauer, D. W., Alfano, J. R., Loniello, A. O., He, S. Y., and Collmer, A. (1996). Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (Hrp) secretion system in eliciting genotype-specific hypersensitive cell death. *Plant Cell* **8**, 1095-105.

Grant, M. R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Sattler, A., Innes, R. W., and Dangl, J. L. (1995). Structure of the *Arabidopsis* RPM1 gene enabling dual specificity disease resistance. *Science* **269**, 843-6.

Greenberg, J. (1997). Programmed cell death in plant-pathogen interactions. *Annual review of plant physiology and plant molecular biology* **48**, 525-545.

Greenberg, J. T., Guo, A., Klessig, D. F., and Ausubel, F. M. (1994). Programmed cell death in plants: a pathogen-triggered response activated coordinately with multiple defense functions. *Cell* **77**, 551-63.

Grimm, C., Aufsatz, W., and Panopoulos, N. J. (1995). The *hrpRS* locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. *Mol Microbiol* **15**, 155-65.

Grimm, C., and Panopoulos, N. J. (1989). The predicted protein product of a pathogenicity locus from *Pseudomonas syringae* pv. *phaseolicola* is homologous to a highly conserved domain of several procaryotic regulatory proteins. *J Bacteriol* **171**, 5031-8.

Hacker, J., Blum-Oehler, G., Muhldorfer, I., and Tschape, H. (1997). Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol Microbiol* **23**, 1089-97.

Ham, J. H., Bauer, D. W., Fouts, D. E., and Collmer, A. (1998). A cloned *Erwinia chrysanthemi* Hrp (type III protein secretion) system functions in *Escherichia coli* to deliver *Pseudomonas syringae* Avr signals to plant cells and to secrete Avr proteins in culture. *Proc Natl Acad Sci U S A* **95**, 10206-11.

Hammond-Kosack, K. E., and Jones, J. D. G. (1997). Plant disease resistance genes. *Annu Rev Plant Physiol and Plant Mol Biol* **48**, 575-607.

He, S. Y. (1998). Type III protein secretion systems in plant and animal pathogenic bacteria. *Annu Rev Phytopathol* **36**, 363-392.

He, S. Y., Huang, H. C., and Collmer, A. (1993). *Pseudomonas syringae* pv. *syringae* harpin$_{Pss}$: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell* **73**, 1255-66.

Heu, S., and Hutcheson, S. W. (1993). Nucleotide sequence and properties of the *hrmA* locus associated with the *Pseudomonas syringae* pv. *syringae* 61 *hrp* gene cluster. *Mol Plant Microbe Interact* **6**, 553-64.

Huang, H. C., Schuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutcheson, S. W., and Collmer, A. (1988). Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants. *J Bacteriol* **170**, 4748-56.

Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* **62**, 379-433.

Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. (1989). Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science* **245**, 1374-7.

Innes, R. W., Bent, A. F., Kunkel, B. N., Bisgrove, S. R., and Staskawicz, B. J. (1993). Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. *J Bacteriol* **175**, 4859-69.

Jarvis, K. G., Giron, J. A., Jerse, A. E., McDaniel, T. K., Donnenberg, M. S., and Kaper, J. B. (1995). Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proc Natl Acad Sci U S A* **92**, 7996-8000.

Johal, G. S., and Briggs, S. P. (1992). Reductase activity encoded by the *HM1* disease resistance gene in maize. *Science* **258**, 985-7.

Jones, D. A., Thomas, C. M., Hammond-Kosack, K. E., Balint-Kurti, P. J., and Jones, J. D. (1994). Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science* **266**, 789-93.

Kearney, B., and Staskawicz, B. J. (1990). Widespread distribution and fitness contribution of *Xanthomonas campestris* avirulence gene *avrBs2*. *Nature* **346**, 385-6.

Kobayashi, D. Y., Tamaki, S. J., and Keen, N. T. (1990). Molecular characterization of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. *Mol Plant Microbe Interact* **3**, 94-102.

Lagudah, E. S., Moullet, O., and Appels, R. (1997). Map-based cloning of a gene sequence encoding a nucleotide-binding domain and a leucine-rich region at the *Cre3* nematode resistance locus of wheat. *Genome* **40**, 659-65.

Lamb, C., and Dixon, R. (1997). The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol and Plant Mol Biol* **48**, 251-275.

Lawrence, G. J., Finnegan, E. J., Ayliffe, M. A., and Ellis, J. G. (1995). The L^6 gene for flax rust resistance is related to the *Arabidopsis* bacterial resistance gene *RPS2* and the tobacco viral resistance gene *N*. *Plant Cell* **7**, 1195-206.

Leach, J., and White, F. (1996). Bacterial avirulence genes. *Annu Rev Phytopathol* **34**, 153-179.

Lee, C. A. (1997). Type III secretion systems: machines to deliver bacterial proteins into eukaryotic cells? *Trends Microbiol* **5**, 148-56.

Leister, R. T., Ausubel, F. M., and Katagiri, F. (1996). Molecular recognition of pathogen attack occurs inside of plant cells in plant disease resistance specified by the *Arabidopsis* genes *RPS2* and *RPM1*. *Proc Natl Acad Sci U S A* **93**, 15497-502.

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Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **79**, 583-93.

Lindgren, P. B., Frederick, R., Govindarajan, A. G., Panopoulos, N. J., Staskawicz, B. J., and Lindow, S. E. (1989). An ice nucleation reporter gene system: identification of inducible pathogenicity genes in *Pseudomonas syringae* pv. *phaseolicola*. *EMBO J* **8**, 1291-301.

Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. (1986). Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity of nonhost plants. *J Bacteriol* **168**, 512-22.

Lonetto, M. A., Brown, K. L., Rudd, K. E., and Buttner, M. J. (1994). Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase sigma factors involved in the regulation of extracytoplasmic functions. *Proc Natl Acad Sci U S A* **91**, 7573-7.

Lorang, J. M., and Keen, N. T. (1995). Characterization of *avrE* from *Pseudomonas syringae* pv. *tomato*: a *hrp*-linked avirulence locus consisting of at least two transcriptional units. *Mol Plant Microbe Interact* **8**, 49-57.

Lorang, J. M., Shen, H., Kobayashi, D., Cooksey, D., and Keen, N. T. (1994). *avrA* and *avrE* in *Pseudomonas syringae* pv. *tomato* PT23 play a role in virulence on tomato plants. *Mol Plant Microbe Interact* **7**, 508-515.

Mansfield, J., Jenner, C., Hockenhull, R., Bennett, M. A., and Stewart, R. (1994). Characterization of *avrPphE*, a gene for cultivar-specific avirulence from *Pseudomonas syringae* pv. *phaseolicola* which is physically linked to *hrpY*, a new *hrp* gene identified in the halo-blight bacterium. *Mol Plant Microbe Interact* **7**, 726-39.

Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganal, M. W., Spivey, R., Wu, T., Earle, E. D., and Tanksley, S. D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* **262**, 1432-6.

Menard, R., Dehio, C., and Sansonetti, P. J. (1996). Bacterial entry into epithelial cells: the paradigm of Shigella. *Trends Microbiol* **4**, 220-6.

Meyers, B. C., Chin, D. B., Shen, K. A., Sivaramakrishnan, S., Lavelle, D. O., Zhang, Z., and Michelmore, R. W. (1998). The major resistance gene cluster in lettuce is highly duplicated and spans several megabases. *Plant Cell* **10**, 1817-32.

Mills, D. M., Bajaj, V., and Lee, C. A. (1995). A 40 kb chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent from the corresponding region of the *Escherichia coli* K- 12 chromosome. *Mol Microbiol* **15**, 749-59.

Mindrinos, M., Katagiri, F., Yu, G. L., and Ausubel, F. M. (1994). The *A. thaliana* disease resistance gene *RPS2* encodes a protein containing a nucleotide-binding site and leucine-rich repeats. *Cell* **78**, 1089-99.

Mittler, R., u Rutgers, T. S. U. o. N. J. P. N. J., Shulaev, V., and Lam, E. (1995). Coordinated activation of programmed cell death and defense mechanisms in transgenic tobacco plants expressing a bacterial proton pump. *Plant Cell* **7**, 29-42.

Morett, E., and Segovia, L. (1993). The sigma 54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. *J Bacteriol* **175**, 6067-74.

Mudgett, M. B., and Staskawicz, B. J. (1999). Characterization of the *Pseudomonas syringae* pv. *tomato* AvrRpt2 protein: demonstration of secretion and processing during bacterial pathogenesis. *Mol Microbiol* **32**, 927-41.

Ori, N., Eshed, Y., Paran, I., Presting, G., Aviv, D., Tanksley, S., Zamir, D., and Fluhr, R. (1997). The *I2C* family from the wilt disease resistance locus *I2* belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. *Plant Cell* **9**, 521-32.

Parker, J. E., Coleman, M. J., Szabo, V., Frost, L. N., Schmidt, R., van der Biezen, E. A., Moores, T., Dean, C., Daniels, M. J., and Jones, J. D. (1997). The *Arabidopsis* downy mildew resistance gene *RPP5* shares similarity to the toll and interleukin-1 receptors with *N* and L^6. *Plant Cell* **9**, 879-94.

Pirhonen, M. U., Lidell, M. C., Rowley, D. L., Lee, S. W., Jin, S., Liang, Y., Silverstone, S., Keen, N. T., and Hutcheson, S. W. (1996). Phenotypic expression of *Pseudomonas syringae avr* genes in *E. coli* is linked to the activities of the *hrp*-encoded secretion system. *Mol Plant Microbe Interact* **9**, 252-60.

Preston, G., Huang, H. C., He, S. Y., and Collmer, A. (1995). The HrpZ proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* are encoded by an operon containing *Yersinia ysc* homologs and elicit the hypersensitive response in tomato but not soybean. *Mol Plant Microbe Interact* **8**, 717-32.

Pugsley, A. P. (1993). The complete general secretory pathway in gram-negative bacteria. *Microbiol Rev* **57**, 50-108.

Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. (1992). Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*. *J Bacteriol* **174**, 3499-507.

Ritter, C., and Dangl, J. L. (1995). The *avrRpm1* gene of *Pseudomonas syringae* pv. *maculicola* is required for virulence on *Arabidopsis*. *Mol Plant Microbe Interact* **8**, 444-53.

Ryerson, D., and Heath, M. (1996). Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. *Plant Cell* **8**, 393-402.

Salmeron, J. M., Oldroyd, G. E., Rommens, C. M., Scofield, S. R., Kim, H. S., Lavelle, D. T., Dahlbeck, D., and Staskawicz, B. J. (1996). Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. *Cell* **86**, 123-33.

Salmond, G. P., and Reeves, P. J. (1993). Membrane traffic wardens and protein secretion in gram-negative bacteria. *Trends Biochem Sci* **18**, 7-12.

Salmond, G. P. C. (1994). Secretion of extracellar virulence factors by plant pathogenic bacteria. *Annu Rev Phytopathol* **32**, 181-200.

Sansonetti, P. J., Kopecko, D. J., and Formal, S. B. (1982). Involvement of a plasmid in the invasive ability of *Shigella flexneri*. *Infect Immun* **35**, 852-60.

Schulte, R., and Bonas, U. (1992). Expression of the *Xanthomonas campestris* pv. *vesicatoria hrp* gene cluster, which determines pathogenicity and hypersensitivity on and tomato, is plant inducible. *J Bacteriol* **174**, 815-23.

Scofield, S. R., Tobias, C. M., Rathjen, J. P., Chang, J. H., Lavelle, D. T., Michelmore, R. W., and Staskawicz, B. J. (1996). Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science* **274**, 2063-5.

Song, W. Y., Wang, G. L., Chen, L. L., Kim, H. S., Pi, L. Y., Holsten, T., Gardner, J., Wang, B., Zhai, W. X., Zhu, L. H., and *et al.* (1995). A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. *Science* **270**, 1804-6.

Sory, M. P., Boland, A., Lambermont, I., and Cornelis, G. R. (1995). Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the *cyaA* gene fusion approach. *Proc Natl Acad Sci U S A* **92**, 11998-2002.

Staskawicz, B. J., Ausubel, F. M., Baker, B. J., Ellis, J. G., and Jones, J. D. (1995). Molecular genetics of plant disease resistance. *Science* **268**, 661-7.

Swarup, S., Yang, Y., Kingsley, M. T., and Gabriel, D. W. (1992). An *Xanthomonas citri* pathogenicity gene, *pthA*, pleiotropically encodes gratuitous avirulence on nonhosts. *Mol Plant Microbe Interact* **5**, 204-13.

Tang, X., Frederick, R. D., Zhou, J., Halterman, D. A., Jia, Y., and Martin, G. B. (1996). Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* **274**, 2060-3.

Van den Ackerveken, G., Marois, E., and Bonas, U. (1996). Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host plant cell. *Cell* **87**, 1307-16.

van Kan, J. A., Joosten, M. H., Wagemakers, C. A., van den Berg-Velthuis, G. C., and de Wit, P. J. (1992). Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. *Plant Mol Biol* **20**, 513-27.

Vos, P., Simons, G., Jesse, T., Wijbrandi, J., Heinen, L., Hogers, R., Frijters, A., Groenendijk, J., Diergaarde, P., Reijans, M., Fierens-Onstenk, J., de Both, M., Peleman, J., Liharska, T., Hontelez, J., and Zabeau, M. (1998). The tomato *Mi-1* gene confers resistance to both root-knot nematodes and potato aphids. *Nat Biotechnol* **16**, 1365-9.

Warren, R. F., Henk, A., Mowery, P., Holub, E., and Innes, R. W. (1998). A mutation within the leucine-rich repeat domain of the *Arabidopsis* disease resistance gene *RPS5* partially suppresses multiple bacterial and downy mildew resistance genes. *Plant Cell* **10**, 1439-52.

Wattiau, P., Bernier, B., Deslee, P., Michiels, T., and Cornelis, G. R. (1994). Individual chaperones required for Yop secretion by *Yersinia*. *Proc Natl Acad Sci U S A* **91**, 10493-7.

Wattiau, P., and Cornelis, G. R. (1993). SycE, a chaperone-like protein of *Yersinia enterocolitica* involved in the secretion of YopE. *Mol Microbiol* **8**, 123-31.

Wei, Z. M., and Beer, S. V. (1993). HrpI of *Erwinia amylovora* functions in secretion of harpin and is a member of a new protein family. *J Bacteriol* **175**, 7958-67.

Wei, Z. M., and Beer, S. V. (1995). *hrpL* activates *Erwinia amylovora hrp* gene transcription and is a member of the ECF subfamily of sigma factors. *J Bacteriol* **177**, 6201-10.

Wei, Z. M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. (1992). Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* **257**, 85-8.

Wei, Z. M., Sneath, B. J., and Beer, S. V. (1992). Expression of *Erwinia amylovora hrp* genes in response to environmental stimuli. *J Bacteriol* **174**, 1875-82.

Whitham, S., Dinesh-Kumar, S. P., Choi, D., Hehl, R., Corr, C., and Baker, B. (1994). The product of the tobacco mosaic virus resistance gene *N*: similarity to toll and the interleukin-1 receptor. *Cell* **78**, 1101-15.

Xiao, Y., Heu, S., Yi, J., Lu, Y., and Hutcheson, S. W. (1994). Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling expression of *Pseudomonas syringae* pv. *syringae Pss*61 *hrp* and *hrmA* genes. *J Bacteriol* **176**, 1025-36.

Xiao, Y., and Hutcheson, S. W. (1994). A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae. J Bacteriol* **176**, 3089-91.

Xiao, Y., Lu, Y., Heu, S., and Hutcheson, S. W. (1992). Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. *J Bacteriol* **174**, 1734-41.

Yang, Y., De Feyter, R., and Gabriel, D. (1994). Host-specific symptoms and increased release of *Xanthomonas citri* and *X. campestris* pv. *malvacearum* from leaves are determined by the 102-bp tandem repeats of *pthA* and *avrb6*, respectively. *Mol Plant Microbe Interact* **7**, 345-355.

Yang, Y., and Gabriel, D. (1995). *Xanthomonas* avirulence/pathogenicity gene family encodes functional plant nuclear targeting signals. *Mol Plant Microbe Interact* **8**, 627-631.

Yuan, J., and He, S. Y. (1996). The *Pseudomonas syringae* Hrp regulation and secretion system controls the production and secretion of multiple extracellular proteins. *J Bacteriol* **178**, 6399-402.

Chapter 2

THE *HRPA* GENE ENCODES THE STRUCTURAL PROTEIN OF A BACTERIAL SURFACE APPENDAGE ASSOCIATED WITH TYPE III PROTEIN SECRETION IN *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000

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2.1.

2.1. ABSTRACT

Hypersensitive response and pathogenicity (*hrp*) genes control the ability of major groups of plant pathogenic bacteria to elicit the hypersensitive response (HR) in resistant plants and to cause disease in susceptible plants. A number of Hrp proteins share significant similarities with components of the type III secretion system and the flagellar assembly apparatus in animal pathogenic bacteria. The *hrpA* gene of *Pseudomonas syringae* pv. *tomato* DC3000 encodes an extracellular protein with unknown function. A non-polar *hrpA* mutant was constructed by replacing the *hrpA* gene open reading frame (ORF) with the *aph* (encoding the aminoglycoside phosphotransferase) ORF. The HrpA protein was not produced in the *hrpA* mutant. pHRPA, a pUCP18 derivative carrying the wild type *hrpA* gene together with its native promoter, restored production and secretion of the HrpA protein in the *hrpA* mutant. In collaboration with Dr. Martin Romantschuk's group, we identified a filamentous surface appendage, called the Hrp pilus, from DC3000 grown in a solid minimal medium. Using the non-polar *hrpA* mutant, I demonstrated that the *hrpA* gene encodes the major structural protein of the Hrp pilus. Transmission electron microscopy (TEM) showed that *hrpA* mutant did not form any Hrp pilus, whereas pHRPA restored the formation of Hrp pili in the *hrpA* mutant. In addition, the *hrpA* mutant was unable to cause disease in *Arabidopsis thaliana* or to elicit HR in the non-host tobacco or resistant tomato plants carrying the *Pto* gene. These results suggest that the *hrpA* plays a critical role in plant-*Pst* DC3000 interactions.

2.2. INTRODUCTION

Major groups of Gram-negative plant pathogenic bacteria belonging to the genera *Erwinia, Pseudomonas, Ralstonia,* and *Xanthomonas* contain hypersensitive reaction and pathogenicity (*hrp*) genes. These genes control the ability of these bacteria to initiate interactions with plants, including elicitation of the hypersensitive reaction (HR) and causation of disease (Bonas, 1994; He, 1996).

hrp genes specify the type III protein secretion system in pathogenic phytobacteria, and many of them encode proteins sharing high sequence similarity with components of the type III system of animal pathogenic bacteria, such as *ysc* genes in *Yersinia* spp. (Cornelis and Wolf-Watz, 1997). In *Yersinia*, extracellular proteins that traverse the type III protein secretion system are involved in the translocation of effector proteins into host cells. For example, it has been suggested that two type III secreted hydrophobic proteins, YopB and YopD, form a translocation pore in the host cytoplasmic membrane (Hakansson *et al.*, 1996; Lee and Schneewind, 1999). Other extracellular proteins, such as YopN, LcrG, and TyeA, are involved in sensing of host cells by *Yersinia* (Iriarte *et al.*, 1998).

The functional dependence of Avr proteins on *hrp* genes suggests possible transport of Avr proteins into the interior of the plant cell via the Hrp secretion system. Indeed, using transgenic and transient expression of AvrB of *P. syringae* in leaves of *Arabidopsis thaliana*, Gopalan *et al.* showed that the site of activity of AvrB is inside the plant cell and that the Hrp system is necessary for the putative delivery of AvrB into plant cells during infection (Gopalan *et al.*, 1996).

The mechanism by which the Hrp secretion system putatively delivers proteins through the plant cell wall is not known. To send bacterial proteins into plant cells, bacteria must overcome the thick plant cell wall (>100 nm). Although it is not clear which proteins are involved in forming the hypothetical translocation structure, they are likely secreted outside the bacteria via the Hrp system.

Pst DC3000 produces several major extracellular proteins (EXPs) in a minimal medium that induces *hrp* genes. Five EXPs (EXP-60, EXP-45, EXP-43, EXP-22, and EXP-10) are under the control of the Hrp secretion system (Yuan and He, 1996). Immunoblot analysis with the $HrpZ_{Pss}$ antibody identified EXP-45 as the $HrpZ_{Pss}$ homolog in DC3000, named the $HrpZ_{Pst}$ protein. The amino-terminal sequence analysis revealed that EXP-10 is encoded by the *hrpA* gene, located upstream of *hrpZ* in the same operon (Yuan and He, 1996).

In this study, experiments were carried out to determine the function of the *hrpA* gene in plant-*Pst* DC3000 interactions. For this purpose, a non-polar *hrpA* mutant was constructed. Work with the *hrpA* mutant allowed us to demonstrate that the *hrpA* gene is essential for *Pst* DC3000 to initiate pathogenesis and to elicit the HR in plants, as well as for the function of AvrB when produced in *Pst* DC3000. In collaboration with Dr. Martin Romantschuk's group, we showed that *Pst* DC3000 produces a thin, pilus-like structure on solid *hrp*-inducing media. HrpA was found associated with the Hrp pilus. By using the *hrpA* mutant, I provided evidence that the *hrpA* gene encodes the major structural protein of this Hrp pilus.

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2.3. MATERIALS AND METHODS

2.3.1. Culture Conditions

For detection of bacterial extracellular proteins (EXPs) in liquid cultures, bacteria were first grown at 28°C to an OD_{600} of 0.8-1.0 in 50 ml King's medium B broth (King *et al.*, 1954), supplemented with 100 μg/ml rifampicin. Bacteria were then pelleted and resuspended in 50 ml *hrp*-inducing broth [NaCl 0.1%, KH_2PO_4 0.17%, $MgSO_4 \cdot 7H_2O$ 0.07%, potassium citrate 0.324%, $(NH_4)_2SO_4$ 0.4%, pH5.5; 10 mM fructose added after autoclaving] (He *et al.*, 1993; Innes *et al.*, 1993) or King's medium B broth, and then incubated with shaking (250 rpm) at room temperature (21-23°C) for 24 hr. For preparation of bacterial surface-associated proteins, bacteria were grown on solid *hrp*-inducing medium (He *et al.*, 1993) at room temperature (21-23°C) for 2 days.

2.3.2. Analysis of Bacterial Extracellular Proteins

For preparation of bacterial secreted proteins, bacteria were removed from liquid cultures by centrifugation at 10,000 × g for 10 min. The supernatant was concentrated 50-fold using centricon concentrators with a molecular weight cutoff of 3,000 daltons (Amicon) and 10 μl was analyzed by 15% SDS/PAGE followed by staining with 0.025% Coomassie Brilliant Blue R-250. Immunoblot analysis of HrpA was carried out using rabbit antibody raised against the HrpA protein. For preparation of bacterial surface-associated proteins, bacteria from a 76-mm agar plate containing solid *hrp*-inducing medium were resuspended in 1 ml of 10 mM sodium phosphate (pH 5.5), pelleted by centrifugation at 13,000 × g for 10 min to partially remove proteins not associated with

cell surface structures, and then resuspended in 0.2 ml of 10 mM sodium phosphate (pH 5.5). The bacterial suspension was forced through a 25 G needle 4 to 5 times to shear surface structures and proteins (e.g., flagella and pili) from the bacteria, and was then centrifuged at 13,000 × g for 10 min at 4°C. Twenty microliters of the supernatant was used for SDS/PAGE analysis.

2.3.3. Construction of the *hrpA* Mutant

For construction of the nonpolar *hrpA* mutants, a 5.1-kb *Eco*RI fragment of pCPP2201 (Preston *et al.*, 1995) containing *hrpRS*, *hrpAZBCDE*, and the 5' half of *hrpF* of *Pst* DC3000 was subcloned into pBluescript II SK(-) (Stratagene) and used as the template for PCR. A 1.5-kb DNA fragment, 5' of the *hrpA* start codon, was amplified by PCR using the following oligonucleotide set: 5'-GGAAACAGCTATGACCATG-3' (pBSSK reverse primer) and 5'-GGGGTACCCCTTAAGATTTACCAGCGTGATTGC-3' (containing a *Kpn*I site at the 5' end). A 3.8-kb fragment 3' of the *hrpA* stop codon was amplified using the following primer set: 5'-CGGGATCCCGTTGCCCCCTCATC-AGAGG-3' (containing a *Bam*HI site at the 5' end) and 5'-GTAAAACGACGGCCAGT-3' (M13-20 primer). The 0.8-kb open reading frame (ORF) of the *aph* gene (encoding aminoglycoside phosphotransferase) without its own promoter or terminator was amplified from mini-Tn5 *xylE* (de Lorenzo *et al.*, 1990) using the following oligonucleotides: 5'-GGGGTACCCTGTTATGAGCCATATTCAACG-3' (containing a *Kpn*I site at the 5' end) and 5'-CGGGATCCCGTTAGAAAAACTCATCGAGCATC-3' (containing a *Bam*HI site at the 5' end). Through multiple cloning steps, the three PCR products were ligated (*hrpRS-aph-hrpZBCDEF*) and cloned into pBluescript II SK(-).

The *hrpA* ORF was precisely replaced by the *aph* ORF without changing the promoter or Shine-Dalgarno sequences upstream of the *hrpA* ORF, or the sequence and spacing between *hrpA* and *hrpZ*. For marker exchange mutagenesis, the *hrpRS-aph-hrpZBCDEF* fragment was cloned into pRK415 (Keen *et al.*, 1988), which was then electroporated into *Pst* DC3000. Marker exchange events were selected following a standard procedure (Huang *et al.*, 1991), except that *hrp*-inducing medium was used for activating the expression of the *aph* gene. The junction sequences of the *aph* gene in the marker-exchanged *hrpA* mutant were cloned, sequenced, and found to be correct. See Fig. 2.1 for details of *hrpA* non-polar mutant construction. For complementation, the *hrpA* ORF and its native promoter were amplified using the following primers: 5'-TTGCAAAGA-CGCTGGAACCGTATCGC-3' and 5'-GGGGTACCTCCTCAAGGTAGCGGCCCCC-TC-3'. The PCR product was cloned into the *Sma*I site of pUCP18 (Schweizer, 1991), resulting in pHRPA.

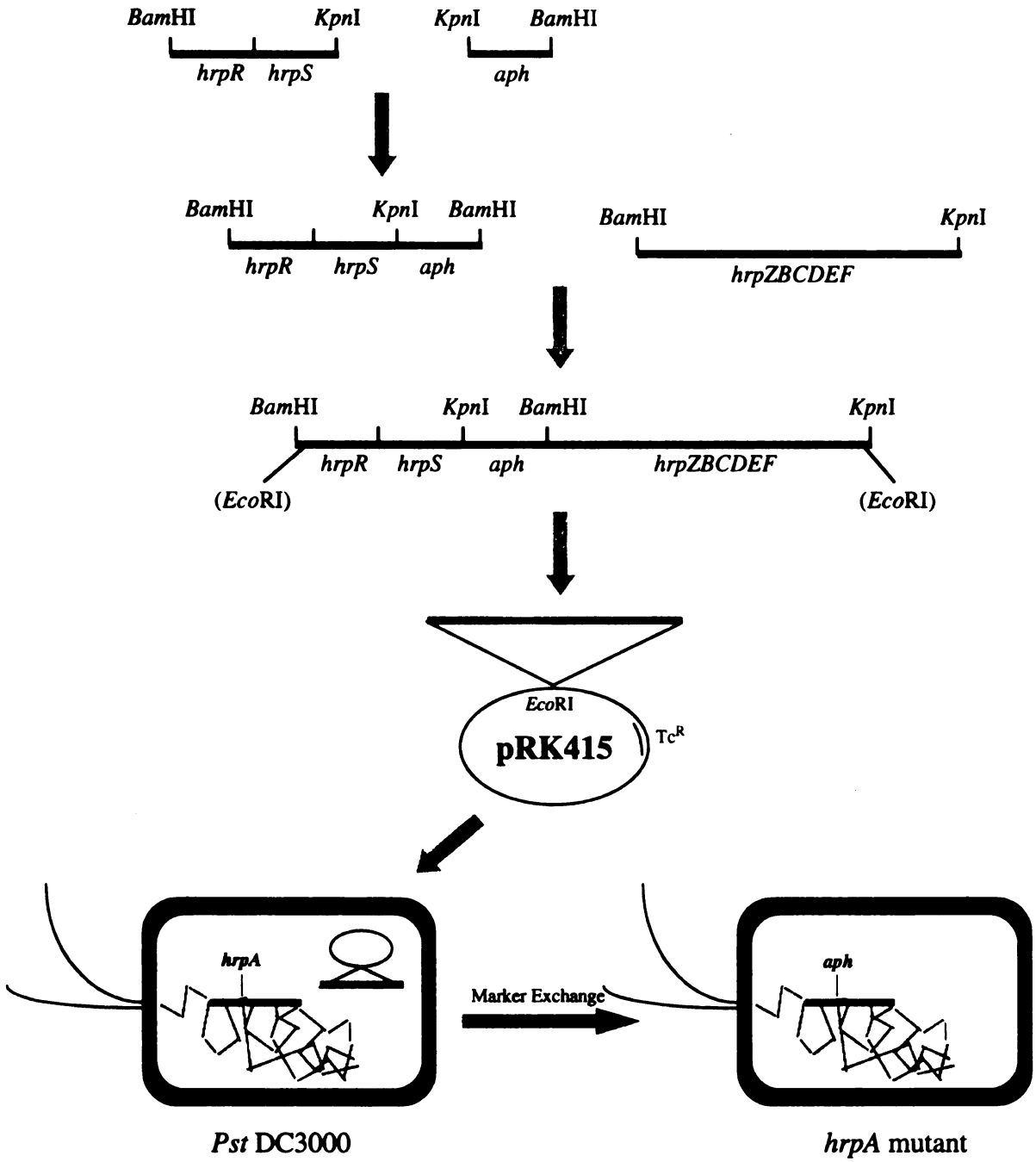

Figure 2.1. A flow chart of construction of a non-polar *hrpA* mutant in *Pst* DC3000.

2.3.4. Partial Purification of Bacterial Surface Structures (Flagella and Hrp Pili)

(conducted by Dr. Martin Romantschuk's group)

All purification steps were performed at 4°C. Bacterial surface structures and proteins were sheared off as described above and subjected to ultracentrifugation in a Beckman Ti45 rotor at 100,000 × g for 3 hr. The pellet was resuspended in 10 mM Tris·HCl (pH 7.5) to which sodium deoxycholate was added to a final concentration of 0.5% (wt/vol). After overnight incubation at 4°C, proteins were subjected to ultracentrifugation in a 10-60% (wt/wt) sucrose gradient in a Beckman SW27 rotor at 80,000 × g for 20 hr. Ten fractions were taken from the gradient and dialyzed against 10 mM Tris·HCl (pH 7.5) and then against water. The pellet of the gradient was resuspended in 10 mM Tris·HCl (pH 7.5), repelleted to remove the sucrose (in a Beckman Ti50 rotor at 125,000 × g for 3 hr), and finally resuspended in 10 mM Tris·HCl (pH 7.5). Dialyzed gradient fractions and the pellet were then used for transmission electron microscopy (TEM) and for SDS/PAGE analysis.

2.3.5. Transmission Electron Microscopy (TEM) Analysis

For TEM observation, a drop of bacterium or flagellum plus pilus suspension was applied to a copper grid coated with pioloform and carbon, followed by staining with 1% potassium phosphotungstic acid adjusted to pH 6.5 with potassium hydroxide. The grids were then examined with a transmission electron microscope.

2.3.6. Pathogenesis Assays

Bacteria were grown in King's B broth to an OD_{600} of 0.6-0.8. Bacterial suspensions in distilled water were infiltrated into leaves of tomato (*Lycopersicon esculentum* cultivar Rio Grande-PtoR), *Arabidopsis thaliana* ecotype Columbia (Col), and tobacco (*Nicotiana tabacum* cultivar Samsun NN) using needleless syringes. The concentrations of bacteria used were 2×10^8 colony-forming units (cfu)/ml and 2×10^6 cfu/ml for HR and pathogenesis assays, respectively. Plant responses were recorded at 24 hr (for HR assay) or 4 days (for pathogenesis assay) post-infiltration. HR is indicated by rapid, localized tissue collapse in the infiltrated area within 24 hr. Disease symptoms caused by *Pst* DC3000 and complemented *hrpA* mutants in tomato and *Arabidopsis* leaves were characterized by slowly developing necrosis and spreading tissue chlorosis, usually observed 3 days after infiltration. Strain DC3000 and its *hrpA* and *hrpS* mutants contain *avrPto* in the chromosome (Ronald *et al.*, 1992). Plasmid pAVRB contains *P. syringae* pv. *glycinea avrB* in pDSK609 (kindly provided by N. T. Keen, University of California). The tobacco and tomato plants were grown in greenhouses. *Arabidopsis* plants were grown in growth chambers at 20°C with 70% relative humidity and a 12-hr photoperiod. HR assays with tobacco and tomato plants were performed at room temperature in the laboratory. Pathogenesis assays with *Arabidopsis* plants were performed at 20°C in growth chambers with 70% relative humidity and a 12-hr photoperiod.

2.4. RESULTS

2.4.1. Mutational Analysis of the *hrpA* Gene

To study the function of HrpA protein, a nonpolar *hrpA* mutation was constructed by precisely replacing the *hrpA* open reading frame (ORF) with the *aph* (conferring kanamycin resistance) ORF, which confers kanamycin resistance (de Lorenzo *et al.*, 1990) (Fig. 2.1). For complementation, pHRPA (containing the *hrpA* gene with its native promoter; see MATERIALS AND METHODS) was introduced into the nonpolar *hrpA* mutant. SDS-PAGE was used to examine bacterial surface-associated proteins. A 10-kDa protein was secreted in both DC3000 and the *hrpA* mutant carrying pHRPA; however, it was absent in the *hrpA* mutant (Fig. 2.2A). Immunoblot analysis using an HrpA antibody showed that HrpA was not detectable at the surface of the *hrpA* mutant, while pHRPA restored the production of HrpA at the bacterial surface (Fig. 2.2B). These results confirmed that I had obtained a non-polar *hrpA* mutant.

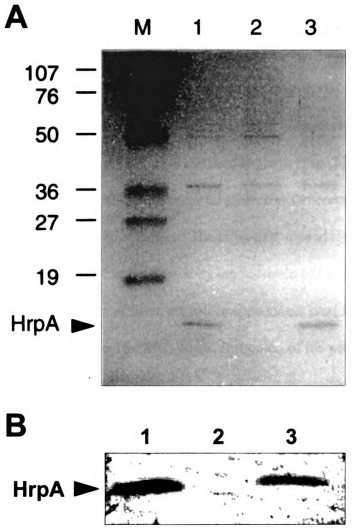

Figure 2.2. (A) A 15% SDS/PAGE and (B) an immunoblot analysis of protein samples prepared from surfaces of DC3000 (lane 1), *hrpA* mutant (lane 2), and *hrpA* mutant containing pHRPA (lane 3) grown on solid *hrp*-inducing medium. Lane M, molecular weight markers (Bio-Rad) in kDa. The gel (A) was stained with 0.025% Coomassie Brilliant Blue R-250. The immunoblot analysis was carried out using an anti-HrpA antibody.

2.4.2. Formation of an *hrp* Gene-Dependent Pilus by DC3000 on Solid *hrp*-Inducing Medium

Examination of bacteria grown on solid *hrp*-inducing medium by TEM revealed that DC3000 produced two or three polar flagella (15-18 nm in diameter) on both King's medium B (Fig. 2.3A) and *hrp*-inducing agar plates (Fig. 2.3B). In addition, DC3000 also produces many pilus-like appendages (6-8 nm in diameter) on solid *hrp*-inducing medium (Fig. 2.3B), but not on King's medium B plates (Fig. 2.3A). These pilus-like appendages were found both on the bacterial surface with no consistent distribution pattern, and as detached pilus clusters. The pilus-like appendages were easily fragmented during sample preparation, as evidenced by the presence of many short pieces of pili (Fig. 2.3B); therefore, the length of these pilus-like appendages could not be determined. The effect of temperature (from 16-28°C) on the formation of the pilus-like appendages was examined. The number of pili observed decreased dramatically when incubation temperature exceeded 25°C. This result suggests that the pilus-like structure is unstable in high temperature.

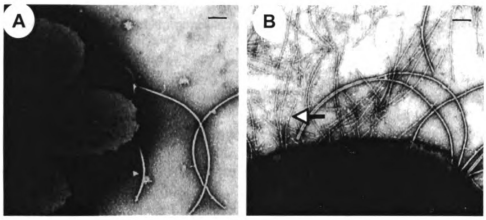

Figure 2.3. Detection of the Hrp pilus on the surface of *Pst* DC3000. (A) DC3000 grown on King's medium B agar plates, and (B) DC3000 mutants grown on solid *hrp*-inducing medium were examined with a transmission electron microscope after staining with 1% potassium phosphotungstic acid (pH 6.5). One to three polar flagella of 15-18 nm in diameter are present on most rod-shaped bacteria (surrounded by dark shadows) in samples (A and B); in *B*, many Hrp pili of 6-8 nm in diameter are also present (indicated by arrows). (Scale bars = 200 nm).

2.4.3. Identification of Proteins Associated with the DC3000 Hrp Pilus

(carried out mainly by Dr. M. Romantschuk's group; independently confirmed by SY He)

The presence of the Hrp pilus is specifically correlated with the appearance of a 10-kDa extracellular protein. Only DC3000 grown on solid *hrp*-inducing medium produced the 10-kDa protein (Fig. 2.2A & B, lane 1). Neither DC3000 grown on King's B agar medium, nor the *hrcC* mutant bacteria grown on solid *hrp*-inducing medium, produced the protein (Roine *et al.*, 1997).

To determine the identity of the Hrp pilus, a preparation of cell surface structures (flagella and Hrp pili) from DC3000 grown on solid M9 minimal medium was subjected to ultracentrifugation in a 10-60% sucrose gradient. Hrp pili together with flagella were found in the pellet (Fig. 2.4A). SDS/PAGE analysis of the pellet fraction revealed three major proteins of 50, 36, and 10 kDa in size, respectively, associated with these filamentous structures (Fig. 2.4C, lane 1). A minor, contaminating, 100-kDa protein was also present in some, but not all, preparations (Fig. 2.4C, lane 1). Another fraction from the middle of the sucrose gradient contained only flagella (Fig. 2.4B). When this fraction was analyzed by SDS/PAGE, only the 36-kDa protein, but not the 10- or 50-kDa protein, was found. This result suggests that the 36-kDa protein is flagellin that makes up flagella, and that the 10- and 50-kDa proteins are associated with the Hrp pilus.

The N-terminal sequences of the 36-, 10-, and 50-kDa proteins were determined. The N-terminal sequence (ALTVNTNVASLNVQKNLGRASDALST) of the 36-kDa protein, from both the pellet fraction containing flagella and Hrp pili (Fig. 2.4C, lane 1) and the flagella fraction (Fig. 2.4C, lane 2), is almost identical to those of flagellins of *Pseudomonas aeruginosa* and *Pseudomonas putida* (Totten and Lory, 1990; Winstanley

et al., 1994), confirming that the 36-kDa protein is the DC3000 flagellin. The first 35 amino acids (VAFAGLTSKLTNLGNSAVGGVGGALQGVNTVASNA) of the 10-kDa protein matched exactly that of HrpA encoded by *hrpA* (Preston *et al.*, 1995). The sequence of the first 16 amino acids (ASPITSTTGLGSGLAI) of the N terminus of the 50-kDa protein does not show any significant similarity to any proteins in the current gene/protein databases.

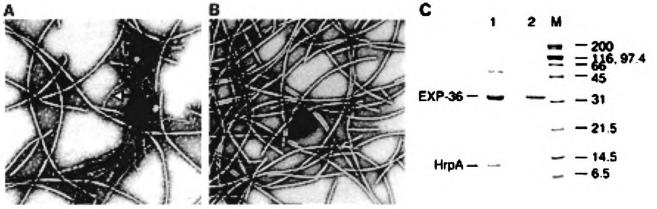

Figure 2.4. Purification of *Pst* DC3000 extracellular appendages (flagella and Hrp pili). (*A*) Electron micrograph of the pellet fraction containing flagella and Hrp pili (indicated by an arrow). (*B*) Electron micrograph of a fraction from the middle of the gradient containing only flagella. (*C*) SDS/PAGE analysis of proteins from the pellet fraction (lane 1) or a fraction from the middle of the gradient containing only flagella (lane 2). The gel was stained with 0.025% Coomassie Brilliant Blue R-250. Lane M, molecular mass markers (Bio-Rad) in kDa.

2.4.4. No Hrp Pilus can be Detected in the *hrpA* Mutant

Since the HrpA protein is associated with the Hrp pilus, it is important to know whether HrpA is required for the assembly of the Hrp pilus. Examination of bacteria grown on solid *hrp*-inducing medium by TEM revealed that the *hrpA* mutant produces only two to three polar flagella (15-18 nm in diameter), and no pilus-like structure could be detected (Fig. 2.5A). pHRPA (containing the *hrpA* gene with its native promoter) enabled the *hrpA* mutant to produce Hrp pili on the bacterial surface (Fig. 2.5B). Therefore we conclude that HrpA is likely the major structural protein of Hrp pili.

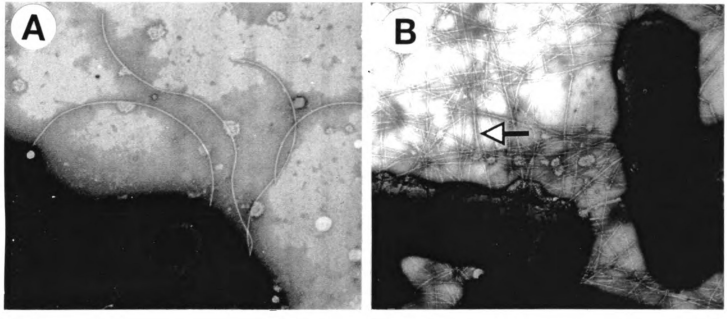

Figure 2.5. Transmission electron microscopic examination of surface appendages on *Pst* DC3000 (A) *hrpA* mutant and (B) *hrpA* mutant containing pHRPA grown on solid *hrp*-inducing medium. Polar flagella of 15-18 nm in diameter are present on most cells of the *hrpA* mutant and *hrpA* mutant containing pHRPA. Flagella were not seen in the field shown in panel B. In panel B, many Hrp pili of 6-8 nm in diameter are presented (indicated by arrow). (Scale bars = 200 nm)

2.4.5. Pathogenesis Assays

To determine the biological role of the Hrp pilus in interactions of plant and *Pst* DC3000, the effect of the *hrpA* mutation on bacterial pathogenesis and HR elicitation was examined. The results are summarized in Table 2.1. The *hrpA* mutant was unable to elicit the HR in leaves of the nonhost tobacco or to cause disease symptoms (tissue chlorosis and necrosis) in leaves of the host *Arabidopsis thaliana*. The responses of tobacco and *A. thaliana* leaves to the *hrpA* mutant were very similar to the responses to a *hrpS* mutant, which is a typical *hrp* mutant unable to initiate any *hrp*-mediated plant responses. Plasmid pHRPA restored the ability of the *hrpA* mutant to elicit HR necrosis in tobacco leaves and to cause disease symptoms in *A. thaliana* leaves. HrpA was also required for two well-characterized *P. syringae avr* genes, *avrB* and *avrPto*, to trigger genotype-specific HR (Table 2.1). DC3000 contains *avrPto*, which mediates the elicitation of an HR on tomato cv. Rio Grande-PtoR containing the *Pto* resistance gene (Martin *et al.*, 1993; Ronald *et al.*, 1992). *avrB* was originally cloned from *P. syringae* pv. *glycinea* and later was found to mediate the elicitation of an *RPM1*-dependent HR and resistance in *A. thaliana* (Bisgrove *et al.*, 1994). Therefore, the Hrp pilus structure gene, *hrpA*, is critical for DC3000 in both pathogenesis and HR elicitation in non-host plants or in host plants carrying the proper *avr* gene(s).

Table 2.1. Plant reactions to DC3000 and the *hrpA* mutant.

Bacteria	*Arabidopsis* (Col)	Tobacco (Samsun NN)	Tomato (Rio Grande-PtoR)
DC3000, *avrPto*$^+$	D	HR	HR
hrpA, *avrPto*$^+$	NR	NR	NR
hrpA/pHRPA, *avrPto*$^+$	D	HR	HR
DC3000/pAVRB	HR	HR	HR
hrpA/pAVRB	NR	NR	NA

HR: rapid, localized tissue collapse in the infiltrated area; D: tissue chlorosis and necrosis typical of disease symptoms caused by DC3000; NR: no visible reaction; NA: not assayed. DC3000 and its derivative, *hrpA* mutant, contain *avrPto* in the chromosome (Ronald *et al.*, 1992).

2.5. DISCUSSION

In this study, I (in collaboration with Dr. Martin Romantschuk's group) showed that *Pst* DC3000 produced a novel pilus-like structure on the cell surface when bacteria are grown on solid *hrp*-inducing medium at low temperature. Furthermore, I have provided genetic evidence that HrpA is a structural protein of this Hrp pilus. Finally, I have shown that a nonpolar *hrpA* mutant strain cannot initiate pathogenesis or elicit the HR in plants.

The nucleic acid sequence of the *Pst* DC3000 *hrpA* gene, the first gene of the *hrpZ* operon, was previously determined by Preston *et al.* (1995). *hrpA* codes for a hydrophilic protein with a predicted molecular weight of 11 kDa (Preston *et al.*, 1995). The primary amino acid sequence of the HrpA protein does not show any significant homology to those of characterized pilin proteins. However, our computer analysis using the PROPSEARCH program (Hobohm and Sander, 1995), which identifies structural similarities between proteins without looking for primary amino acid sequence homology, indicates that HrpA is structurally similar to several pilin proteins, especially to the AF/R1 pilus chain A precursor of *E. coli* (Wolf and Boedeker, 1990). This analysis is in agreement with our results showing that HrpA is the structural protein of the Hrp pilus.

We do not know the role, if any, of the 50-kDa protein in the assembly of the Hrp pilus. The 50-kDa protein was produced by *Pst* DC3000 growing in King's B medium, which represses *hrp* gene expression (Yuan and He, 1996). Furthermore, the *hrpA* mutant, which does not produce the Hrp pilus, still produces the 50-kDa protein (Fig. 2.2A). These two observations suggest that the production and secretion of this protein is

independent of the Hrp secretion system. It is possible that the 50-kDa protein is involved in the formation of some surface structure independent of the Hrp pilus.

To our knowledge, this is the first study showing that the Hrp pathway is involved in the formation of a pilus structure. Our finding is consistent with the observation that many Hrp proteins are structurally related to those that participate in the construction of bacterial flagella (Kihara *et al.*, 1989), suggesting an involvement of Hrp proteins in the assembly of an extracellular macromolecular structure. *Salmonella*, *Shigella*, and *Yersinia*, all of which contain type III secretion systems, secrete proteins required for pathogenesis (Hueck *et al.*, 1995; Michiels *et al.*, 1990; Parsot *et al.*, 1995; Rosqvist *et al.*, 1994), (Persson *et al.*, 1995). Both *Salmonella typhimurium* and *Shigella flexneri* are enteroinvasive pathogens. *S. typhimurium* transiently produces filamentous surface appendages of 60 nm in diameter upon contact with epithelial cells during its invasion of host cells (Ginocchio *et al.*, 1994). The structural components of these appendages have not been identified. *Yersinia* spp. are not intracellular pathogens, but during infection they secrete virulence proteins through contact zones between bacteria and host cells (Rosqvist *et al.*, 1994). The secreted proteins of *S. flexneri* and *Yersinia* spp. form various aggregates and protein complexes in liquid, stationary-phase cultures (Michiels *et al.*, 1990; Parsot *et al.*, 1995). However, the relationship between these protein aggregates and possible formation of surface appendages in these bacteria remains to be determined.

Agrobacterium tumefaciens has been shown to produce pili involved in T-DNA transfer (Fullner *et al.*, 1996). Pili of 3.5 nm in diameter were formed under *vir* gene-inducing conditions. These pili were proposed to function as conjugation pili in T-DNA

transfer between bacteria and plant cells. The protein components of the T-DNA secretion pathway encoded by *virB* genes share sequence similarities with proteins involved in the assembly of conjugative pili, but not with protein components of the type III secretion system. The structural proteins of the *A. tumefaciens* pilus have yet to be identified.

Morphologically, the Hrp pilus of *Pst* DC3000 characterized in this study resembles most closely the pilus produced by *A. tumefaciens*. Both pili are much thinner than the surface appendages of *S. typhimurium*. This similarity may reflect an adaptation of the two bacteria in the infection of wall-bound plant cells. Conditions for pilus production by the two bacteria are also very similar. Like the Hrp pilus, far fewer *A. tumefaciens* pili are produced at higher temperatures (e.g., 28°C) than at lower temperatures (e.g., 19°C). Furthermore, formation of the Hrp pilus requires solid growth medium, the condition used also for growing *A. tumefaciens* for pilus production (Fullner *et al.*, 1996). This may reflect the instability of the pilus under mechanical shaking condition.

Recent results strongly suggest that the action sites of *P. syringae* pv. *glycinea* AvrB and possibly *X. campestris* pv. *malvacearum* Avr/Pth proteins are inside the plant cell. A previous study showed that close bacterial contact is required for bacterial elicitation of HR (Stall and Cook, 1979). The requirement of the Hrp pilus structural gene *hrpA* in the phenotypic expression of *avrB*, as demonstrated in this study, suggests that the Hrp pilus may be involved in the delivery of AvrB and possibly other virulence and avirulence proteins into the plant cell (conduit model, Fig. 2.6). Alternatively, it may be involved in mediating contact between bacteria and plant cells in the plant intercellular

space (contact model, Fig. 2.6). The exact function of the Hrp pilus in protein transfer or cell-cell contact remains to be determined.

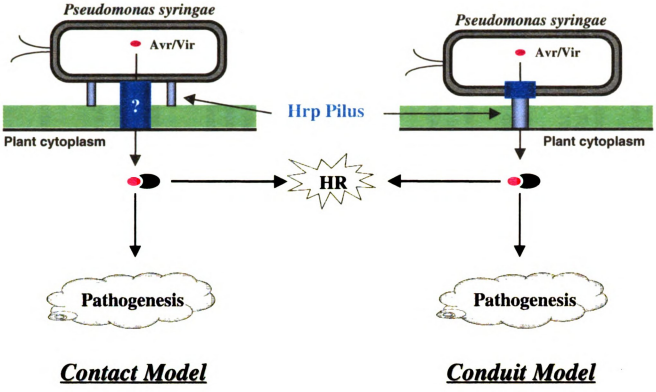

Figure 2.6. Models for the function of the Hrp pilus in the transport of *Pst* DC3000 virulence or avirulence factors from the bacterium to the plant.

2.6. REFERENCES

Bisgrove, S. R., Simonich, M. T., Smith, N. M., Sattler, A., and Innes, R. W. (1994). A disease resistance gene in *Arabidopsis* with specificity for two different pathogen avirulence genes. *Plant Cell* **6**, 927-33.

Bonas, U. (1994). Bacterial pathogenesis of plants and animals-molecular and cellular mechanisms. *Current Topics in Microbiology and Immunology*, J. L. Dangl, ed. (Springer, Berlin) pp. 79-98.

Cornelis, G. R., and Wolf-Watz, H. (1997). The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. *Mol Microbiol* **23**, 861-7.

de Lorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K. N. (1990). Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J Bacteriol* **172**, 6568-72.

Fullner, K. J., Lara, J. C., and Nester, E. W. (1996). Pilus assembly by *Agrobacterium* T-DNA transfer genes. *Science* **273**, 1107-9.

Ginocchio, C. C., Olmsted, S. B., Wells, C. L., and Galan, J. E. (1994). Contact with epithelial cells induces the formation of surface appendages on *Salmonella typhimurium*. *Cell* **76**, 717-24.

Gopalan, S., Bauer, D. W., Alfano, J. R., Loniello, A. O., He, S. Y., and Collmer, A. (1996). Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (Hrp) secretion system in eliciting genotype-specific hypersensitive cell death. *Plant Cell* **8**, 1095-105.

Hakansson, S., Schesser, K., Persson, C., Galyov, E. E., Rosqvist, R., Homble, F., and Wolf-Watz, H. (1996). The YopB protein of *Yersinia pseudotuberculosis* is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. *EMBO J* **15**, 5812-23.

He, S. Y. (1996). Elicitation of plant hypersensitive response by bacteria. *Plant Physiol* **112**, 865-869.

He, S. Y., Huang, H. C., and Collmer, A. (1993). *Pseudomonas syringae* pv. *syringae* harpin$_{Pss}$: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell* **73**, 1255-66.

Hobohm, U., and Sander, C. (1995). A sequence property approach to searching protein databases. *J Mol Biol* **251**, 390-9.

Huang, H. C., Hutcheson, S. W., and Collmer, A. (1991). Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and TnphoA tagging of genes encoding exported or membrane-spanning Hrp proteins. *Mol Plant Microbe Interact* **4**, 469-476.

Hueck, C. J., Hantman, M. J., Bajaj, V., Johnston, C., Lee, C. A., and Miller, S. I. (1995). *Salmonella typhimurium* secreted invasion determinants are homologous to *Shigella* Ipa proteins. *Mol Microbiol* **18**, 479-90.

Innes, R. W., Bent, A. F., Kunkel, B. N., Bisgrove, S. R., and Staskawicz, B. J. (1993). Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. *J Bacteriol* **175**, 4859-69.

Iriarte, M., Sory, M. P., Boland, A., Boyd, A. P., Mills, S. D., Lambermont, I., and Cornelis, G. R. (1998). TyeA, a protein involved in control of Yop release and in translocation of *Yersinia* Yop effectors. *EMBO J* **17**, 1907-18.

Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. (1988). Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* **70**, 191-7.

Kihara, M., Homma, M., Kutsukake, K., and Macnab, R. M. (1989). Flagellar switch of *Salmonella typhimurium*: gene sequences and deduced protein sequences. *J Bacteriol* **171**, 3247-57.

King, E. O., Ward, M. K., and Raney, D. E. (1954). *J Lab Med* **22**, 301-307.

Lee, V. T., and Schneewind, O. (1999). Type III machines of pathogenic *Yersiniae* secrete virulence factors into the extracellular milieu. *Mol Microbiol* **31**, 1619-29.

Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganal, M. W., Spivey, R., Wu, T., Earle, E. D., and Tanksley, S. D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* **262**, 1432-6.

Michiels, T., Wattiau, P., Brasseur, R., Ruysschaert, J. M., and Cornelis, G. (1990). Secretion of Yop proteins by *Yersiniae*. *Infect Immun* **58**, 2840-9.

Parsot, C., Menard, R., Gounon, P., and Sansonetti, P. J. (1995). Enhanced secretion through the *Shigella flexneri* Mxi-Spa translocon leads to assembly of extracellular proteins into macromolecular structures. *Mol Microbiol* **16**, 291-300.

Persson, C., Nordfelth, R., Holmstrom, A., Hakansson, S., Rosqvist, R., and Wolf-Watz, H. (1995). Cell-surface-bound *Yersinia* translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. *Mol Microbiol* **18**, 135-50.

Preston, G., Huang, H. C., He, S. Y., and Collmer, A. (1995). The HrpZ proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* are encoded by an operon containing *Yersinia ysc* homologs and elicit the hypersensitive response in tomato but not soybean. *Mol Plant Microbe Interact* **8**, 717-32.

Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E. L., Kalkkinen, N., Romantschuk, M., and He, S. Y. (1997). Hrp pilus: an *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. *Proc Natl Acad Sci U S A* **94**, 3459-64.

Ronald, P. C., Salmeron, J. M., Carland, F. M., and Staskawicz, B. J. (1992). The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. *J Bacteriol* **174**, 1604-11.

Rosqvist, R., Magnusson, K. E., and Wolf-Watz, H. (1994). Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. *EMBO J* **13**, 964-72.

Schweizer, H. P. (1991). *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* **97**, 109-21.

Stall, R. E., and Cook, A. A. (1979). Evidence that bacterial contact with the plant cell is necessary for the hypersensitive reaction but not the susceptible reaction *Xanthomonas vesicatoria*, pepper. *Physiol Plant Pathol* **14**, 77-84.

Totten, P. A., and Lory, S. (1990). Characterization of the type a flagellin gene from *Pseudomonas aeruginosa* PAK. *J Bacteriol* **172**, 7188-99.

Winstanley, C., Morgan, J. A., Pickup, R. W., and Saunders, J. R. (1994). Molecular cloning of two *Pseudomonas* flagellin genes and basal body structural genes. *Microbiology* **140**, 2019-31.

Wolf, M. K., and Boedeker, E. C. (1990). Cloning of the genes for AF/R1 pili from rabbit enteroadherent *Escherichia coli* RDEC-1 and DNA sequence of the major structural subunit. *Infect Immun* **58**, 1124-8.

Yuan, J., and He, S. Y. (1996). The *Pseudomonas syringae* Hrp regulation and secretion system controls the production and secretion of multiple extracellular proteins. *J Bacteriol* **178**, 6399-402.

Chapter 3

ROLE OF THE HRP PILUS IN TYPE III PROTEIN SECRETION IN *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000

3.1. ABSTRACT

Bacterial surface appendages called pili are often required for DNA and/or protein transfer between cells. The exact function of pili in the transfer process is not understood and is a matter of considerable debate. The Hrp pilus is assembled by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000. In this study, we show that the *hrpA* gene, which encodes the major subunit of the Hrp pilus, is required for expression of all examined genes that are associated with type III secretion. HrpA-mediated gene regulation occurs through activation of two previously characterized regulatory genes (*hrpR* and *hrpS*) at the RNA level. Ectopic expression of the *hrpRS* gene operon restored gene expression in the *hrpA* mutant. Three single amino acid mutations at the HrpA carboxyl terminus were identified that affect the regulatory function of the HrpA protein. In addition to being required for gene regulation, a functional HrpA protein was found to be necessary for extracellular secretion of putative virulence proteins, such as HrpW and AvrPto. These results define an essential role of the Hrp pilus structural protein in protein secretion and global regulation of a major protein secretion system in Gram-negative bacteria.

3.2. INTRODUCTION

Many Gram-negative bacterial pathogens, including *Pst* DC3000, possess a unique protein secretion system called the type III protein secretion system that is necessary for transfer of virulence proteins directly into the host cell (Galan and Collmer, 1999; He, 1998; Hueck, 1998). This system has been shown to play a critical role in bacterial infection of plants, and animals. Genes involved in type III protein secretion have been characterized extensively in several model human, animal and plant bacteria (Galan and Collmer, 1999; He, 1998; Hueck, 1998). Several general features of type III protein secretion have been revealed: i) type III protein secretion appears to be activated fully upon contact with the host cells *in vivo* (Pettersson *et al.*, 1996); ii) extracellular filamentous appendages are often associated with type III protein secretion (Ginocchio *et al.*, 1994; Knutton *et al.*, 1998; Roine *et al.*, 1997); iii) a secretion signal is localized in the 5' region of the mRNA of the secreted protein (Anderson and Schneewind, 1997; Anderson and Schneewind, 1999); and iv) the secretion apparatus is genetically and morphologically similar to the bacterial flagellum (Kubori *et al.*, 1998).

In plant pathogenic bacteria, the type III protein secretion system is encoded by *hrp* (for hypersensitive reaction and pathogenicity) genes (He, 1998; Lindgren, 1997; Lindgren *et al.*, 1986). Nine *hrp* genes have been renamed *hrc* (for *hrp* conserved) genes because of their broad conservation among all bacteria that harbor type III protein secretion systems (Bogdanove *et al.*, 1996). The Hrp secretion system of *Pseudomonas syringae* has been shown to secrete two families of proteins that elicit host responses: Harpins, such as HrpZ and HrpW (Charkowski *et al.*, 1998; He *et al.*, 1993; Yuan and He, 1996), and Avr proteins (Alfano *et al.*, 1997; Gopalan *et al.*, 1996; Scofield *et al.*,

1996; Tang *et al.*, 1996); (Mudgett and Staskawicz, 1999; Stevens *et al.*, 1998; van Dijk, 1999). The expression of *P. syringae hrc/hrp* genes is tightly controlled. Most *hrp* genes are expressed at a very low level in standard nutrient-rich media. The expression of *hrc/hrp* genes is induced in infected plant tissues or in artificial *hrp*-inducing minimal media that presumably mimic the *in planta* conditions (Huynh *et al.*, 1989; Rahme *et al.*, 1992; Xiao *et al.*, 1992). The mechanism by which host and other signals induce *hrc/hrp* genes is not clear. Three intracellular positive regulatory proteins are required: HrpR and HrpS, which belong to the NtrC family of two-component regulatory proteins (Grimm *et al.*, 1995; Grimm and Panopoulos, 1989; Xiao *et al.*, 1994), and HrpL, a member of the ECF family of alternate sigma factors (Xiao and Hutcheson, 1994). The HrpS, HrpR, and HrpL proteins appear to function as a regulatory cascade in which HrpS and HrpR activate the expression of HrpL in response to a signal in host tissue or in *hrp*-inducing minimal medium (Grimm *et al.*, 1995; Xiao *et al.*, 1994). HrpL is presumed to activate all *hrp* and *avr* genes by recognizing a consensus sequence motif ("harp box") present in the upstream regions of many *hrp* and *avr* genes (Xiao *et al.*, 1994; Xiao and Hutcheson, 1994). Recently, a putative negative regulator encoded by the *hrpV* gene has been identified in *P. syringae* (Preston *et al.*, 1998). In *hrp*-inducing minimal medium, overexpression of the *hrpV* gene down-regulates *hrp/hrc* gene expression, whereas a *hrpV* mutant is elevated in *hrp/hrc* gene expression (Preston *et al.*, 1998).

In a previous study, we found that *P. syringae* pv. *tomato* strain DC3000 assembles a *hrp*-dependent pilus (the Hrp pilus) (Roine *et al.*, 1997). We showed that the structural protein of the Hrp pilus, HrpA, is required for *P. syringae* pv. *tomato* (*Pst*) DC3000 to cause disease in *Arabidopsis* and to elicit the hypersensitive response (HR) in

tobacco and tomato (Roine *et al.*, 1997). The exact function of the Hrp pilus in plant-*Pst* DC3000 interactions, however, is not clear. Pili have also been shown to be required for bacterial conjugation (Willetts and Skurray, 1980) and for transfer of T-DNA to plant cells by *Agrobacterium tumefaciens* (Fullner *et al.*, 1996). The conjugative F pilus plays a major role in mediating contact between donor and recipient bacteria during mating (Willetts and Skurray, 1980). However, whether pili have other functions in protein and/or DNA transfer is not understood and is a matter of considerable debate. Specifically, it is not known whether these pili are involved in the actual transfer of DNA or protein or only in mediating cell-cell contact. In this study, we show that the Hrp pilus structural protein HrpA plays a key role in secretion of putative virulence proteins and global regulation of the *Pst* DC3000 Hrp system.

3.3. MATERIALS AND METHODS

3.3.1. Bacterial Strains, Plasmids and Growth Conditions

Pst DC3000 and four *hrp* mutant derivatives (*hrpA*, *hrpS*, *hrcC*, and *hrcChrpTV*) were used in this study. The *hrpA* and *hrcChrpTV* (formerly *hrpH*) mutants were described before (Roine *et al.*, 1997; Yuan and He, 1996). The *hrcC* mutant was constructed by replacing the *hrcC* gene with the terminatorless *nptII* gene (kindly provided by Dr. Alan Collmer's laboratory). The *hrpS* mutant construction is described below. *Pst* strains were grown at 28°C in Luria-Bertani (LB) broth (Sambrook *et al.*, 1989). For induction of *hrp* genes, bacteria were grown at 20°C in *hrp*-inducing minimal medium (NaCl 0.1%, KH_2PO_4 0.17%, $MgSO_4 \cdot 7H_2O$ 0.07%, potassium citrate 0.324%, $(NH_4)_2SO_4$ 0.4%, pH 5.5, and 10 mM fructose added after autoclaving) (Innes *et al.*,

1993). *E. coli* DH5α, grown in LB medium at 37°C, was used for all cloning experiments. pHRPA contains the *Pst* DC3000 *hrpA* gene under the control of its native promoter (Roine *et al.*, 1997). pHRPRS1 carries the *P. syringae* pv. *syringae* 61 *hrpRS* ORFs under the control of the salicylic acid-inducible P_G promoter in pKMY299 (Yen, 1991). pHRPRS2 carries the *Pst* DC3000 *hrpRS* ORFs under the control of P_{lac} in pUCP18 (Schweizer, 1991). pHRPCT contains the *hrcC* and *hrpT* genes downstream of the *lac* promoter of pUCP18 (Schweizer, 1991) and it was able to restore the ability of *hrcChrpTV* mutant to elicit an HR in tobacco and to cause disease in *Arabidopsis* (data not shown). All plasmids were introduced into *P. syringae* by electroporation. The antibiotics used were: rifampicin, 100 mg/l; ampicillin, 100 mg/l; chloramphenicol, 34 mg/l; and kanamycin, 50 mg/l.

3.3.2. Construction of the *hrpS* Mutant

An *hrpS* mutant was constructed through transposon-mediated mutagenesis by using Tn5*Sp/Sm* carried on the suicide plasmid pUT (Herrero *et al.*, 1990) in *E. coli* strain SM10 λpir (Taylor *et al.*, 1989). Previously, the *Pst* DC3000 *hrp* gene cluster was cloned in two plasmids (pDC3000-1 and pDC3000-2) by Sheng Yang He, using pUCP19 as the cloning vector (Schweizer, 1991). Hybridization with individual ^{32}P-labeled *Pss* 61 *hrp* gene probes allowed me to determine that the 10.9-kb insert in pDC3000-1 contains the *hrcC*, *hrpZ*, and *hrpRS* operons. Ms. J. Yuan (in our laboratory) subcloned the 10.9-kb insert in pDC3000-1 into the pRK415 vector (Keen *et al.*, 1988), and the resulting plasmid was designated pRK3000-1. In order to mutagenize the *hrpS* gene, donor (pUT-Tn5*Sp/Sm* in SM10 λpir) and recipient cells (pRK3000-1 in DH5α) were

plated together on LB agar plates and incubated at 37°C for 8-10 hours. The bacterial mixture was subsequently plated on LB plates containing 10 μg/ml nalidixic acid (selecting for DH5α), 10 μg/ml tetracycline (selecting for pRK3000-1), and 50 μg/ml spectinomycin (selecting for Tn5*Sp/Sm*). The pRK3000-1 plasmid DNA was isolated from the surviving transconjugants, transformed en masse into DH5α, and plated on LB plates containing tetracycline and spectinomycin (selecting for pRK3000-1 carrying the sTn5*Sp/Sm* insertion). Individual transformant was examined by polymerase chain reaction (PCR) analysis to identify Tn5*Sp/Sm* insertions in the *hrpRS* operon. The following two oligonucleotide primers were used to amplify the *hrpR* and *hrpS* ORFs: 5'-GGGGTACCTGCTCTTGTCCCGGATCACAGC-3' (annealing to the promoter sequence of the *hrpR/S* gene) and 5'-GCTCTAGATCAGATCTGCAATTCTTTGATG-CG-3' (annealing to the *hrpS* 3' end). One pRK3000-1 carrying a Tn5*Sp/Sm* insertion in the *hrpRS* operon was isolated and electroporated into *Pst* DC3000. The *hrpRS* gene operon in the DC3000 chromosome was replaced with the mutated *hrpRS* copy by standard marker-exchange mutagenesis (de Lorenzo *et al.*, 1990). The *hrpS* mutant was verified by PCR analysis using the above two primers and confirmed by its failure to elicit HR on tobacco and to cause disease on *Arabidopsis thaliana* (data not shown). Furthermore, the *hrpS* mutant could be complemented by both pHRPRS1 and pHRPRS2 (data not shown).

3.3.3. Protein Immunoblot Analysis

For immunoblot analysis of Hrp/Hrc proteins in culture, bacteria were first grown at 28°C to an OD_{600} of 0.8 to 1.0 in 5 ml of LB broth supplemented with appropriate antibiotics. Bacteria were then pelleted and resuspended in 5 ml of *hrp*-inducing broth or LB broth and incubated with shaking (250 rpm) at 20°C for 6 hrs. Cultures were then separated into cell (C) and supernatant (S) fractions by centrifugation. The cell fraction was resuspended in 0.5 ml of sterile water, whereas the supernatant fraction was concentrated 10-fold with Microcon 10 microconcentrators (Amicon) or the 30,000 NMWL filter unit of Ultrafree-MC (Millipore Corporation). Hrp/Hrc proteins in these fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with appropriate antibodies. For immunoblot analysis of Hrp/Hrc proteins expressed *in planta*, bacteria were grown in LB broth at 28°C to an OD_{600} of 0.8. Bacterial suspensions (OD_{600} of 1.5) prepared in distilled water were infiltrated into leaves of tobacco using needleless syringes. Four hours after infiltration, the infiltrated leaf tissue was excised and bacteria were expelled from the infiltrated leaf tissue by re-infiltrating the leaf tissue with an excess amount of distilled water. Bacteria were collected by centrifugation and the levels of Hrp/Hrc proteins in the expelled bacteria were analyzed by immunoblotting with appropriate antibodies. The protocol of immunoblot analysis of AvrPto secretion is as follows (conducted by Wen-Ling Deng in Alan Collmer's lab): Bacteria were first grown at 30°C to an OD_{600} of 0.8-1.0 in 40 ml of LM broth supplemented with appropriate antibiotics. The bacteria were then pelleted, washed with 10- to 15-ml *hrp*-inducing minimal medium (MM) twice, and resuspended in 100-ml *hrp*-inducing MM to an OD_{600} of 0.22-0.23. Cultures were

incubated at 20°C with shaking (180 rpm) until the optical density reached 0.4 (*Pst* DC3000 took about 24 hr to reach 0.4; different strains took different lengths of time). Bacterial cultures were then separated into cell and supernatant fractions by centrifugation (in a 45-ml Oakridge tube) (12,000 x g, 10 min, to pellet the bacteria first; then the supernatant was transferred to a clean tube for another spin at the same speed for another 10 min). After the second centrifugation, the upper 15-ml of the supernatant was carefully withdrawn to a clean tube, and proteins were precipitated with 20% (w/w) TCA at 4°C for at least 1 hr. The precipitated proteins were washed once with cold acetone once, and resuspended in 60 ml SDS sample buffer. In all immunoblotting experiments, gel staining with Coomassie Brilliant Blue R-250 was used to ensure equal loading of the protein samples.

3.3.4. RNA Blot Analysis

For determination of steady-state levels of *hrpW*, *hrcC*, *avrPto,* and *hrpRS* transcripts, total RNA was first isolated from bacteria according to the following protocol: Bacteria were first grown at 28°C to an OD_{600} of 0.3-0.6 in 10 ml of LB broth supplemented with appropriate antibiotics. The bacteria were then pelleted, washed with 10 ml of 10 mM $MgCl_2$, and resuspended into 5 ml of LB or *hrp*-inducing MM, and incubated with shaking for another 3 hr. 1.5 ml of bacterial culture was pelleted in a microtube. The cell pellet was resuspended in 0.5 ml lysis buffer (50mM Tris-HCl, pH 9.0, 50 mM EDTA, 300 mM NaOAc, 0.625% [w/v] sodium dodecyl sulfate), incubated in boiling water for 30 sec, extracted with phenol twice at 65°C, and extracted with chloroform twice. The supernatant was added to 0.5 ml of isopropyl alcohol, and the

visible clumps of DNA were taken out. The rest of the solution was then centrifuged for 5 min. The pellet was resuspended in 0.5 ml DEPC-treated water, incubated with 0.5 ml 4M lithium acetate for 60 min on ice, and then centrifuged for 5 min. The pellet was resuspended in 0.5 ml DEPC-treated water, incubated with 0.5 ml 4M lithium acetate for 60 min on ice, and then centrifuged for 5 min. The pellet was resuspended in 0.5 ml of DEPC-treated water and precipitated with 1 ml cold (0°C) 100% ethanol. The pellet was washed with 70% ethanol, dried, and dissolved in 25 ml of DEPC-treated water. Five μg of total RNA from each bacterial strain were analyzed by northern blotting (Sambrook *et al.*, 1989).

3.3.5. Mutagenesis of the *hrpA* Gene (by Anne Plovanich-Jones and Sheng Yang He)

For site-directed mutagenesis, the *hrpA* gene from pHRPA (Roine *et al.*, 1997) was cloned into pAlter1 (Promega). Six amino acid residues (G^{23}, A^{54}, K^{93}, D^{95}, I^{101}, and I^{111}) that are conserved between the HrpA proteins of *P. syringae* pv. *tomato* and *Erwinia amylovora* and/or among the HrpA proteins of *P. syringae* pvs. *tomato*, *syringae*, and *glycinea* (Kim *et al.*, 1997; Preston *et al.*, 1995) were individually replaced by residues with different properties (i.e., A^{23}, E^{54}, I^{93}, S^{95}, T^{101}, and Pro^{111}). The mutagenized *hrpA* inserts were then recloned into pUCP18 (Schweizer, 1991). For random mutagenesis of the *hrpA* gene, pHRPA was transformed into the *E. coli* mutator strain XL1-Red (Stratagene). After being subcultured four times, each for 12 hr at 37°C, pHRPA was isolated from XL1-Red and introduced into the *Pst* DC3000 *hrpA* nonpolar mutant *en masse* by electroporation. Transformants were individually grown in LB broth in microtiter plates overnight at 28°C (OD_{600} > 1.0). The bacterial cultures were then

diluted ten-fold in distilled water to an OD_{600} of 0.1 to 0.2 and infiltrated into tobacco and *Arabidopsis* leaves for HR and pathogenesis tests (see (Roine *et al.*, 1997), for procedures).

3.3.6. Pathogenesis Assays

Bacteria were grown in LB broth to an OD_{600} of 0.6-0.8. Bacterial suspensions in distilled water were infiltrated into leaves of *Arabidopsis thaliana* ecotype Columbia (Col), and tobacco (*Nicotiana tabacum* cultivar Samsun NN) using needleless syringes. The concentrations of bacteria used were 2×10^8 colony-forming units (cfu)/ml and 2×10^6 cfu/ml for HR and pathogenesis assays, respectively. Plant responses were recorded at 24 hr (for HR assay) or 4 days (for pathogenesis assay). The HR is characterized by rapid, localized tissue collapse in the infiltrated area within 24 hr. Disease symptoms were characterized by slowly developing necrosis and spreading tissue chlorosis, usually observed 3 days after infiltration. Tobacco plants were grown in greenhouses. *Arabidopsis* plants were grown in a growth chamber at 20°C with 70% relative humidity and a 12-hr photoperiod. HR assays with tobacco plants were performed at room temperature in the laboratory. Pathogenesis assays with *Arabidopsis* plants were performed at 20°C in growth chambers with 70% relative humidity and a 12-hr photoperiod.

3.4. RESULTS

3.4.1. Effect of a *hrpA* Deletion Mutation on the Production of HrpW

To examine the role of the Hrp pilus in protein secretion, I investigated the effect of a *hrpA* deletion mutation on secretion of HrpW in culture, in which a possible involvement of the Hrp pilus in mediating bacterial attachment to host cells is excluded. The cellular distribution of HrpW in *Pst* DC3000 and *hrpA*, *hrcC*, *hrcChrpTV,* and *hrpS* mutants was determined. As shown in Fig. 3.1A, HrpW was produced and secreted in DC3000 and in the *hrpA* mutant complemented by pHRPA, which carries the wild-type *hrpA* gene (Roine *et al.*, 1997). HrpW was produced but not secreted in the *hrcC* and *hrcChrpTV* secretion mutants, and was not detectable in the *hrpS* regulatory mutant. In the *hrpA* deletion mutant, however, HrpW was not detectable in the medium and was only barely detectable in the cells (Fig. 3.1A). The higher level of HrpW (and other Hrp, Hrc, and Avr proteins; see below) in the *hrcChrpTV* mutant, compared with that in the wild-type DC3000, may be due to the polar effect of the Tn*5Cm*-induced mutation in the *hrcC* gene on the downstream *hrpV* gene, which encodes a putative negative regulator (Preston *et al.*, 1998). HrpW was not overproduced in the nonpolar *hrcC* mutant, but it was overproduced in the *hrcChrpTV* mutant carrying pHRPCT, which contains *hrcC* and *hrpT* genes driven by P_{lac} promoter (Fig. 3.1A).

3.4.2. *hrpA* is Required for Expression of Other Hrc/Hrp Proteins

The unexpected down-regulation of the secreted protein, HrpW, in the *hrpA* mutant prompted me to examine a possible effect of the *hrpA* mutation on the expression of other *hrc/hrp* genes. Specifically, I examined expression of two membrane-bound components (HrcC and HrcJ) of the Hrp secretion machinery (Deng and Huang, 1999; Huang *et al.*, 1992). In *hrp*-inducing medium HrcJ was highly expressed in DC3000, the *hrcC* and *hrcChrpTV* mutants, and the *hrpA* mutant complemented by pHRPA, but not in the *hrpS* mutant. However, the expression of HrcJ was significantly suppressed in the *hrpA* mutant (Fig. 3.1A). The same expression pattern was observed for HrcC, except that HrcC was not detected in the *hrcC* or *hrcChrpTV* mutant (Fig. 3.1A). To rule out the possibility that the effect of the *hrpA* mutation on expression of *hrc/hrp* genes is due to the difference between *hrp*-inducing culture and the actual environment *in planta*, I determined the expression patterns of HrpW, HrcJ, and HrcC in DC3000 and the *hrp* mutants *in planta*. I found an expression pattern similar to that in *hrp*-inducing culture (Fig. 3.1B). Specifically, HrpW, HrcJ, and HrcC were produced in large amounts in DC3000, but not in the *hrpS* mutant. The amounts of these proteins were very low in the *hrpA* mutant. pHRPA restored the expression of these proteins in the *hrpA* mutant (Fig. 3.1B). Thus, I conclude that the pilus structural gene *hrpA* is required for expression of secreted proteins (HrpW) and components of the Hrp secretion machinery (HrcC and HrcJ) in both *hrp*-inducing minimal medium and *in planta*.

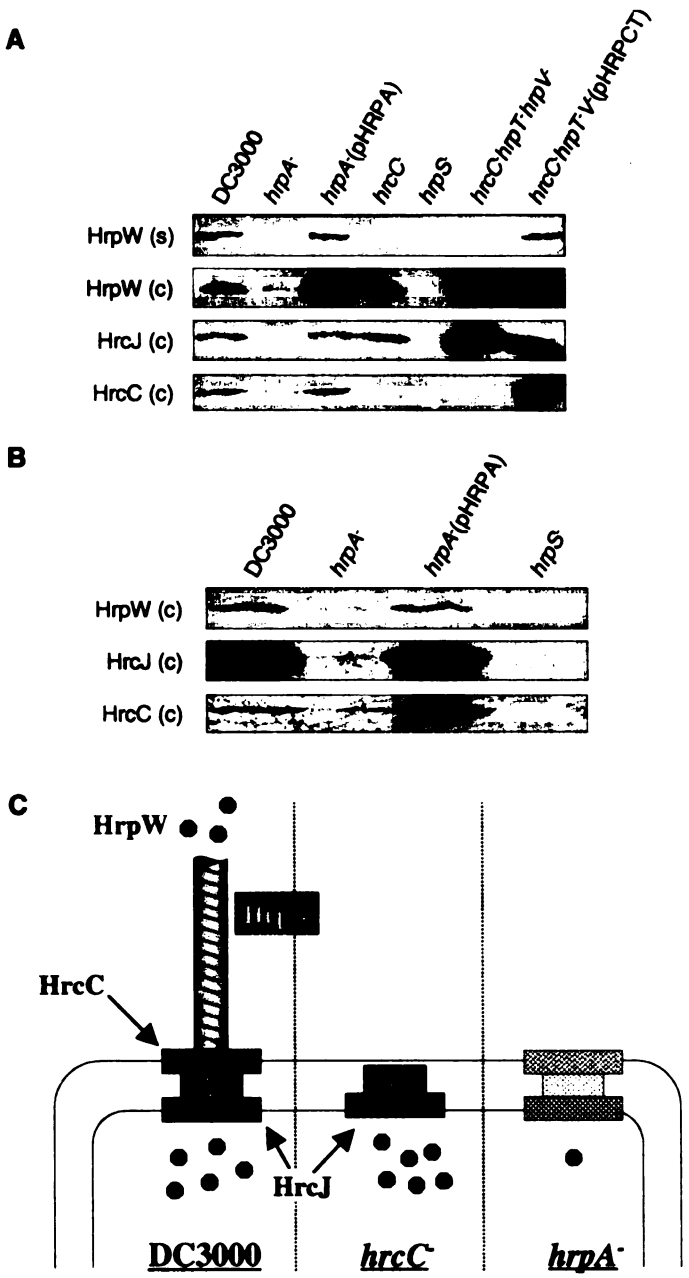

Figure 3.1. Immunoblot analysis of HrpW, HrcJ, and HrcC proteins in *Pst* DC3000 and *hrp* mutants in *hrp*-inducing medium (A) and *in planta* (B). For analysis of HrpW expression in *hrp*-inducing cultures, the levels of HrpW in cell-associated (c) and supernatant (s) fractions were examined. (C) A diagram of Hrp/Hrc protein production and secretion in *Pst* DC3000, *hrcC* mutant, and *hrpA* mutant.

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3.4.3. *hrpA* Controls the Expression of *hrp*, *hrc*, and *avr* Genes at the RNA Level

To determine whether the *hrpA* gene controls the expression of *hrc*/*hrp* genes at the RNA or protein level, I directly measured the steady-state RNA level of the *hrpW* operon in DC3000 and various mutants. Consistent with the immunoblot results, this operon was significantly down-regulated in the *hrpA* and *hrpS* mutants, compared with that in DC3000, in the *hrpA* deletion mutant complemented by pHRPA, and in the *hrcChrpTV* mutant (Fig. 3.2). I also determined the effect of the *hrpA* mutation on the expression of *avrPto*, which encodes a protein that is probably delivered into the plant cell via the Hrp secretion system (Scofield *et al.*, 1996; Tang *et al.*, 1996). As shown in Fig. 3.2, the steady-state RNA level of *avrPto* was also significantly suppressed in the *hrpA* and *hrpS* mutants. The higher level of steady-state RNA in the *hrcChrpTV* mutant is likely due to the absence of the negative regulator HrpV. The same result was obtained for the *hrcC* operon (Fig. 3.2). Thus, both the negative effect of the *hrpA* mutation and the positive effect of the *hrpV* mutation on the expression of *hrc*, *hrp*, and *avr* genes in *hrp*-inducing medium are at the RNA level.

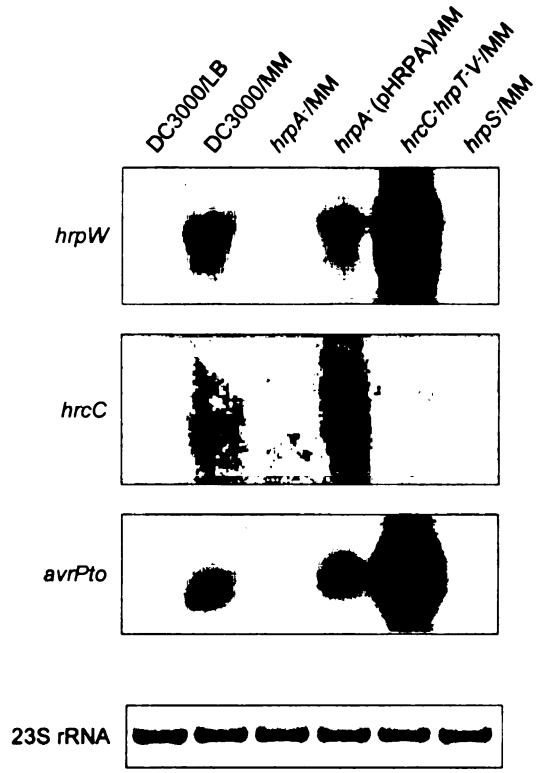

Figure 3.2. RNA blot analysis of *hrpW*, *hrcC* and *avrPto* transcripts in *Pst* DC3000 and *hrp* mutants. The genes indicated on the left were used as probes. The 23S rRNA visualized after ethidium bromide staining was used as a loading control. RNA was isolated from bacteria grown in *hrp*-repressing Luria-Bertani (LB) broth or *hrp*-inducing minimal medium (MM).

3.4.4. Three Amino Acid Residues Located at the Carboxyl Terminus Are Essential for HrpA-Mediated Gene Regulation

We next attempted to define amino acid substitution mutations that affect the regulatory function of the HrpA protein. An HR assay was used to screen for these mutations. As reported previously, the wild-type *hrpA* gene cloned in pHRPA restored the ability of an *hrpA* deletion mutant to elicit an HR in tobacco (Roine *et al.*, 1997). We reasoned that a mutation that affects the regulatory function of the HrpA protein would eliminate the ability of the *hrpA* gene (pHRPA) to complement the genomic *hrpA* deletion mutation. We used both site-directed and random mutagenesis procedures in the screen. Site-directed mutagenesis was facilitated by the relatively few residues (six were substituted in this study) conserved among the hypervariable HrpA proteins of *P. syringae* pathovars and *E. amylovora* (Kim *et al.*, 1997; Preston *et al.*, 1995). Residue substitutions in two positions (D^{95} to S^{95} and I^{111} to P^{111}) were found to eliminate the ability of the *hrpA* gene to complement the *hrpA* mutation for the elicitation of HR in tobacco (Fig. 3.3A). Random mutagenesis enabled us to identify a third mutant *hrpA* gene that fails to complement the *hrpA* mutation. Sequence analysis revealed an amino acid substitution at position 94 (from E^{94} to K^{94}) in the HrpA protein (Fig. 3.3A). All three mutant *hrpA* genes were found to be defective in positive regulation of *hrc/hrp* expression (Fig. 3.3B).

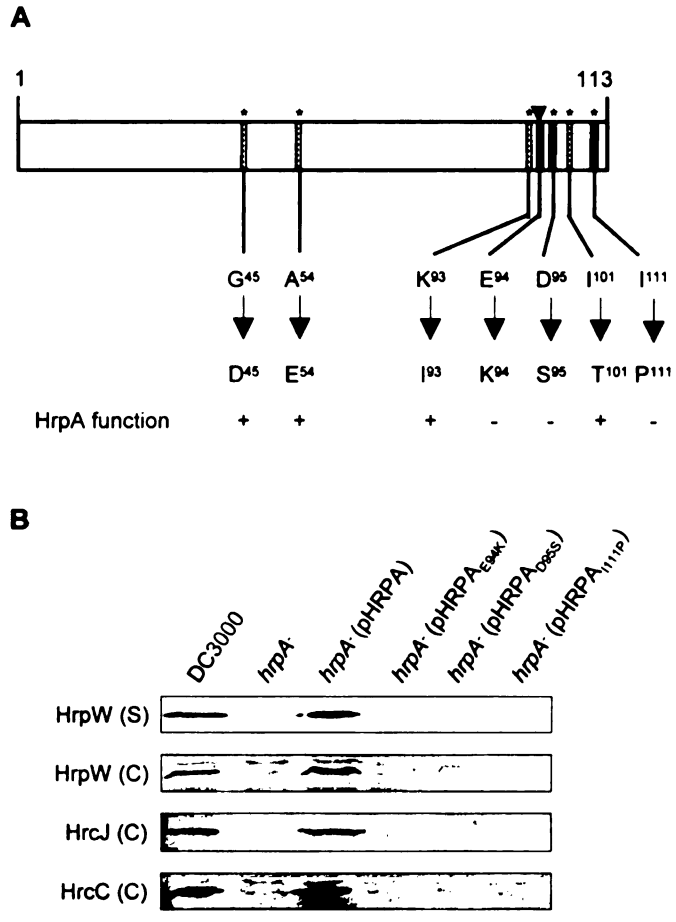

Figure 3.3. (A) A diagram of the *P. syringae* pv. *tomato* DC3000 HrpA protein (113 amino acids in length). Six amino acid residues (*) conserved among HrpA proteins of *P. syringae* pvs. *tomato*, *syringae*, and *glycinea* and *Erwinia amylovora* were mutated by site-directed mutagenesis. The E^{94} to K^{94} mutation, indicated by an arrowhead, was obtained by random mutagenesis. Amino acid residue substitutions that did not affect the HrpA function are indicated with hatched bars. Those substitutions that eliminated the ability of pHRPA to complement the genomic *hrpA* mutation are indicated with filled bars. The HrpA function was assayed by the ability (+) or inability (-) of the corresponding pHRPA derivatives to complement the genomic *hrpA* deletion mutation for HR elicitation in tobacco leaves and disease causation in *Arabidopsis thaliana* leaves. (B) Immunoblot analysis of the effect of single amino acid mutations of HrpA on the production of HrpW, HrcJ, and HrcC in *hrp*-inducing medium. Both cell-associated (C) and supernatant (S) fractions were analyzed.

3.4.5. HrpA-Mediated Gene Regulation is Through Activation of *hrpS* and *hrpR* at the RNA Level

To examine the relationship between *hrpA* gene and other regulatory genes, I first determined the steady-state message levels of the *hrpRS* positive regulatory operon in DC3000 and the *hrpA* mutant. As expected, the expression of the *hrpRS* operon was induced when DC3000 and the *hrcChrpTV* mutant were grown in *hrp*-inducing minimal medium, whereas this operon was barely expressed in DC3000 grown in LB or in the *hrpS* mutant grown in the *hrp*-inducing medium. I found significant down-regulation of the *hrpRS* operon in the *hrpA* mutant, whereas overexpression of *hrpA* from pHRPA elevated the expression of the *hrpRS* operon (Fig. 3.4A). Furthermore, when the *hrpRS* operon was expressed ectopically from a plasmid, *hrc/hrp* gene expression in the *hrpA* mutant was completely restored. Specifically, HrpW and HrcC were produced in both DC3000 and the *hrpA* mutant when *hrpRS* genes were ectopically expressed in the otherwise *hrp*-repressing LB broth (Fig. 3.4B). Similarly, ectopic expression of the *hrpRS* operon allowed expression of HrpW and HrcC proteins in the *hrpA* mutant *in planta* (Fig. 3.4C). The same results were obtained for HrcJ protein (Fig. 3.4C). These experiments suggest that *hrpR* and *hrpS* genes act downstream of *hrpA* in regulation of *hrc*, *hrp*, and *avr* genes. A previous study, however, has clearly shown that the expression of the *hrpA* gene is under the control of *hrpR* and *hrpS* (Xiao *et al.*, 1994). Therefore, the activation of the type III protein secretion system in *P. syringae* involves a closed regulatory network consisting of the extracellular pilus protein HrpA and at least three intracellular positive regulators, HrpS, HrpR, and HrpL.

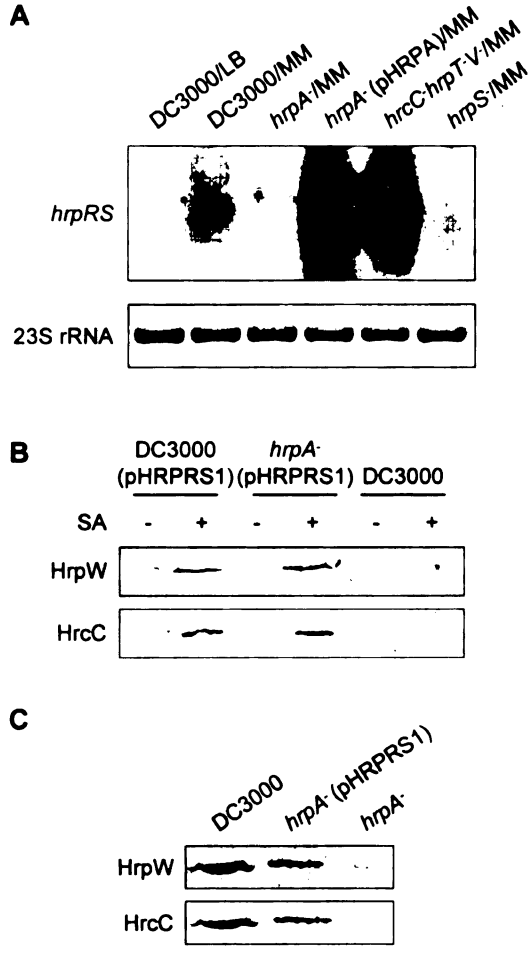

Figure 3.4. (A) Effect of the *hrpA* mutation on the steady-state message abundance of *hrpRS*. The procedures for bacterial growth and RNA preparation and blotting are the same as in the legend to Fig. 2, except that the RNA gel blot was hybridized to the *hrpRS* gene probe. (B) Effect of ectopic expression of *hrpRS* on the accumulation of Hrp/Hrc proteins in the *hrpA* mutant. Bacteria were grown in LB broth supplemented with (+) or without (-) 35 μM salicylic acid (SA). The original culture was directly used for analysis by SDS-PAGE followed by immunoblot analysis with antibodies against HrpW and HrcC, respectively, without further fractionation. pHRPRS1 carries the *P. syringae* pv. *syringae* 61 *hrpRS* open reading frames under the control of the SA-inducible P_G promoter in pKMY299 (Yen, 1991). (C) Effect of ectopic expression of *hrpRS* on the accumulation of Hrp/Hrc proteins in the *hrpA* mutant *in planta*. Bacteria were supplemented with 35 μM SA before infiltration. Ectopic expression of *Pst* DC3000 *hrpRS* genes also restored the expression of HrpW and HrcC in the *hrpA* mutant (see Fig. 3.5).

3.4.6. *hrpA* is Required for Secretion of HrpW and AvrPto

The restoration of gene expression in the *hrpA* mutant by the ectopically expressed *hrpRS* operon enabled the testing of a direct role of HrpA in protein secretion. As shown in Fig. 3.5A, when grown in the *hrp*-inducing medium, DC3000 produced and secreted HrpW to the medium with or without ectopic expression of the *hrpRS* gene operon, whereas HrpW was not detected in the culture medium of the *hrpA* mutants even when the *hrpRS* gene operon was constitutively expressed. Instead, the expressed HrpW was recovered exclusively in the cell fraction. Similarly, secretion of AvrPto was completely blocked by both mutation of *hrpA* and $hrpA_{D95S}$ (Fig. 3.5B). Consistent with a defect in protein secretion, the *hrpA* mutant ectopically expressing the *hrpRS* genes did not elicit HR in tobacco or cause disease in *Arabidopsis* (Table 3.1). Thus, in addition to its critical role in regulation of the Hrp secretion system, the Hrp pilus structural protein is required for secretion of HrpW and AvrPto.

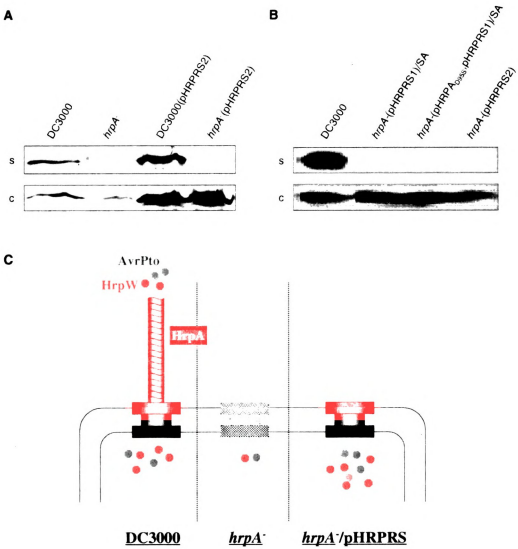

Figure 3.5. Effect of ectopic expression of the *hrpRS* operon in the *hrpA* mutant on HrpW and AvrPto secretion. Bacteria were grown in *hrp*- inducing minimal medium. Bacterial supernatant (s) and cell (c) fractions were analyzed by SDS-PAGE followed by immunoblotting with HrpW (A) or AvrPto antibody (B). pHRPRS2 carries the *Pst* DC3000 *hrpRS* open reading frames under the control of P_{lac} in pUCP18. (C) Diagram of protein production and secretion in *Pst* DC3000, *hrpA* mutant, and *hrpA* mutant carrying pHRPRS1 or pHRPRS2.

Table 3.1. Plant reactions to DC3000 and various *hrp* mutants.

Bacteria	*Arabidopsis thaliana* (ecotype Columbia)	Tobacco (cv. Samsun NN)
DC3000 ± SA	D	HR
hrpS$^-$	NR	NR
hrpA$^-$	NR	NR
hrpA$^-$/pHRPA	D	HR
hrpS$^-$/pHRPRS1 + SA	D	HR
hrpS$^-$/pHRPRS2	D	HR
hrpA$^-$/pHRPRS1 + SA	NR	NR
hrpA$^-$/pHRPRS2	NR	NR
hrpA$^-$/pHRPA$_{D95S}$, pHRPRS1 +SA	NR	NR

HR, rapid, localized tissue collapse in the infiltrated area within 24 hours. D, disease symptoms (slowly developing necrosis and spreading tissue chlorosis) observed 3 days after infiltration; NR, no visible reaction; SA, 0.35 mM salicylic acid.

3.5. DISCUSSION

In this study, I have attempted to define the function of the Hrp pilus structural protein HrpA in type III protein secretion in *P. syringae* pv. *tomato* DC3000. I found that the HrpA protein is required for expression of all examined genes that are associated with type III protein secretion. I showed that the positive regulatory function of HrpA on all *hrc*, *hrp*, and *avr* genes is achieved through activation of the *hrpR* and *hrpS* regulatory genes at the RNA level. Ectopic expression of the *hrpRS* gene operon can completely restore gene expression in the *hrpA* mutant. We identified three amino acid residues at the carboxyl terminus that affect the regulatory function of the HrpA protein. Finally, we showed that a functional HrpA protein is required not only for gene expression, but also for secretion of HrpW and AvrPto in culture. To our knowledge, this is the first demonstration of participation of a pilus structural protein in global regulation of a major protein secretion system and control of protein secretion in bacteria. We suggest that the Hrp pilus is an integral component of a supramolecular protein secretion structure that enables *Pst* DC3000 to turn on virulence genes and inject virulence proteins at the right place and time during bacterial infection of plants.

Pathogenic bacteria devote a large number of genes to type III protein secretion. In the *P. syringae hrp* gene cluster alone, for example, about 27 genes are co-regulated and they encode either regulatory, secretion, or effector proteins (Huang *et al.*, 1995). In addition, several *hrp*-regulated genes that encode effector proteins have been shown to be unlinked to the *hrp* gene cluster (Collmer, 1998; Leach and White, 1996). Thus, turning on the type III protein secretion system is an energy-consuming process. Because the final outcome of activating the type III secretion system is delivery of some virulence

proteins into the host cell, it would be beneficial for bacteria to prevent full induction of type-III-secretion-associated genes until host cells are available for protein injection. This prediction is consistent with the observed "contact-dependent activation" (transcriptional or posttranscriptional) of type III protein secretion in *Yersinia pseudotuberculosis*, *Salmonella typhimurium*, *Shigella flexneri*, and *E. coli* (Ginocchio *et al.*, 1994; Knutton *et al.*, 1998; Menard *et al.*, 1994; Pettersson *et al.*, 1996; Rosqvist *et al.*, 1994). Expression of *hrp* genes has also been shown to be induced in infected plant tissues (Huynh *et al.*, 1989; Rahme *et al.*, 1992; Xiao *et al.*, 1992). Host-bacterium contact appears to be important for bacterial elicitation of an HR (Stall and Cook, 1979), although direct evidence for contact-dependent activation of the Hrp system has yet to be obtained. The molecular basis of bacterial sensing of host-bacterium contact is not understood in any bacterium. In *Yersinia*, the current hypothesis for contact-dependent activation assumes that host-bacterium contact removes bacterial surface sensors, such as YopN (Forsberg *et al.*, 1991), LcrG (Sarker *et al.*, 1998; Skryzpek and Straley, 1993), and TyeA (Iriarte *et al.*, 1998), that normally prevent secretion (Cornelis, 1998). However, an involvement of secreted proteins (e.g. LcrV) as part of an extracellular secretion appendage in positive regulation of Yop secretion is a possibility (Cornelis, 1998).

The significant down-regulation of *hrp*, *hrc*, and *avr* genes in the *hrpA* mutants *in vivo* makes the Hrp pilus a strong candidate for mediating the presumed contact-dependent regulation of the Hrp system in *Pst* DC3000. Although bacterial sensors in the inner membrane or within the cytoplasm are often used to detect diffusible signals, they would not be suitable for detection of extracellular physical contact. Pili, on the other

hand, because of their extracellular location and supramolecular nature, appear to be suitable for sensing extracellular physical contact. In support of a role of pili in contact-based gene regulation, the adhesive P pilus of *E. coli* has been shown to sense the host-bacterium contact signal, resulting in the induction of bacterial iron starvation response genes (Zhang and Normark, 1996). The growing Hrp pilus may therefore be used by *Pst* DC3000 to sense the availability of host cells *in vivo*. In *Yersinia* spp., an artificial minimal medium has been shown to mimic the host-bacterium contact signal to induce the Ysc type III secretion system (Cornelis, 1998; Rosqvist *et al.*, 1994). It is therefore likely that the composition of the *hrp*-inducing minimal medium and growth conditions *in vitro* (usually with constant shaking, resulting in frequent pilus breakage) also mimic the plant-bacterium contact signal *in vivo*.

Whether pili are involved directly in the transfer of DNA or protein or indirectly in mediating cell-cell contact has been a long-standing and unresolved question in any system. We show here that in addition to its involvement in gene regulation, the HrpA protein is necessary for secretion of HrpW and AvrPto in culture, in which the possible function of pilus-mediated bacterial attachment to the host cell is excluded. The demonstrated role of HrpA in secretion of AvrPto and HrpW is therefore consistent with a hypothesis that the Hrp pilus is an integral component of the Hrp secretion structure. The Hrp pilus could be the functional equivalent of the extracellular part (called the short needle extension) of the *S. typhimurium* type III secretion supramolecular structure (Kubori *et al.*, 1998), providing a conduit for protein secretion and/or maintenance of the integrity of the Hrp secretion structure. Assembly of the longer pilus in *Pst* DC3000 may reflect a need for this bacterium to deliver proteins through the thick plant cell wall,

which is lacking in animal cells. Alternatively, the HrpA protein may function as a chaperone protein that pilots HrpW, AvrPto, and other secreted proteins through the Hrp secretion machinery (and along the Hrp pilus).

The observed dual function of HrpA in gene regulation and protein secretion strongly suggests that HrpA-mediated gene regulation is linked to the HrpA function in protein secretion. One explanation would be that HrpA (and hence the Hrp pilus) is also required for secretion of a negative regulator. Gene regulation based on export of a negative regulator (i.e., LcrQ) via the type III protein secretion system, but not involving a pilus, has been shown in *Yersinia pseudotuberculosis* (Pettersson *et al.*, 1996). According to this model, a negative regulator would accumulate in the cytoplasm of any type III secretion mutant (e.g., the *hrpA* mutant), thus repressing the expression of *hrc*, *hrp*, and *avr* genes. In support of this mechanism, a putative negative regulatory gene, *hrpV*, has recently been identified in *P. syringae* pv. *syringae* strain 61 (Preston *et al.*, 1998). However, this simple model does not appear to be consistent with the observed normal gene expression in the *hrcC* (Figs. 1 and 2) and other secretion mutants in *P. syringae* (Preston *et al.*, 1998). In fact, the apparently different effects of secretion mutations on gene expression are the key differences in the regulation of the type III protein secretion systems of *Y. pseudotuberculosis* and *P. syringae*. The molecular basis for this difference remains to be determined. However, the fact that HrpW, HrcC, and HrcJ proteins are expressed in much higher levels in the *hrcC* mutant than in the *hrpA* mutant suggests that HrpA accumulated inside the *hrcC* mutant causes a high level of gene expression. The HrpA-mediated positive regulatory function in the *hrcC* mutant may reflect a normal function of HrpA in the wild-type bacteria, assuming that there is a

pool of intracellular HrpA protein in *Pst* DC3000. Alternatively, the aberrant, intracellular localization of HrpA in the *hrcC* mutant may directly or indirectly interfere with the action of a negative regulator, such as HrpV.

The requirement of the *hrpA* gene for expression of the *hrpRS* operon argues that transcriptional activation of the Hrp secretion system in *P. syringae* involves a 'closed' regulatory circuit consisting of the extracellular pilus structural protein HrpA and three intracellular positive regulators, HrpS, HrpR and HrpL. Where is the start point of this regulatory circuit? The experimental results shown in Figs. 1, 3, and 5 suggest a two-phase activation model. Specifically, we found that in both *hrp*-inducing medium and *in vivo*, the levels of HrpW, HrcJ, and HrcC expression were slightly higher in the *hrpA* mutant than in the *hrpS* mutant. In most experiments we observed detectable levels of HrpW, HrcC, and HrcJ in the *hrpA* mutant (Figs. 3.1, 3.3, and 3.5), whereas we have never been able to detect any HrpW, HrcC, or HrcJ protein in the *hrpS* mutant. The HrpS and HrpR positive regulators must therefore be involved in detection of both a *hrpA*-dependent signal and a *hrpA*-independent signal in the plant apoplast. Perhaps the *hrpA*-independent signal triggers the first phase, low-level activation of *hrp* gene operons in the plant apoplast, as revealed in the *hrpA* mutant. The low-level expression of *hrp* genes initiates the growth of Hrp pili, which are then required for the second-phase, *hrpA* (and presumably contact)-dependent induction of *hrc*, *hrp*, and *avr* genes. The increased *hrc*/*hrp*/*avr* gene expression would enable further assembly of the Hrp secretion structure on top of or along with the existing Hrp pilus to penetrate the plant cell wall and host membrane, which eventually leads to injection of expressed virulence proteins into the host cytoplasm.

3. 6. REFERENCES

Alfano, J. R., Klm, H. S., Delaney, T. P., and Collmer, A. (1997). Evidence that the *Pseudomonas syringae* pv. *syringae hrp*-linked *hrmA* gene encodes an Avr-like protein that acts in an *hrp*-dependent manner within tobacco cells. *Mol Plant Microbe Interact* **10**, 580-8.

Anderson, D. M., and Schneewind, O. (1997). A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. *Science* **278**, 1140-1143.

Anderson, D. M., and Schneewind, O. (1999). Type III machines of Gram-negative pathogens: injecting virulence factors into host cells and more. *Curr Opin Microbiol* **2**, 18-24.

Bogdanove, A. J., Beer, S. V., Bonas, U., Boucher, C. A., Collmer, A., Coplin, D. L., Cornelis, G. R., Huang, H. C., Hutcheson, S. W., Panopoulos, N. J., and Van Gijsegem, F. (1996). Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. *Mol Microbiol* **20**, 681-3.

Charkowski, A. O., Alfano, J. R., Preston, G., Yuan, J., He, S. Y., and Collmer, A. (1998). The *Pseudomonas syringae* pv. *tomato* HrpW protein has domains similar to harpins and pectate lyases and can elicit the plant hypersensitive response and bind to pectate. *J Bacteriol* **180**, 5211-7.

Collmer, A. (1998). Determinants of pathogenicity and avirulence in plant pathogenic bacteria. *Curr Opin Plant Biol* **1**, 329-35.

Cornelis, G. R. (1998). The *Yersinia* deadly kiss. *J Bacteriol* **180**, 5495-504.

de Lorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K. N. (1990). Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J Bacteriol* **172**, 6568-72.

Deng, W. L., and Huang, H. C. (1999). Cellular locations of *Pseudomonas syringae* pv. *syringae* HrcC and HrcJ proteins, required for harpin secretion via the type III pathway. *J Bacteriol* **181**, 2298-301.

Forsberg, A., Viitanen, A. M., Skurnik, M., and Wolf-Watz, H. (1991). The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. *Mol Microbiol* **5**, 977-86.

Fullner, K. J., Lara, J. C., and Nester, E. W. (1996). Pilus assembly by *Agrobacterium* T-DNA transfer genes. *Science* **273**, 1107-9.

Galan, J. E., and Collmer, A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**, 1322-8.

Ginocchio, C. C., Olmsted, S. B., Wells, C. L., and Galan, J. E. (1994). Contact with epithelial cells induces the formation of surface appendages on *Salmonella typhimurium*. *Cell* **76**, 717-24.

Gopalan, S., Bauer, D. W., Alfano, J. R., Loniello, A. O., He, S. Y., and Collmer, A. (1996). Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (Hrp) secretion system in eliciting genotype-specific hypersensitive cell death. *Plant Cell* **8**, 1095-105.

Grimm, C., Aufsatz, W., and Panopoulos, N. J. (1995). The *hrpRS* locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. *Mol Microbiol* **15**, 155-65.

Grimm, C., and Panopoulos, N. J. (1989). The predicted protein product of a pathogenicity locus from *Pseudomonas syringae* pv. *phaseolicola* is homologous to a highly conserved domain of several procaryotic regulatory proteins. *J Bacteriol* **171**, 5031-8.

He, S. Y. (1998). Type III protein secretion systems in plant and animal pathogenic bacteria. *Annu Rev Phytopathol* **36**, 363-392.

He, S. Y., Huang, H. C., and Collmer, A. (1993). *Pseudomonas syringae* pv. *syringae* harpin$_{Pss}$: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell* **73**, 1255-66.

Herrero, M., de Lorenzo, V., and Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol* **172**, 6557-67.

Huang, H. C., He, S. Y., Bauer, D. W., and Collmer, A. (1992). The *Pseudomonas syringae* pv. *syringae* 61 *hrpH* product, an envelope protein required for elicitation of the hypersensitive response in plants. *J Bacteriol* **174**, 6878-85.

Huang, H. C., Lin, R. H., Chang, C. J., Collmer, A., and Deng, W. L. (1995). The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpin$_{Pss}$ secretion that are arranged colinearly with *Yersinia ysc* homologs. *Mol Plant Microbe Interact* **8**, 733-46.

Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* **62**, 379-433.

Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. (1989). Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science* **245**, 1374-7.

Innes, R. W., Bent, A. F., Kunkel, B. N., Bisgrove, S. R., and Staskawicz, B. J. (1993). Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. *J Bacteriol* **175**, 4859-69.

Iriarte, M., Sory, M. P., Boland, A., Boyd, A. P., Mills, S. D., Lambermont, I., and Cornelis, G. R. (1998). TyeA, a protein involved in control of Yop release and in translocation of *Yersinia* Yop effectors. *EMBO J* **17**, 1907-18.

Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. (1988). Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* **70**, 191-7.

Kim, J., Wei, Z., and Beer, S. (1997). The *hrpA* and *hrpC* operons of *Erwinia amylovora* encode components of a type III pathway that secretes harpin. *Journal of bacteriology* **179**, 1690-1697.

Knutton, S., Rosenshine, I., Pallen, M. J., Nisan, I., Neves, B. C., Bain, C., Wolff, C., Dougan, G., and Frankel, G. (1998). A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *EMBO J* **17**, 2166-76.

Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galan, J. E., and Aizawa, S. I. (1998). Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* **280**, 602-5.

Leach, J., and White, F. (1996). Bacterial avirulence genes. *Annu Rev Phytopathol* **34**, 153-179.

Lindgren, P. B. (1997). The role of *hrp* genes during plant-bacterial interactions. *Annu Rev Phytopathol* **35**, 129-52.

Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. (1986). Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity of nonhost plants. *J Bacteriol* **168**, 512-22.

Menard, R., Sansonetti, P., and Parsot, C. (1994). The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD. *EMBO J* **13**, 5293-302.

Mudgett, M. B., and Staskawicz, B. J. (1999). Characterization of the *Pseudomonas syringae* pv. *tomato* AvrRpt2 protein: demonstration of secretion and processing during bacterial pathogenesis. *Mol Microbiol* **32**, 927-41.

Pettersson, J., Nordfelth, R., Dubinina, E., Bergman, T., Gustafsson, M., Magnusson, K. E., and Wolf-Watz, H. (1996). Modulation of virulence factor expression by pathogen target cell contact. *Science* **273**, 1231-3.

Preston, G., Deng, W. L., Huang, H. C., and Collmer, A. (1998). Negative regulation of *hrp* genes in *Pseudomonas syringae* by HrpV. *J Bacteriol* **180**, 4532-7.

Preston, G., Huang, H. C., He, S. Y., and Collmer, A. (1995). The HrpZ proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* are encoded by an operon containing *Yersinia ysc* homologs and elicit the hypersensitive response in tomato but not soybean. *Mol Plant Microbe Interact* **8**, 717-32.

Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. (1992). Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*. *J Bacteriol* **174**, 3499-507.

Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E. L., Kalkkinen, N., Romantschuk, M., and He, S. Y. (1997). Hrp pilus: an *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. *Proc Natl Acad Sci U S A* **94**, 3459-64.

Rosqvist, R., Magnusson, K. E., and Wolf-Watz, H. (1994). Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. *EMBO J* **13**, 964-72.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). *Molecular cloning : a laboratory manual 2nd ed.*

Sarker, M. R., Sory, M. P., Boyd, A. P., Iriarte, M., and Cornelis, G. R. (1998). LcrG is required for efficient translocation of *Yersinia* Yop effector proteins into eukaryotic cells. *Infect Immun* **66**, 2976-9.

Schweizer, H. P. (1991). *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* **97**, 109-21.

Scofield, S. R., Tobias, C. M., Rathjen, J. P., Chang, J. H., Lavelle, D. T., Michelmore, R. W., and Staskawicz, B. J. (1996). Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science* **274**, 2063-5.

Skryzpek, E., and Straley, S. C. (1993). LcrG, a secreted protein involved in negative regulation of the low-calcium response in *Yersinia pestis*. *J Bacteriol* **175**, 3520-8.

Stall, R. E., and Cook, A. A. (1979). Evidence that bacterial contact with the plant cell is necessary for the hypersensitive reaction but not the susceptible reaction *Xanthomonas vesicatoria*, pepper. *Physiol Plant Pathol* **14**, 77-84.

Stevens, C., Bennett, M. A., Athanassopoulos, E., Tsiamis, G., Taylor, J. D., and Mansfield, J. W. (1998). Sequence variations in alleles of the avirulence gene *avrPphE.* R2 from *Pseudomonas syringae* pv. *phaseolicola* lead to loss of recognition of the AvrPphE protein within bean cells and a gain in cultivar-specific virulence. *Mol Microbiol* **29**, 165-77.

Tang, X., Frederick, R. D., Zhou, J., Halterman, D. A., Jia, Y., and Martin, G. B. (1996). Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* **274**, 2060-3.

Taylor, R. K., Manoil, C., and Mekalanos, J. J. (1989). Broad-host-range vectors for delivery of TnphoA: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae. J Bacteriol* **171**, 1870-8.

van Dijk, K., Fouts, D. E., Rehm, A. H., Hill, A. R., Collmer, A., & Alfano, J. R. (1999). *J. Bacteriol. In press.*

Willetts, N., and Skurray, R. (1980). The conjugation system of F-like plasmids. *Annu Rev Genet* **14**, 41-76.

Xiao, Y., Heu, S., Yi, J., Lu, Y., and Hutcheson, S. W. (1994). Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling expression of *Pseudomonas syringae* pv. *syringae Pss*61 *hrp* and *hrmA* genes. *J Bacteriol* **176**, 1025-36.

Xiao, Y., and Hutcheson, S. W. (1994). A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae. J Bacteriol* **176**, 3089-91.

Xiao, Y., Lu, Y., Heu, S., and Hutcheson, S. W. (1992). Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. *J Bacteriol* **174**, 1734-41.

Yen, K. M. (1991). Construction of cloning cartridges for development of expression vectors in gram-negative bacteria. *J Bacteriol* **173**, 5328-35.

Yuan, J., and He, S. Y. (1996). The Pseudomonas syringae Hrp regulation and secretion system controls the production and secretion of multiple extracellular proteins. *J Bacteriol* **178**, 6399-402.

Zhang, J. P., and Normark, S. (1996). Induction of gene expression in *Escherichia coli* after pilus-mediated adherence. *Science* **273**, 1234-6.

Chapter 4

IDENTIFICATION OF COTTON TARGETS OF *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM* AVR/PTH FAMILY OF PROTEINS

4.1. ABSTRACT

The function of *avr* genes is dependent on *hrp* genes, which are involved in the assembly of a type III secretion system. Recent studies have shown that Avr proteins are probably translocated into plant cells to trigger host responses. One unique Avr family of proteins, Avr/Pth, from the cotton pathogen *Xanthomonas campestris*, shares very high sequence similarity among each other. Members of this family differ primarily in the multiplicity of a tandemly repeated 102-base pair motif within the central portions of the genes. The repeated regions have been shown to confer specificity in *avr-R* (Resistance) gene interactions. Their C-terminal regions contain eukaryotic nuclear localization signals (NLSs), which have been demonstrated to be capable of delivering reporter fusion proteins into the plant nucleus, and are critical for their avirulence functions. Some members (e.g. Avrb6) of this family are also responsible for the virulence of *X. campestris* pv. *malvacearum* (*Xcm*) in susceptible cotton. This chapter describes experiments aimed at identifying cotton proteins that interact with Avrb6, a member of the Avr/Pth family of proteins. The yeast two-hybrid system was employed for this purpose. After screening over 1.8×10^8 clones, I confirmed that more than 152 of them encode proteins that specifically bind to the bait 6ΔNC, the central repeat region of

Avrb6. Sequencing analysis revealed three interesting clones: a serine/threonine kinase homologous to Pto in tomato, a calcineurin B-like protein, and a putative transcription factor with a zinc-finger motif. The functional significance of these putative target proteins in *Xcm* avirulence and virulence and their interaction with the entire length Avrb6 protein remain to be determined.

4.2. INTRODUCTION

Bacterial pathogens are responsible for numerous diseases in higher plants, animals and humans. Plants have evolved a variety of mechanisms to defend themselves from pathogens (Lamb *et al.*, 1989; Lamb, 1994). Among them, the gene-for-gene type resistance response is being studied intensively. According to the gene-for-gene hypothesis (Flor, 1971), disease resistance is conditioned by the presence of a single dominant or semidominant disease resistance (*R*) gene in the host and a corresponding avirulence (*avr*) gene in the pathogen. The absence of an *avr* gene in the pathogen or its matching *R* gene in the plant, or both, results in a compatible interaction and leads to plant disease.

A type III protein secretion system found in many bacteria transports virulence or avirulence proteins to host cells. In animal pathogens, the type III secreted proteins affect host cell signaling. For example, *Yersinia* species secrete several Yops (*Yersinia* Outer Proteins), including a tyrosine phosphatase (YopH), an actin cytotoxin (YopE), and a Ser/Thr kinase (YpkA), into the host cell following host cell contact (Galyov *et al.*, 1993; Sory *et al.*, 1995).

Indirect evidence suggests that bacterial virulence and avirulence proteins are also transported into plant cell via type III protein secretion system. The first line of evidence was provided by Gopalan *et al.* (1996) from our laboratory. By using transgenic and transient expression of AvrB of *P. syringae* in leaves of *Arabidopsis thaliana*, they showed that the site of activity of AvrB is inside the plant cell and that the Hrp secretion system is necessary for the delivery of AvrB into plant cells during infection. Similar indirect evidence supporting transport of Avr proteins has independently been obtained

for *P. syringae* pv. *tomato* AvrPto (Scofield *et al.*, 1996; Tang *et al.*, 1996) and AvrRpt2 (Leister *et al.*, 1996). In the case of the *avrPto/Pto* system, the Pto protein appears to be a cytoplasmic protein. It has been shown that AvrPto and Pto physically bind to each other when expressed in yeast (Tang *et al.*, 1996).

Additionally, Yang and Gabriel (Yang and Gabriel, 1995) reported that members of the *Xanthomonas* Avr/Pth family of proteins might be recognized inside the plant cell. PthA contains functional nuclear localization signals (NLSs). Subsequently, Van den Ackerveken *et al.* (1996) showed that the NLSs in AvrBs3, another member of the Avr/Pth family of proteins, were able to target the reporter protein β-glucuronidase (GUS) to the plant nucleus. They also showed that these NLSs are required for AvrBs3 to trigger disease resistance-associated HR, suggesting that some members of the Avr/Pth family not only enter the plant cell, but also enter the plant nucleus. Expression of *avrBs3* in pepper cells, using *Agrobacterium tumefaciens* for gene delivery, resulted in induction of hypersensitive cell death specifically on plants carrying the *avrBs3*-corresponding *R* gene, *Bs3*. This response resembled phenotypically the HR induced by the pathogen *Xanthomonas campestris* pv. *vesicatoria* expressing *avrBs3*.

The research described in this chapter was aimed at identifying cotton proteins that interact with Avrb6. *Xanthomonas campestris* pv. *malvacearum* (*Xcm*) causes bacterial blight of cotton (*Gossypium hirsutum* L.). The disease symptoms include severe water soaking and eventual necrosis. *Xcm* also elicits HR in resistant cotton cultivars. At least 16 genetically characterized resistance genes against *Xcm* have been identified in cotton. Many of these *R* genes have been introduced into a common (Acala-44) genetic background by repeated backcrossing (Brinkerhoff *et al.*, 1984; De Feyter and Gabriel,

1991), resulting in the resistant line named IM216. A large avirulence (*avr*)/pathogenicity (*pth*) gene family has been found in *Xcm* (Yang *et al.*, 1996). The published members of the *avr/pth* gene family include *avrB4, avrb6, avrB102, avrB101, avrBIn*, and *avrb7* of *X. campestris* pv. *malvacearum* (De Feyter and Gabriel, 1991; De Feyter *et al.*, 1993). All family members sequenced to date are 95 to 98% identical to each other and to the prototype of this Avr protein family, AvrBs3 (Bonas *et al.*, 1993; De Feyter *et al.*, 1993; Hopkins *et al.*, 1992; Yang and Gabriel, 1995). They differ primarily in the multiplicity of a tandemly repeated 102-base pair motif within the central portions of the genes, repeated from 14 to 23 times in members of this gene family. The changes in the repeat region can give rise to different specificity. This strongly suggests that the Avr/Pth protein is the actual elicitor molecule that is being recognized by the plant, rather than functioning in the bacterium as an enzyme required for the production of elicitor molecules (De Feyter *et al.*, 1993).

By constructing chimeric genes among *pthA, avrB4, avrb6, avrb7, avrBIn, avrB101,* and *avrB102*, the 102-bp tandem repeats of the genes were found to determine gene-for-gene specificity (Yang *et al.*, 1994). *avrb6* was also found to be required for *Xcm* to cause severe water-soaking and subsequent necrosis in susceptible Acala-44 cotton lines (Yang *et al.*, 1994). Using Avrb6, it should therefore be possible to find the receptors responsible for both plant resistance and susceptibility.

The yeast two-hybrid assay approach was used for identifying cotton proteins that interact with Avrb6. The yeast two-hybrid system provides a sensitive method for detecting relatively weak and transient protein-protein interactions. Such interactions may not be biochemically detectable, but may be critical for the proper functioning of

complex biological systems (Estojak *et al.*, 1995). In addition, because the two-hybrid assay is performed *in vivo*, the proteins are most likely to be in their native conformations, which may lead to increased sensitivity and accuracy of detection. For the purpose of identifying cotton receptors of Avrb6, I chose the GAL4 MATCHMAKER Two-hybrid System 2 from CLONTECH Company. The GAL4 systems utilize two reporter genes (*HIS3* and *lacZ*) under the control of a GAL4-responsive UAS. All reporter cassettes are integrated into the genome of the host strain. Screening His^+ clones for the expression of the *lacZ* reporter, which has a promoter other than the *HIS3* reporter, eliminates many of the false positives that arise in a typical two-hybrid library screening.

4.3. MATERIALS AND METHODS

4.3.1. *Agrobacterium*-Mediated Transient Expression

A. tumefaciens strain GM3850 containing the Ti-plasmid pKYLX71 (Maiti *et al.*, 1993) or individual transconjugants were grown in 2 ml LB medium containing appropriate antibiotics. Following overnight growth at 28°C, bacteria were pelleted by centrifugation and resuspended to an optical density at 600 nm (OD_{600}) equal to 0.25 in induction medium according to Bundock *et al.* (1995), with minor modifications. The medium contained 10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 1.0 g/l $(NH_4)_2SO_4$, 0.5 g/l NaCitrate.$2H_2O$, 1 mM $MgSO_4.H_2O$, 0.2% glucose, 0.5% glycerol, 50 μM acetosyringone (Aldrich), and 10 mM N-morpholino-ethanesulfonic acid (MES) (pH 5.6). After overnight incubation at 28°C, at OD_{600} = 0.5-0.6, bacteria were pelleted by

centrifugation, washed with Murashige and Skoog's medium (MS, Sigma) with 10 mM MES (pH 5.6), and resuspended to OD_{600} = 0.5 in MS-MES medium with 150 μM acetosyringone. Bacterial suspensions were infiltrated into young but fully expanded leaves of 6- to 8-week-old cotton plants using a needleless syringae. After infiltration, plants were immediately covered with transparent plastic bags and placed at 20°C (reported optimal for T-DNA transfer, (Fullner and Nester, 1996)). After 40-48 hr, plastic bags were removed and plants placed at 28°C, 16 hr light/8hr dark, and 80% humidity to allow symptoms to develop (Van den Ackerveken *et al.*, 1996).

4.3.2. Yeast Two-Hybrid System

Yeast two-hybrid screening was conducted according to instructions with the CLONTECH MATCHMAKER GAL4 Two-hybrid system 2 (K1604-1) and Yeast Protocols Handbook (PT3024-1). The pAS2-1 plasmid was used to construct the bait plasmid. Yeast strain CG-1945 was used as the host for the library screening. An alternative *HIS3* "jump-start" procedure (CLONTECH) was also used in which the yeast strain Y190 was used as the host. The "jump-start" procedure permits detection of some interactions in which the affinity is too low to allow the *HIS3* reporter to produce enough HIS3 protein for survival if plated directly on the SD (synthetic dropout)/-His selection medium.

4.3.3. Total RNA Isolation from Cotton Plant

Acala-44 is a susceptible cotton line lacking most of resistance genes. IM216 is a resistant cotton line which has many *R* genes (Brinkerhoff *et al.*, 1984; De Feyter and Gabriel, 1991). Avrb6 proteins are responsible not only for triggering HR in line IM216, but also for causing water-soaking disease in Acala-44. The total cotton RNA was isolated from both IM216 and Acala-44 lines by using a modified hot borate method in order to cope with cotton's high levels of phenolics, terpenes, secondary metabolites, and, in some tissues, polysaccharides (Wan and Wilkins, 1994). Preheated (80°C) RNA extraction buffer (200 mM sodium borate decahydrate (Borax), pH 9.0, 30 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 1% (w/v) sodium dodecyl sulfate (SDS), 10 mM dithiothreitol, 2% (w/v) polyvinylpyrrolidone (PVP)-40,000) was added to frozen ground tissue at a ratio of 5 ml of buffer per gram of fresh weight and homogenized for 2 min. The homogenate was transferred to a 50-ml Oak Ridge tube containing 0.5 mg of proteinase K per milliliter of extraction buffer and incubated with mild agitation on a rotary shaker at 100 rpm for 1.5 hr at 42°C. The homogenate was adjusted to 160 mM potassium chloride and chilled on ice for 1 hr. After centrifugation at 12,000 x g for 20 min, the supernatant was filtered through miracloth and the RNA was precipitated overnight in 2 M LiCl on ice. Following centrifugation as above, the RNA pellet was washed two to three times with 5 ml of cold 2 M LiCl until the supernatant was relatively colorless. The LiCl-RNA pellet was suspended in 2 ml of 10 mM Tris-HCl, pH 7.5, and clarified by centrifugation for 10 min. 200 mM potassium acetate (pH 5.5) was added and following an incubation of 15 min on ice, salt-insoluble material was removed from the supernatant containing the RNA by centrifugation. The RNA was

precipitated overnight with 2.5 x volume of 100% ethanol at –20°C. The RNA was pelleted, washed with 70% cold ethanol, dried briefly under vacuum, and suspended in DEPC (diethylpyrocarbonate)-treated deionized water. Agarose gel electrophoresis was used to examine the quality of the isolated cotton leaf total RNA.

4.3.4. cDNA Library Construction

In order to have the chance to identify both resistance and susceptibility factors, I combined both IM216 and Acala-44 total RNA in 50:50 ratio to make the cDNA library. For construction of an AD library, 3.6 mg of Acala-44 and 5.4 mg of IM216 total RNA was sent to CLONTECH.

Both oligo(dT) and random primers were used for cDNA synthesis. *Eco*RI adapters were ligated to the cDNA before being cloned into the pGAD10 vector. The amplified library had a titer of $\geq 10^8$ cfu/ml (in DH5α), 75% of colonies were estimated to have inserts, and the number of independent clones was 2.5×10^6 with an average insert size of 1.0 kb (ranging from 0.2 - 4.0 kb). Fifteen colonies were randomly picked and subjected to polymerase chain reaction (PCR) analysis; 14 of these contained inserts (data not shown).

4.3.5. Construction of Bait Plasmid

The *avrb6* ORF was amplified by PCR from plasmid pAVRB6-1 (*avrb6* gene clone in pBSSKII(-) from pUFR106 (DeFeyter *et al.*, 1990) using the *Nde*I-tagged upstream primer (5'-CTAGACCACATATGGATCCCATTCGTTCGCGCACGCCAAG-

TC-3'), and *Eco*RI-tagged downstream primer (5'-CGAATTCCCTCACTGAGGCAAT-AGCTCCATCAAC-3'). The PCR product was digested with *Nde*I and *Eco*RI, and cloned into the corresponding sites in vector pAS2-1. The *avrb6* sequence was inserted in the correct reading frame and orientation to make a fusion of Avrb6 and the GAL4 binding domain (BD). This construct was then transformed into yeast strain CG-1945 or Y190 using a small-scale yeast transformation protocol (CLONTECH K1604-1). Transformants were assayed for *lacZ* reporter gene expression.

Sequential deletion of *avrb6* was accomplished by PCR cloning using primers matching the sequences of different regions of the *avrb6* open reading frame. PCR products were cloned into a pAS2-1 and transformed into yeast strains CG-1945 or Y190, as described above. The primers used to amplify the central repeat region of *avrb6* for construction of the bait plasmid 6ΔNC were: *Nde*I-tagged upstream primer (5'-CCCAAGCTTCATATGGTGCAATCGGGTCTGCGGGC-3') and *Eco*RI-tagged downstream primer (5'-GCTCTAGAATTCAAAATGCTTGCGCTGGGTGGGAGT-GGC-3').

4.3.6. DNA Sequencing and Analysis

For sequence analysis of putative clones, the cDNA inserts were amplified by using pAD10 vector primers: 5'AD (5'-TACCACTACAATGGATG-3') and 3'AD (5'-TGCACGATGCACAGTTGAAGTAAC-3'). To obtain full-length sequences, primers were designed according to the internal sequence information. In combination with either the 5'AD or the 3'AD primer, the 5' and 3' end sequences of each cDNA insert were amplified from the AD-cDNA library by using gene-specific primers and 5'AD or

3'AD primer. DNA sequence data were analyzed using the MacVector software (Oxford Molecular Group) and BLAST search program (http://www.ncbi.nlm.nih.gov/blast/). The deduced amino acid sequences were compared with sequences in the current databases (GENBANK, SWISS PROTEIN, and EMBL) using the BLAST, MOTIFS, and PROPSEARCH programs. Sequences were aligned using the BOXSHADE 3.21 program (http://www.ch.embnet.org/software/box_form.html). Protein motifs were examined by using the PROSITE PATTERN search program (http://www.motif.genome.ad.jp/). The protein localization prediction was analyzed by using the PSORT program (http://psort.nibb.ac.jp:8800/form.html).

4.3.7. *Arabidopsis thaliana* Transformation

The full length the *avrb6* gene was amplified from plasmid pAVRB6-1 using the *Hind*III-tagged upstream primer (5'-CCCAAGCTTACCATGGATCCCATTCGTTCGC-GC-3') and *Xba*I-tagged downstream primer (5'-GCTCTAGATCACTGAGGCAATAG-CTCCATCAACC-3'), digested with *Hind*III and *Xba*I, and cloned into the corresponding sites in pKYLX71::35S[2], a plant transformation vector (Maiti *et al.*, 1993). The resulting construct was electroporated into *Agrobacterium tumefaciens* GV3850 and transformed into *Arabidopsis thaliana* (Col) by vacuum infiltration (Bent *et al.*, 1994). Following the same strategy, the *avrB101* gene was also transformed into *Arabidopsis thaliana* (Col). The same *avrb6* and *avrB101* clones in pKYLX71 were also used in an *Agrobacterium*-mediated transient assay in cotton.

4.4. RESULTS

4.4.1. *Agrobacterium*-Mediated Transient Expression of Avrb6 and AvrB101 in Cotton

AvrBs3 of *Xanthomonas campestris* pv. *vesicatoria* has been shown to induce hypersensitive cell death when transiently expressed in pepper leaves that contains Bs3 resistance gene (Van den Ackerveken *et al.*, 1996). This suggests that AvrBs3 functions inside the plant cell. To determine whether the Avrb6 protein also acts inside plant cells, transient expression of *avrb6* in cotton leaves was carried out. The PCR-amplified *avrb6* gene was cloned into the *Hind*III / *Xba*I sites of pKYLX71::35S^2 (Maiti *et al.*, 1993). Using *A. tumefaciens* for gene delivery, *avrb6* was expressed transiently in cotton leaves. As shown in Fig. 4.1, leaves of the cotton resistant line IM216 responded to *avrb6* expression with an HR, whereas those of the susceptible line Acala-44 did not. In the absence of the *avrb6* gene, the HR was not induced by *A. tumefaciens*. Deletion of the central repeat region also abolished ability of Avrb6 to elicit HR in IM216 (Fig. 4.1). These results indicate that Avrb6 by itself is sufficient to trigger HR in the resistant cotton line IM216 when expressed inside plant leaves. The same results were obtained for AvrB101 (Fig. 4.1). Therefore, Avrb6 and AvrB101 appear to function inside cotton cells to trigger HR.

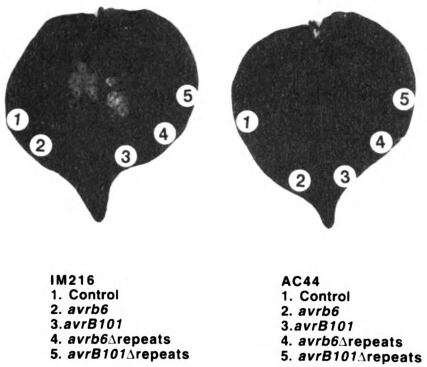

Figure 4.1. *A. tumefaciens*-mediated transient expression in cotton leaves.

A. tumefaciens transconjugants carrying different constructs were grown under *vir* gene-inducing conditions and inoculated into the intercellular space of cotton leaves. Symptoms were photographed 48 hours post-infiltration. *avrb6*, *avrB101*, *avrb6*Δrepeats and *avrB101*Δrepeats (*Msc*I complete digestion to delete the central repeat region) were cloned into *Hind* III / *Xba*I sites of pKYLX71::35S[2]. Transient expression of *avrb6* and *avrB101*, but not the control (pKYLX alone), *avrb6*Δrepeats, or *avrB101*Δrepeats led to hypersensitive cell death in leaves of the resistant cotton Im 216, but not the susceptible line Acala-44, within 48 hours.

4.4.2. Construction and Test of the Bait Plasmid

The full length of the *avrb6* ORF was cloned into the bait vector pAS2-1. The resulting construct was transformed into the yeast strain CG-1945 and assayed for the expression of the *lacZ* reporter gene. To our surprise, this bait construct was able to activate the expression of both reporter genes to a high level even in the absence of any AD protein. Although this result indicates that Avrb6 may function as a transcription activator in plants, the full length of the *avrb6* could therefore not be used as a bait to screen the AD library.

Activation domains of many eukaryotic transcription factors share a common feature, i.e., presence of a net negative charge (Ma and Ptashne, 1988; Remacle *et al.*, 1997; Saha *et al.*, 1993). In fact, *E. coli* genomic DNA fragments that bear an excess number of acidic amino acids can function as yeast transcriptional activators (Ma and Ptashne, 1987; Ruden *et al.*, 1991). By using the DNASTAR Protean program (DNASTAR Inc., Madison, Wisconsin), I defined several acidic domains in Avrb6 (data not shown). There is a small acidic domain in the N-terminus (102-108 residues). At the C-terminus, starting from residue 782, there are several acidic regions (Fig. 4.2). I sequentially deleted different numbers of acidic domain(s) from Avrb6 and tested for the transcription activation activity of the remaining polypeptides. The C-terminal acidic domain alone was capable of activating reporter gene transcription. To my surprise, the N-terminal small acidic sequence also had the ability to turn on reporter gene transcription. When the N-terminal (1-128 amino acid) acidic region was deleted, the rest of Avrb6 had no transcription activation activity despite the presence of the C-terminal acidic domain. Deletion of both the N-terminal and C-terminal acidic domains

also abolished the transcription activation activity (Fig. 4.2). I therefore had two bait choices: the central repeated region plus C-terminal (385-3291 bp), designated 6ΔN, and the central region only (385-2498 bp), designated 6ΔNC. 6ΔNC was chosen as the bait for the library screening. The reason I did not choose to use 6ΔN as the bait will be discussed in the DISCUSSION (4.5).

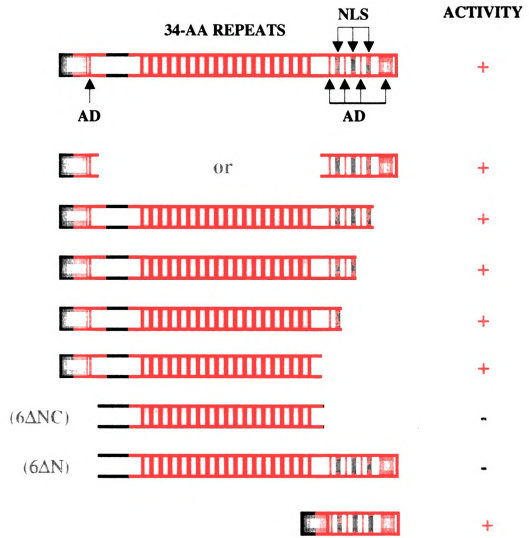

Figure 4.2. Activation of the yeast *Gal1* promoter by different deletion constructs of Avrb6 (AD: acidic domain; NLSs: nuclear localization signals).

Avrb6, when fused with Gal4 BD, activates the transcription of reporter genes (*HIS3* and *lacZ*) in yeast, unless the acidic domain in the N-terminus or those in both N- and C-terminus are deleted.

4.4.3. Yeast Two-Hybrid Screening

Yeast two-hybrid screening was carried out using a standard protocol with CG-1945 as the yeast host strain, and using a "jump-start" protocol with Y190 as the host (CLONTECH K1604-1). To increase the yeast transformation efficiency, I employed a sequential transformation method instead of simultaneous transformation because my bait protein was not toxic to the yeast and sequential transformation was 10 times more efficient (data not shown).

The number of independent clones of the cotton cDNA library was about 2.5 x 10^6. To identify rare clones, at least 10 times the number of independent clones in the library should be screened according to the "jump-start" protocol. After screening 1.8 x 10^8 clones, I obtained over one thousand His^+ clones, among which 372 clones were both His^+ and $LacZ^+$. After eliminating false positive clones (see CLONTECH Manual K1604-1), at least 152 clones were confirmed to specifically bind to 6ΔNC (Fig. 4.3).

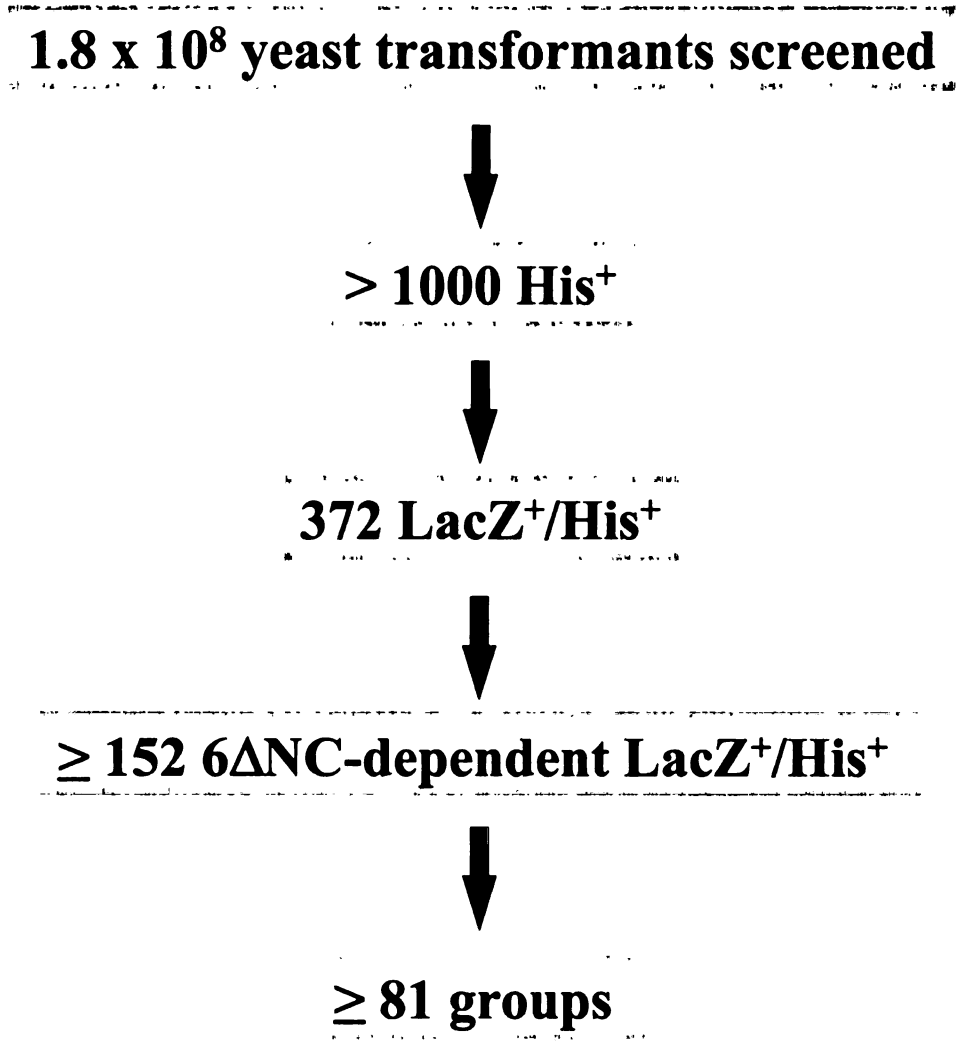

Figure 4.3. Summary of yeast two-hybrid screening of cotton cDNA library.

4.4.4. Sequencing Analysis of cDNA Clones

Putative clones were further sorted to eliminate duplicates. For this purpose, AD plasmids from the $His^+/LacZ^+$ yeast transformants were isolated. The AD library inserts were characterized by restriction digestion with the frequent-cutting restriction enzyme, *Alu*I. Clones sharing the same digestion pattern were sorted into the same group. The 152 putative clones were found to form 81 groups.

One or two clones from each group were sequenced. The sequencing data are summarized in Table 4.1. Most of the sequences turned out to be clearly uninteresting, such as Rubisco gene, rRNA genes, etc. However, proteins encoded by three genes were found to be of interest: Cpk (for Cotton Pto-like Kinase), a serine/threonine kinase highly homologous to Pto (a disease resistance gene product identified from tomato); Ccb (for Cotton Calcineurin B-like protein), a calcineurin B homologous protein; and Ctf (for Cotton Transcription Factor), a putative transcription factor containing a zinc-finger motif. The full-length sequences were obtained for all three genes. Their deduced amino acid sequences and the alignment with the homologous gene products are shown in Figs. 4.4-4.6.

Table 4.1. Cotton cDNA clones encoding proteins specifically binding to Avrb6ΔNC.

Clones (No.)	Encoded Protein	Notes
JS005 (1)	novel	named Ctf, with NLSs and C_2H_2-type Zinc finger motif
JS175 (1)	serine/threonine kinase	named Cpk, homologous to Pto kinase
YBB24 (1)	calcineurin B	named Ccb, homologous to calcineurin B-like protein from *Arabidopsis thaliana*
JS013 (1)	eIF-2 β subunit	homologous to eukaryotic translation initiation factor β subunit
JS050 (1)	K^+ channel protein	glutathione-regulated potassium-efflux system protein
JS160 (1)	putative phosphatase	
JS192 (1)	tenascin precursor	
JS094 (1)	cyclophilin	
JS157 (1)	heat-shock protein	
JS279 (1)	EF-1α	translation elongation factor α
JS200 (1)	K^+ transporter	similar to high affinity potassium transporter
JS297 (1)	metallothionein-like protein	from *Gossypium hirsutum*
Y6-72 (1)		homologous to bcr/C-abl oncogene protein
YBB140 (1)		ATP synthase delta chain, mitochondrial precursor
(19)	Rubisco	ribulose 1,5-bisphosphate carboxylase/oxygenase, rbcL or rbcS gene product
(23)	PS I/II or CAB	photosystem I/II reaction centre subunit, PS II 10 KD polypeptide precursor, or chlorophyll A/B-binding protein
JS084 (1)	FBPASE	fructose-1,6-bisphosphatase, cytosolic
(46)	rRNA	16s, 18s, 23s, 26s
(58)	misc.	short peptide

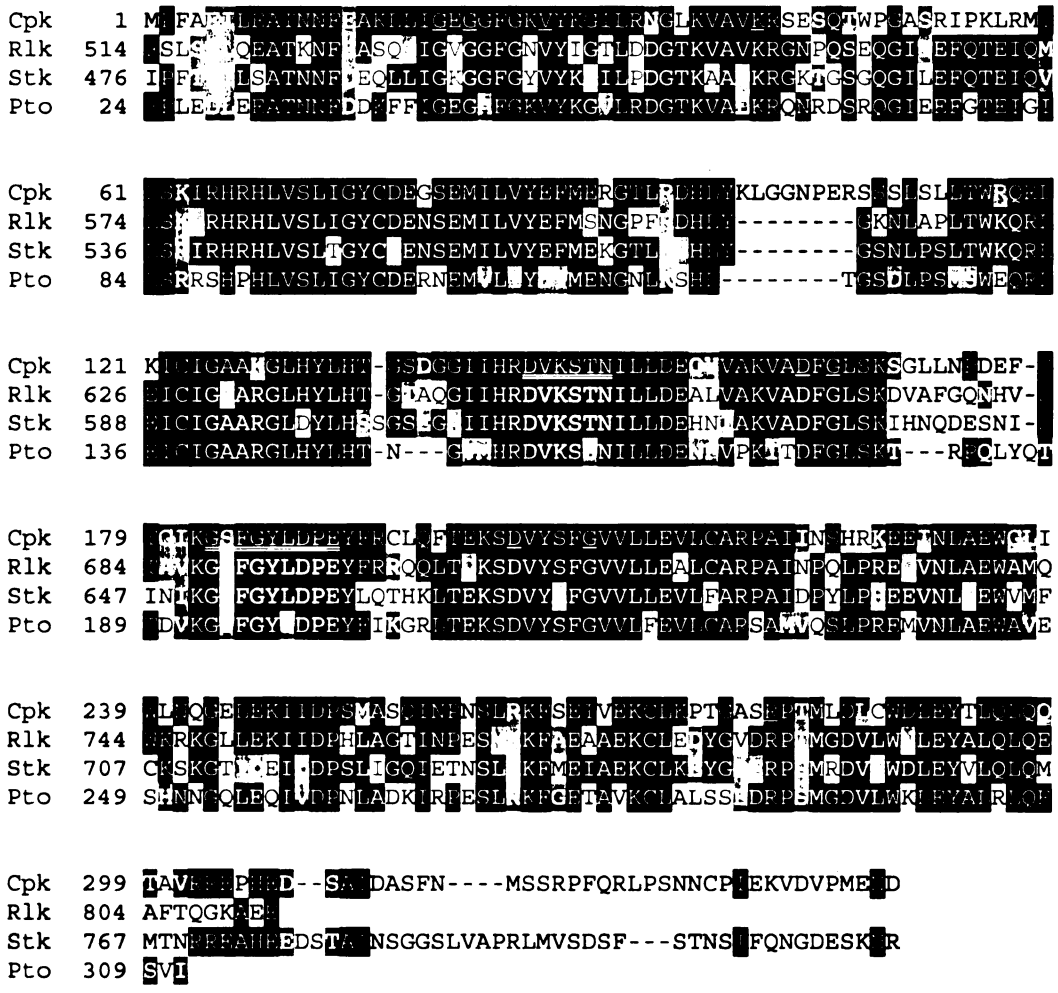

Figure 4.4. Amino acid sequence comparison of Cpk with three other protein kinases from plants.

The kinases include one receptor-like protein kinase from *Arabidopsis thaliana* (Rlk, accession AL050351), one putative serine/threonine protein kinase from *A. thaliana* (Stk, accession AC002391), and serine/threonine kinase Pto from tomato (accession U59316). Amino acids identical with Cpk are highlighted in black, and conservative sequences are highlighted in gray. The invariant residues conserved in all protein kinases are underlined, and residues that indicate serine-threonine specificity are double-underlined (in Cpk sequence).

Figure 4.5. Amino acid sequence comparison of Ccb with three calcineurin B-like proteins from *Arabidopsis thaliana*.

The calcineurin B-like proteins include Cnb-3 (accession AF076253), Cnb-2 (accession AF076252), and Cnb-1 (accession AF076251). Amino acids identical with Ccb are highlighted in black, and conservative sequences are highlighted in gray.

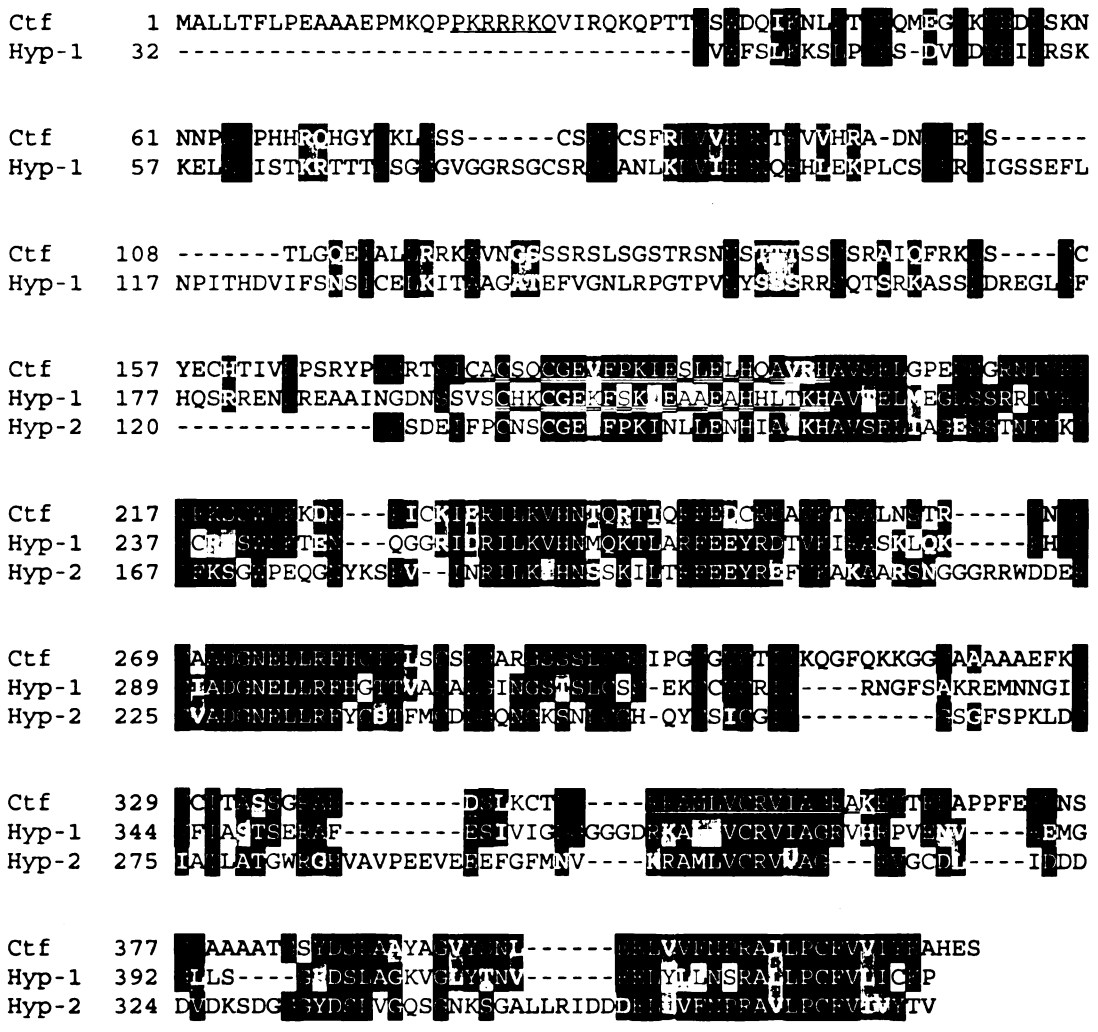

Figure 4.6. Amino acid sequence comparison of Ctf with two hypothetical proteins from *Arabidopsis thaliana*.

The hypothetical proteins are from the *Arabidopsis* genome sequencing project, including Hyp-1 (accession AL030978.1) and Hyp-2 (accession AC005496.1). Amino acids identical with Ctf are highlighted in black, and conservative sequences are highlighted in gray. The putative zinc finger motifs (C_2H_2 type) are double-underlined. The putative nuclear localization signals are underlined.

4.4.5. Functional Study

The cotton RNA preparation for cDNA library construction included RNA from both the resistant line IM216 and susceptible line Acala-44 for the purpose of having the opportunity to fish out both resistance and susceptibility factors. The putative clones therefore could be proteins involved in either resistance or susceptibility. To distinguish these two possibilities, transient assays were conducted by co-expressing the *avrb6* gene with the putative target genes in IM216 and Ac44 cotton leaves. The *Cpk* and *Ccb* genes were cloned into pKYLX71::35S[2]. Transient co-expression of *avrb6* with *cpk* or *ccb* did not result in an HR in cotton Acala-44 (susceptible line) leaves (data not shown). However, this experiment was very preliminary, and needs to be repeated with appropriate modifications. Thus, no conclusions can be drawn at this point.

I also transformed the *avrb6* gene into *Arabidopsis thaliana* (Col). The T2 seeds were collected. Once the homozygous transgenic plants are obtained, the putative target gene will be introduced into this transgenic plant to examine the phenotypic symptoms resulting from the interactions between Avrb6 and the putative cotton target protein.

4.5. DISCUSSION

Two constructs were used to screen the yeast two-hybrid library. I first tried the longer bait (6ΔN) in library screening because I thought that the longer protein might exhibit a closer conformation to the native protein. By using yeast strain CG-1945, a total of 3.35×10^6 clones were screened. Among them, about 700 clones were both His^+ and $LacZ^+$. However, in the subsequent verification analysis (Chien *et al.*, 1991), none of

the AD clones was able to bind to the original bait protein. One possible explanation could be that the "positive" clones were due to spontaneous mutations in the bait (6ΔN). In fact, it was reported by De Feyter *et al.* (1993) that spontaneous race-change mutations in *X. c.* pv. *malvacearum* involved *avr* genes and were readily obtained at unusually high frequencies of 10^{-3} to 10^{-4}. To confirm if this indeed was the cause, I designed the following experiments.

"Positive" clones were grown in SD/-Trp+Leu plates that select only the BD plasmid so that the AD plasmids would be randomly lost. After obtaining the yeast colonies carrying only the bait (BD) plasmid, I tested whether the yeast retained the ability to turn on reporter gene transcription in the absence of AD protein. All of the clones tested retained the transcription activation activity. Therefore, those "positive" clones were due to spontaneous mutations in the bait. The mutation frequency was about 2.3×10^{-4}, which is consistent with De Feyter's report that the central repeat regions mutate at a high rate (De Feyter *et al.*, 1993). The C-terminal region of Avrb6 alone, as we tested before, had transcriptional activation activity. This activity was curiously absent in 6ΔN. One explanation is that the acidic-rich activation domains were buried inside the original fusion protein, but became exposed outside when spontaneous mutations occurred in the repeat regions.

For this reason, the central repeat region of Avrb6 (6ΔNC) was used as bait in the subsequent screening. Following the "jump-start" protocol, I screened over 1.8×10^8 clones, from which about 152 clones were shown to specifically bind to the bait protein. Further verification and sequencing analysis revealed three clones of interest, i.e., Cpk, (a

serine-threonine kinase homologous to Pto), Ccb (a calcineurin B-like protein), and Ctf (a putative transcription factor with zinc-finger motif).

Pto is a disease resistance gene product identified from tomato, which confers resistance to bacterial pathogens carrying *avrPto* (Martin *et al.*, 1993). It has been shown that AvrPto and Pto physically bind to each other in the yeast nucleus (Tang *et al.*, 1996). Like Pto, Cpk is predicted to be cytoplasmic based on its sequencing information. Avrb6 contains functional NLSs and is predicted to act inside the plant nucleus. It is possible, however, that Avrb6 binds to Cpk first in the cytoplasm, and then Avrb6 carries Cpk into the nucleus. Alternatively, Cpk could phosphorylate Avrb6 first to expose its NLSs to aid the nuclear-importing process. The function of Cpk in Avrb6-mediated host response remains to be determined.

Calcineurin is a ubiquitously expressed serine/threonine phosphatase that exists as a heterodimer comprised of a 59-kDa calmodulin-binding catalytic A subunit and a 19-kDa Ca^{2+}-binding regulatory B subunit (Stemmer and Klee, 1994). Ccb shares significant homology with an *Arabidopsis thaliana* calcineurin B protein (about 92% identity and 95% similarity). Activation of calcineurin is mediated by binding of Ca^{2+} and calmodulin to the regulatory and catalytic subunits, respectively. It has been found that calcineurin is involved in many eukaryotic cell signal transduction pathways (Crabtree, 1999), programmed cell death (Wang *et al.*, 1999), and pathogenesis (Alspaugh *et al.*, 1998; Odom *et al.*, 1997). For example, the calcium-dependent phosphatase calcineurin has been found to induce cardiac hypertrophy in response to numerous pathologic stimuli (Molkentin *et al.*, 1998). It will be interesting to determine if Ccb plays any role in cotton/*Xcm* interactions, especially in pathogenesis.

Ctf is a nuclear protein with a C_2H_2 type zinc-finger motif. Since Avrb6 contains functional NLSs, and is believed to be targeted into the plant nucleus, it was predicted to bind to a nuclear protein. Ctf contains typical NLSs that could target the protein to the nucleus. Again, a functional study is required to determine if Ctf plays any role in cotton disease resistance and/or response to pathogens.

Since the bait I used for library screening is the central repeated portion of Avrb6, the question remains whether the truncated sequence retained the native protein conformation so that the interaction between 6ΔNC and its putative receptor(s) are biological significant. It is important to determine whether Cpk, Ccb, and Ctf also bind to the full length Avrb6 protein. One way to test this is to use these proteins as bait and to express *avrb6* in the AD vector. This assumes that AD-Avrb6 by itself does not turn on reporter gene transcription.

Alternatively, biochemical approaches, e.g., affinity capillary electrophoresis (Chu *et al.*, 1994) and affinity chromatography (Olson *et al.*, 1991), could be used to examine interactions between Avrb6 and these three proteins.

One way to test the biological function of the identified genes is to make transgenic cotton plants (cultivars IM216 or Acala-44) that express the genes either in sense or antisense. Cotton plants are transformable (Estruch *et al.*, 1997; John, 1996; John and Crow, 1992; Millar and Dennis, 1996; Rinehart *et al.*, 1996). However, it takes a relatively long time to get transgenic plants. Two alternative methods may be considered for the functional test: one is the transient expression assay as described above; and the second is to sequentially introduce *avrb6* and a cotton target gene into *Arabidopsis thaliana* to see if the interaction between the two gene products resulted in

any phenotypic change in the transgenic plant. The advantage of using *Arabidopsis* is that it is much easier to transform. However, the genetic differences between cotton and *Arabidopsis* may make this approach unrealistic.

It is worth mentioning that the transcriptional activation activity of Avrb6 in yeast nucleus might reflect its role in plants. Recently, Zhu *et al.* (1998) showed that the C-terminal coding region of AvrXa10, another member of the Avr/Pth family, has transcriptional activation activity in both yeast and *Arabidopsis thaliana*, and the transcription activation activity was critical for its avirulence activity. Therefore, Avrb6 and other members of the Avr/Pth family may function as transcription factors during pathogenesis. Avrb6 may bind to other transcription factor(s) to activate target gene transcription (Figure 4.7, model 2), or it may work alone by binding to the promoter region of target genes in the host (Figure 4.7, model 1).

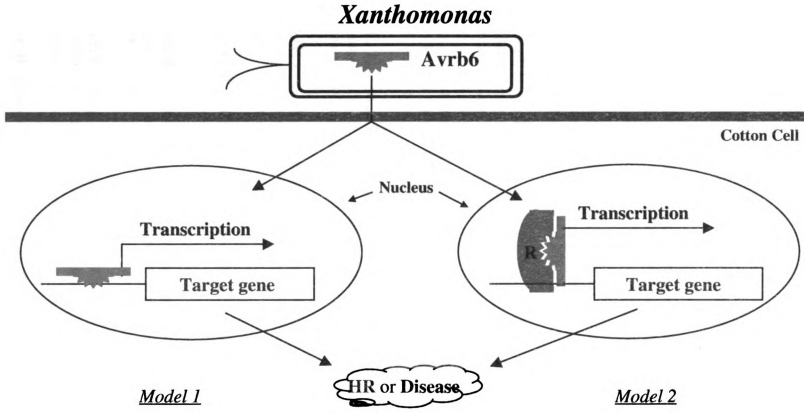

Figure 4.7. Models for the role of Avrb6 in the cotton nucleus. After injection into the plant cell through the type III secretion system, Avrb6 enters the plant nucleus. Avrb6 may directly bind to the promoter region of the target gene to induce the gene transcription (Model 1), or bind to another transcription factor to activate target gene expression (Model 2).

4.6. REFERENCES

Alspaugh, J. A., Perfect, J. R., and Heitman, J. (1998). Signal transduction pathways regulating differentiation and pathogenicity of *Cryptococcus neoformans*. *Fungal Genet Biol* **25**, 1-14.

Bent, A. F., Kunkel, B. N., Dahlbeck, D., Brown, K. L., Schmidt, R., Giraudat, J., Leung, J., and Staskawicz, B. J. (1994). *RPS2* of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. *Science* **265**, 1856-60.

Bonas, U., Conrads-Strauch, J., and Balbo, I. (1993). Resistance in tomato to *Xanthomonas campestris* pv. *vesicatoria* is determined by alleles of the pepper-specific avirulence gene *avrBs3*. *Mol Gen Genet* **238**, 261-9.

Brinkerhoff, L., Verhalen, L., Johnson, W., Essenberg, M., and Richardson, P. (1984). Development of immunity to bacterial blight of cotton and its implications for other diseases. *Plant Disease* **68**, 168-173.

Bundock, P., den Dulk-Ras, A., Beijersbergen, A., and Hooykaas, P. J. (1995). Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. *EMBO J* **14**, 3206-14.

Chien, C. T., Bartel, P. L., Sternglanz, R., and Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc Natl Acad Sci U S A* **88**, 9578-82.

Chu, Y. H., Lees, W. J., Stassinopoulos, A., and Walsh, C. T. (1994). Using affinity capillary electrophoresis to determine binding stoichiometries of protein-ligand interactions. *Biochemistry* **33**, 10616-21.

Crabtree, G. R. (1999). Generic signals and specific outcomes: signaling through Ca^{2+}, calcineurin, and NF-AT. *Cell* **96**, 611-4.

De Feyter, R., and Gabriel, D. (1991). At least six avirulence genes are clustered on a 90-kilobase plasmid in *Xanthomonas campestris* pv. *malvacearum*. *Mol Plant Microbe Interact* **4**, 423-432.

De Feyter, R., Yang, Y., and Gabriel, D. W. (1993). Gene-for-genes interactions between cotton *R* genes and *Xanthomonas campestris* pv. *malvacearum avr* genes. *Mol Plant Microbe Interact* **6**, 225-37.

DeFeyter, R., Kado, C. I., and Gabriel, D. W. (1990). Small, stable shuttle vectors for use in *Xanthomonas*. *Gene* **88**, 65-72.

Estojak, J., Brent, R., and Golemis, E. A. (1995). Correlation of two-hybrid affinity data with in vitro measurements. *Mol Cell Biol* **15**, 5820-9.

Estruch, J. J., Carozzi, N. B., Desai, N., Duck, N. B., Warren, G. W., and Koziel, M. G. (1997). Transgenic plants: an emerging approach to pest control. *Nat Biotechnol* **15**, 137-41.

Flor, H. H. (1971). Current status of the gene-for-gene concept. *Annu Rev Phytopathol* **9**, 275-296.

Fullner, K. J., and Nester, E. W. (1996). Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens*. *J Bacteriol* **178**, 1498-504.

Galyov, E. E., Hakansson, S., Forsberg, A., and Wolf-Watz, H. (1993). A secreted protein kinase of *Yersinia pseudotuberculosis* is an indispensable virulence determinant. *Nature* **361**, 730-2.

Gopalan, S., Bauer, D. W., Alfano, J. R., Loniello, A. O., He, S. Y., and Collmer, A. (1996). Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (Hrp) secretion system in eliciting genotype-specific hypersensitive cell death. *Plant Cell* **8**, 1095-105.

Hopkins, C. M., White, F. F., Choi, S. H., Guo, A., and Leach, J. E. (1992). Identification of a family of avirulence genes from *Xanthomonas oryzae* pv. *oryzae*. *Mol Plant Microbe Interact* **5**, 451-9.

John, M. E. (1996). Structural characterization of genes corresponding to cotton fiber mRNA, E6: reduced E6 protein in transgenic plants by antisense gene. *Plant Mol Biol* **30**, 297-306.

John, M. E., and Crow, L. J. (1992). Gene expression in cotton (*Gossypium hirsutum* L.) fiber: cloning of the mRNAs. *Proc Natl Acad Sci U S A* **89**, 5769-73.

Lamb, C., Lawton, M., Dron, M., and Dixon, R. (1989). Signals and transduction mechanisms for activation of plant defenses against microbial attack. *Cell* **56**, 215-224.

Lamb, C. J. (1994). Plant disease resistance genes in signal perception and transduction. *Cell* **76**, 419-22.

Leister, R. T., Ausubel, F. M., and Katagiri, F. (1996). Molecular recognition of pathogen attack occurs inside of plant cells in plant disease resistance specified by the *Arabidopsis* genes *RPS2* and *RPM1*. *Proc Natl Acad Sci U S A* **93**, 15497-502.

Ma, J., and Ptashne, M. (1988). Converting a eukaryotic transcriptional inhibitor into an activator. *Cell* **55**, 443-6.

Ma, J., and Ptashne, M. (1987). A new class of yeast transcriptional activators. *Cell* **51**, 113-9.

Maiti, I. B., Murphy, J. F., Shaw, J. G., and Hunt, A. G. (1993). Plants that express a potyvirus proteinase gene are resistant to virus infection. *Proc Natl Acad Sci U S A* **90**, 6110-4.

Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganal, M. W., Spivey, R., Wu, T., Earle, E. D., and Tanksley, S. D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* **262**, 1432-6.

Millar, A. A., and Dennis, E. S. (1996). The alcohol dehydrogenase genes of cotton. *Plant Mol Biol* **31**, 897-904.

Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* **93**, 215-28.

Odom, A., Muir, S., Lim, E., Toffaletti, D. L., Perfect, J., and Heitman, J. (1997). Calcineurin is required for virulence of *Cryptococcus neoformans*. *EMBO J* **16**, 2576-89.

Olson, S. T., Bock, P. E., and Sheffer, R. (1991). Quantitative evaluation of solution equilibrium binding interactions by affinity partitioning: application to specific and nonspecific protein- heparin interactions. *Arch Biochem Biophys* **286**, 533-45.

Remacle, J. E., Albrecht, G., Brys, R., Braus, G. H., and Huylebroeck, D. (1997). Three classes of mammalian transcription activation domain transcription in *Schizosaccharomyces pombe*. *EMBO J* **16**, 5722-9.

Rinehart, J. A., Petersen, M. W., and John, M. E. (1996). Tissue-specific and developmental regulation of cotton gene *FbL2A*. Demonstration of promoter activity in transgenic plants. *Plant Physiol* **112**, 1331-41.

Ruden, D. M., Ma, J., Li, Y., Wood, K., and Ptashne, M. (1991). Generating yeast transcriptional activators containing no yeast sequences. *Nature* **350**, 250-2.

Saha, S., Brickman, J. M., Lehming, N., and Ptashne, M. (1993). New eukaryotic transcriptional repressors. *Nature* **363**, 648-52.

Scofield, S. R., Tobias, C. M., Rathjen, J. P., Chang, J. H., Lavelle, D. T., Michelmore, R. W., and Staskawicz, B. J. (1996). Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science* **274**, 2063-5.

Sory, M. P., Boland, A., Lambermont, I., and Cornelis, G. R. (1995). Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of

macrophages, using the *cyaA* gene fusion approach. *Proc Natl Acad Sci U S A* **92**, 11998-2002.

Stemmer, P. M., and Klee, C. B. (1994). Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B. *Biochemistry* **33**, 6859-66.

Tang, X., Frederick, R. D., Zhou, J., Halterman, D. A., Jia, Y., and Martin, G. B. (1996). Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* **274**, 2060-3.

Van den Ackerveken, G., Marois, E., and Bonas, U. (1996). Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host plant cell. *Cell* **87**, 1307-16.

Wan, C. Y., and Wilkins, T. A. (1994). A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal Biochem* **223**, 7-12.

Wang, H. G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T. F., and Reed, J. C. (1999). Ca^{2+}-induced apoptosis through calcineurin dephosphorylation of BAD. *Science* **284**, 339-43.

Yang, Y., De Feyter, R., and Gabriel, D. (1994). Host-specific symptoms and increased release of *Xanthomonas citri* and *X. campestris* pv. *malvacearum* from leaves are determined by the 102-bp tandem repeats of *pthA* and *avrb6*, respectively. *Mol Plant Microbe Interact* **7**, 345-355.

Yang, Y., and Gabriel, D. (1995). *Xanthomonas* avirulence/pathogenicity gene family encodes functional plant nuclear targeting signals. *Mol Plant Microbe Interact* **8**, 627-631.

Yang, Y., and Gabriel, D. W. (1995). Intragenic recombination of a single plant pathogen gene provides a mechanism for the evolution of new host specificities. *J Bacteriol* **177**, 4963-8.

Yang, Y., Yuan, Q., and Gabriel, D. W. (1996). Water-soaking function(s) of *Xcm*H1005 are redundantly encoded by members of the *Xanthomonas avr/pth* Gene Family. *MPMI* **9**, 105-113.

Zhu, W., Yang, B., Chittoor, J. M., Johnson, L. B., and White, F. F. (1998). AvrXa10 contains an acidic transcriptional activation domain in the functionally conserved C terminus. *Mol Plant Microbe Interact* **11**, 824-32.

Chapter 5

CONCLUSION AND FUTURE PERSPECTIVES

The research for my doctoral dissertation has mainly been focused on studying the mechanism of type III protein secretion in *Pseudomonas syringae* pv. *tomato* DC3000. Type III secretion is controlled by *hrp* genes in DC3000 and other phytopathogenic bacteria. The bacterial *hrp* system is believed to be responsible for secretion and translocation of virulence and avirulence factors into the plant cell. The actual mechanisms of this interkingdom transfer was previously poorly understood.

By constructing a non-polar *hrpA* mutant of DC3000, I demonstrated that the *hrpA* gene encodes an essential protein for bacterial pathogenesis and HR elicitation. In collaboration with Martin Romantschuk's group, I have characterized a novel pilus-like structure (named the Hrp pilus), assembled by DC3000. The Hrp pili are about 6-8 nm in diameter, and can be easily distinguished from polar flagella, which are about 15-18 nm in diameter. The pilus may function as a bridge that connects the bacterium to the plant cell. The HrpA protein was found to be associated with this structure. By mutant analysis and transmission electron microscopic examination, I demonstrated that HrpA is a structural protein of the Hrp pilus.

A supramolecular structure controlled by the type III system has recently been isolated from *Salmonella typhimurium* (Kubori *et al.*, 1998). This structure, termed the needle complex, spans both the inner and outer membranes of the bacterial envelope. The basal structure resembles the flagellar basal body (Aizawa, 1996) because it contains two upper (or outer) and two lower (or inner) rings. The needle structure itself is a stiff,

straight tube, 80 nm long and 13 nm wide. It was suggested that *S. typhimurium* uses this needle structure to send its effector proteins into host cells. The Hrp pilus is much longer than the needle structure. This may reflect bacterial adaptation to the different surface barriers of the host, because, unlike animal bacteria, phytobacteria have to overcome a thick cell wall (~100 nm). Consistent with the view that the Hrp pilus is involved in protein transfer, another pilus-like structure has also been identified from *Agrobacterium tumefaciens*. The pili varied in length, but pili longer than the length of bacterium were observed. *Agrobacterium* pili, 3.8 nm in diameter, are required for transfer of DNA to plant cells in a process similar to that of bacterial conjugation (Fullner *et al.*, 1996). In contrast, no pilus-like structures are associated with type III secretion in animal pathogenic bacteria.

Since we first reported the finding of the Hrp pilus, similar cell surface appendages have been found in all major Gram-negative bacteria carrying *hrp* genes, including *Erwinia amylovora* (W. Hu and S.Y. He, unpublished results), *Xanthomonas campestris* (U. Bonas, personal communication), and *Ralstonia solanacearum* (C. Boucher, personal communication). Thus, formation of the Hrp pilus appears to be a common function of *hrp* genes.

To define the function of the Hrp pilus, I first examined the effect of the *hrpA* mutation on protein secretion through the Hrp system. Unexpectedly, the *hrpA* mutation was found not only to block type III protein secretion, but also to suppress the expression of other *hrc/hrp* and *avr* genes. Northern analysis showed that *hrpA*-mediated gene activation of *hrpS* and *hrpR* was at the RNA level. Although ectopic expression of *hrpR/S* restored *hrc/hrp* and *avr* gene expression in the *hrpA* mutant, Hrp and Avr protein

secretion was still blocked. However, ectopic expression of *hrpR/S* genes was unable to restore the *hrpA* mutant's ability to cause disease in susceptible *Arabidopsis thaliana* leaves or to elicit an HR in resistant tobacco leaves. These data suggest a dual function of the Hrp pilus in protein secretion and coordinate regulation of the Hrp system. My experimental results are therefore in favor of the conduit model, in which the Hrp pilus functions as an integral part of the Hrp secretion structure.

At least eight type III secretion proteins share sequence similarities with the components of flagellar assembly machinery (Bogdanove *et al.*, 1996). In fact, these eight Hrp proteins are more closely related to components of the flagella machinery than to their counterparts in animal pathogenic bacteria. Thus, it is possible that type III secretion systems first evolved in plant pathogenic bacteria from the flagellar export apparatus in order to secrete proteins other than flagellin (Galan and Collmer, 1999). The *Salmonella typhimurium* flagellar assembly senses structural integrity by export of a negative regulator, FlgM (Hughes *et al.*, 1993). In cells with a functional hook-basal body complex, the flagellin genes are transcribed normally and the negative regulator FlgM is expelled into the growth medium. In strains with a defective hook-basal body structure, the anti-σ^{28} FlgM protein accumulates inside the cell and inhibits flagellin transcription (Fig. 5.1A).

A similar mechanism has been found in *Yersinia pseudotuberculosis* (Pettersson *et al.*, 1996). Microbe-host contact triggers export of LcrQ, a negative regulator of Yop expression, via the Yop-type III secretion system. The intracellular concentration of LcrQ is thereby lowered upon host-bacterial contact, resulting in increased expression of

Yops (Fig. 5.1B). However, the actual molecule or structure that senses host-bacterial contact has not been identified.

Recently, a putative negative regulator, called HrpV, has been identified from *Pseudomonas syringae* pv. *syringae* (Preston *et al.*, 1998). *hrpV* is the last gene in the *hrcC* operon (Fig. 1.1). A *hrpV* mutant produced higher levels of proteins encoded by all three examined *hrp* gene operons—HrcJ (*hrpZ* operon), HrcC (*hrcC* operon), and $HrcQ_B$ (*hrpU* operon)—and constitutive expression of *hrpV* in *trans* abolished the production of each of these proteins. It was further demonstrated that HrpV acts upstream of HrpR and HrpS in the regulation cascade (Preston *et al.*, 1998).

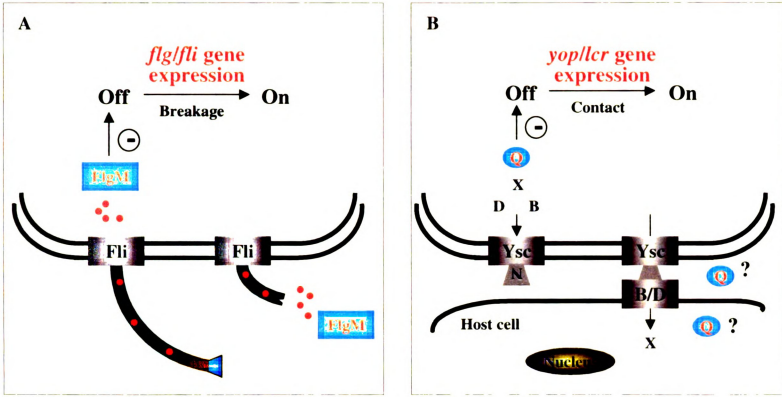

Figure 5.1. Regulation of flagellar assembly in *S. typhimurium* (A) and type III protein secretion in *Y. pseudotuberculosis* (B) by export of negative regulators.

In my own experiments, the *hrcChrpTV* triple mutant of DC3000 overproduced HrpW, HrcJ, and AvrPto. pHRPCT, which contains the *hrcChrpT* genes driven by the P_{lac} promoter, complemented the *hrcChrpTV* triple mutant in HrcC production and pathogenicity *in planta*. However, the protein production was still significantly higher in *hrcChrpTV*/pHRPCT than in DC3000. These data confirmed that the HrpV protein in DC3000 also negatively regulates *hrc/hrp* and *avr* gene expression. Figure 5.2 provides a model of how the Hrp pilus might control gene expression in *Pst* DC3000. We believe that there are two phases of induction for the Hrp system. The HrpS and HrpR positive regulators first detect a signal (nutritional signal?) in the plant apoplast, leading to the first-phase, low-level activation of *hrc/hrp* and *avr* genes, including the *hrpA* gene. The low-level expression of *hrc/hrp* genes initiates the assembly of Hrp pili, which are required for the second-phase, much higher (contact-dependent?) induction of *hrc/hrp* and *avr* genes. The extracellular location of the Hrp pilus appears to be especially suitable for sensing host-bacterial contact. For second phase induction, there are three possible mechanisms: 1) HrpV proteins are normally secreted through the Hrp pilus, either into the plant apoplast or into the plant cell; in the *hrpA* mutant, the secretion of this negative regulator is blocked, thereby repressing *hrp* gene expression (Fig. 5.2. Model 1). 2) HrpA regulates other genes by directly inducing *hrpRS* transcription. The negative regulatory function of HrpV is independent of the HrpA pathway (Fig. 5.2. Model 2). 3) HrpA protein suppresses the negative regulation of HrpV, possibly by physically binding to and inactivating the repression function of HrpV (Fig. 5.2. Model 3).

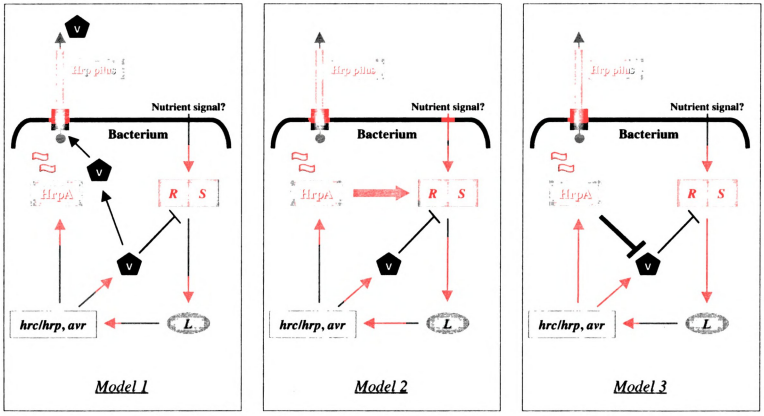

Figure 5.2. Models depicting the regulatory function of HrpA in type III protein secretion in *Pseudomonas syringae* pv. *tomato* DC3000. Red arrows indicate positive regulation, whereas black "T" symbols indicate negative regulation. *R*, *hrpR*; *S*, *hrpS*; *L*, *hrpL*; and V, HrpV.

The first model is consistent with what is known about flagellar assembly in *S. typhimurium* and the negative regulation of type III secretion in *Y. pseudotuberculosis*. To study the possible secretion of HrpV, HrpV has been fused to the 6xHis epitope tag at the C-terminus. I have been unsuccessful so far in identifying the secretion of HrpV using the His-tagged antibody. It is possible that the amount of HrpV is too low to be detected. Alternatively, the 6xHis epitope might have interfered with the function or secretion of HrpV. An antibody raised against the native HrpV may have a better chance of localizing this protein. Another alternative would be to create a *hrpA/hrpV* double mutant. If *hrpA* regulates gene expression exclusively through *hrpV*, I would expect normal gene expression in the *hrpA hrpV* double mutant. An effort has been made to create this double mutant.

According to the negative regulation model, gene expression in any secretion mutant should be suppressed by the retention of HrpV in the cytoplasm. I found, however, that *hrc/hrp* and *avr* gene expression is normal, instead of being suppressed, in the *hrcC* mutant. These results suggest that HrpA has a positive regulation function, by either directly binding to HrpV to prevent it from suppressing *hrpR/S* gene transcription, or indirectly competing with HrpV to bind to the promoter of the *hrpR/S* operon. Based on these results I decided to determine whether the HrpA protein physically binds to HrpV in the yeast two-hybrid system. I have cloned the *hrpV* ORF into both the pAS-2 and pACT-2 vectors to create fusion proteins BD-HrpV and AD-HrpV. The HrpA protein can be fused with the Gal4 activation or DNA binding domain. Using these constructs, a direct interaction between HrpV and HrpA will be examined in yeast.

I am personally in favor of Model 1 and 3. The real mechanism is likely to be the combination of these two mechanisms. Under *hrp*-inducing conditions, HrpV negative regulator is secreted through the Hrp pilus in *Pst* DC3000, and most of HrpA proteins are also secreted to outside the bacterium to form the Pilus. In the *hrcC* mutant, both HrpA and HrpV proteins are accumulated inside bacterium, however, HrpA protein physically binds to HrpV and thus inactivates its repression function. The *hrp* gene expression is therefore normal in the secretion mutant. Only in the absence of functional *hrpA* gene, HrpV is able to suppress *hrp* gene expression. The ratio of HrpA and HrpV seems to be critical because, at least in *Pss* 61, over-expression of *hrpV* resulted in down-regulation of *hrp* gene expression (Preston *et al.*, 1998). This hypothesis remains to be determined.

If the HrpV protein is not directly involved in the HrpA regulatory pathway, it is still possible that there is some other negative regulator. Several of the 28 *hrp/hrc* genes have not been assigned a function. These genes, such as *hrpK* and *hrpJ*, may be candidates for mutant analysis.

To study a possible interaction of type III effector proteins with plant proteins, I carried out the yeast two-hybrid screening experiment. This work is described in Chapter 4. After screening over 1.8×10^8 clones from a cotton AD library, I obtained over a hundred clones that specifically bind to the bait, 6ΔNC, which is the central repeat region of the Avrb6 protein of *Xanthomonas campestris* pv. *malvacearum*. Sequence analysis revealed three clones: Cpk, (a serine-threonine kinase homologous to Pto), Ccb (a calcineurin B-like protein), and Ctf (a putative transcription factor with a zinc-finger motif). A functional assay is still required to determine whether any of these proteins plays a role in *avrb6*-mediated *Xcm*-cotton interactions.

Cotton is transformable, but it is a time-consuming and labor-intensive process (Estruch *et al.*, 1997; John, 1996; John and Crow, 1992; Millar and Dennis, 1996; Rinehart *et al.*, 1996). I have explored the possibility of transforming the *avrb6* and *avrB101* genes into *Arabidopsis thaliana*. The function of the putative target genes could be tested in the homozygous *avrb6* (or *avrB101*) transgenic *Arabidopsis* plants.

Attempts have also been made to transiently express both the *avrb6* and the *Cpk* or *Ccb* genes in cotton leaves using the *Agrobacterium* gene delivery system. Neither Cpk nor Ccb elicited HR in susceptible cotton plant Acala-44 when co-expressed with *avrb6*. However, transient expression is affected by many environmental factors. Future work is needed to optimize the transient expression method.

In addition, it is necessary to verify further binding between Avrb6 and the proteins encoded by the cotton clones. For example, all the putative clones were demonstrated to bind specifically to the central repeat region of Avrb6. It is important to show that such an interaction also occurs between the target proteins and the full-length Avrb6. Additionally, northern analysis should be conducted to examine whether genes of the interacting clones are inducible during *Xcm* infection of cotton plant.

In an effort to identify the interacting protein of Avrb6, I discovered that Avrb6 had transcription activation activity in the yeast nucleus, perhaps due to the acidic domains at both the C- and N-terminal regions. This result suggests that Avrb6 might be a transcription factor in cotton plant cells. The Avrb6 protein may bind to the target gene promoter or dimerize with other transcription factors within the cotton nucleus to induce gene expression. It is attempting to identify those genes whose expression could be

induced by Avrb6. Many powerful techniques could be used to find out these genes, such as differential display and DNA Microarray.

REFERENCES

Aizawa, S. I. (1996). Flagellar assembly in *Salmonella typhimurium*. *Mol Microbiol* **19**, 1-5.

Bogdanove, A. J., Beer, S. V., Bonas, U., Boucher, C. A., Collmer, A., Coplin, D. L., Cornelis, G. R., Huang, H. C., Hutcheson, S. W., Panopoulos, N. J., and Van Gijsegem, F. (1996). Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. *Mol Microbiol* **20**, 681-3.

Estruch, J. J., Carozzi, N. B., Desai, N., Duck, N. B., Warren, G. W., and Koziel, M. G. (1997). Transgenic plants: an emerging approach to pest control. *Nat Biotechnol* **15**, 137-41.

Fullner, K. J., Lara, J. C., and Nester, E. W. (1996). Pilus assembly by *Agrobacterium* T-DNA transfer genes. *Science* **273**, 1107-9.

Galan, J. E., and Collmer, A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**, 1322-8.

Hughes, K. T., Gillen, K. L., Semon, M. J., and Karlinsey, J. E. (1993). Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. *Science* **262**, 1277-80.

John, M. E. (1996). Structural characterization of genes corresponding to cotton fiber mRNA, E6: reduced E6 protein in transgenic plants by antisense gene. *Plant Mol Biol* **30**, 297-306.

John, M. E., and Crow, L. J. (1992). Gene expression in cotton (*Gossypium hirsutum* L.) fiber: cloning of the mRNAs. *Proc Natl Acad Sci U S A* **89**, 5769-73.

Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galan, J. E., and Aizawa, S. I. (1998). Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* **280**, 602-5.

Millar, A. A., and Dennis, E. S. (1996). The alcohol dehydrogenase genes of cotton. *Plant Mol Biol* **31**, 897-904.

Pettersson, J., Nordfelth, R., Dubinina, E., Bergman, T., Gustafsson, M., Magnusson, K. E., and Wolf-Watz, H. (1996). Modulation of virulence factor expression by pathogen target cell contact. *Science* **273**, 1231-3.

Preston, G., Deng, W. L., Huang, H. C., and Collmer, A. (1998). Negative regulation of *hrp* genes in *Pseudomonas syringae* by HrpV. *J Bacteriol* **180**, 4532-7.

Rinehart, J. A., Petersen, M. W., and John, M. E. (1996). Tissue-specific and developmental regulation of cotton gene FbL2A. Demonstration of promoter activity in transgenic plants. *Plant Physiol* **112**, 1331-41.