P. M. HAUCK PH.D. 2004

This is to certify that the
dissertation entitled

UNDERSTANDING THE MOLECULAR BASIS OF DISEASE SUSCEPTIBILITY OF *ARABIDOPSIS* TO *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000.

presented by

Paula Margaret Hauck

has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Genetics

Major Professor's Signature

12-16-04

Date

DATE DUE	DATE DUE	DATE DUE

UNDERSTANDING THE MOLECULAR BASIS OF DISEASE SUSCEPTIBILITY OF *ARABIDOPSIS* TO *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000.

By

Paula Margaret Hauck

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics Program

2004

Abstract

UNDERSTANDING THE MOLECULAR BASIS OF DISEASE SUSCEPTIBILITY OF *ARABIDOPSIS* TO *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000.

By

Paula Margaret Hauck

Plant diseases are widespread and cause devastating crop losses each year. However, little is known about the molecular mechanisms of disease susceptibility to virulent pathogens. Knowledge of disease progression could be vital to designing improved methods for disease control. *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000), an important model for studying plant-pathogen interactions, causes bacterial speck on tomato and *Arabidopsis thaliana*. *Pst* DC3000 enters plants through natural openings such as stomata and wounds. It is an extracellular pathogen that must suppress or evade plant defenses and obtain nutrients from the host to be successful. Disease progression is typified by bacterial multiplication and development of water soaking followed by chlorosis and necrosis in the infected tissues. *Pst* DC3000 relies on the type III secretion system to deliver protein effectors across the plant cell wall into the host cell. These effectors are essential for pathogenesis as demonstrated by the inability of *hrp* mutants to multiply or cause disease on otherwise susceptible hosts.

To gain insight into the function of *Pst* DC3000 effectors in the host cell, I created transgenic *Arabidopsis* plants that express *avrPto*. I showed that transgenic

expression of *avrPto* repressed a set of *Arabidopsis* genes that were also repressed during *Pst* DC3000 infection. In addition, *avrPto* plants permitted enhanced multiplication of a *Pst* DC3000 *hrp* mutant, an avirulent derivative of *Pst* DC3000, and *P. fluorescens* (a non-phytopathogenic bacterium). The increased growth of these bacteria in *avrPto* plants is not correlated with water-extractible nutrients in the apoplastic space, but is associated with impaired host extracellular defense and secretion. *avrPto* plants were unable to deposit defense-related callose in the cell wall. Furthermore, several host proteins that are present in the apoplast of wild-type plants inoculated with an avirulent pathogen were absent in *avrPto* plants. Based on these and other results, we postulate that one virulence function of AvrPto in *Arabidopsis* is to promote pathogenesis by interfering with host trafficking to the extracellular space.

In addition to determining how AvrPto operates in the plant cell, I investigated host components that are involved in disease symptom development. An *Arabidopsis* mutant screen uncovered a mutant that did not develop disease-associated chlorosis in response to *Pst* DC3000 infection. The growth and development of this mutant, *noc1* (no-chlorosis1), is not different from wild-type, but the *noc1* plants lose chlorophyll at a slower rate than wild-type plants during disease development. Both *noc1* and wild-type plants had similar increased transcript levels of *AtClh11* (a gene in the chlorophyll degradation pathway) upon *Pst* DC3000 infection. The *noc1* gene is located on the long arm of chromosome 4.

The information gained from this research may lead to an increased understanding of the molecular processes that occur during *Pst* DC3000 infection of susceptible *Arabidopsis*.

Acknowledgements

I would first like to thank Dr. Sheng Yang He for accepting me in his lab, his support, his guidance, and his enlightening discussions throughout my graduate career. I also would like to thank him for letting me do a six month internship on a project that was unrelated to his research. I would like to thank Dr. Rebecca Grumet, Dr. Gregg Howe, and Dr. Jonathan Walton for agreeing to be on my committee, spending their time with me and giving me their input over the years.

I would like to thank all of the past and current members of the He lab: Jing Yuan, Wensheng Wei, Anne E. Plovanich-Jones, Julie Zwiesler-Vollick, Wenqi Hu, Mingbo Lu, Yong Bum Kwack, Qiaoling Jin, Roger Thilmony, Kinya Nomura, Ola Kolade, Sruti DebRoy, Elena Bray Speth, William Underwood, Yong Hoon Lee, Lori Imboden, Maeli Melotto, Young Nam Lee, Francisco Uribe, Eliana Gonzales-Vigil. In addition, I am grateful for all of our undergrads, but especially Nate Pumplin, Katie McLellen, Marisa Trapp, Beth Rzendzian, Ruth Nantais, and Vusumuzi Ndlovu for help in the *noc1* project.

I would particularly like to thank Roger Thilmony for collaborating with me I on the work described in Chapter 2. I would like to thank Elena Bray Speth, Dr. Sheng Yang He and others for critical reading of this dissertation. I would especially like to thank Julie Zwiesler-Vollick, Anne E. Plovanich-Jones, Sruti DebRoy, and Elena Bray Speth for their support, encouragement, advice, and friendship.

I would like to express my gratitude to all of the support staff at the PRL and the genetics program. They have really helped me survive graduate school and were invaluable in answering my questions. I would like to thank Jim Klug for his excellent care of the growth chambers and helping me keep my plants healthy. I would also like to thank all of my teachers who have provided me with the knowledge base critical for understanding science. I would like thank all of the PRL for making my stay at Michigan State a memorable and rewarding experience. It truly is an environment that is conducive for research.

Lastly, I appreciate all of the love, encouragement, support, reassurances and advice I have received from my Family. They have been there for me through it all. I would also like to thank my husband, Nat, for being patient with me and for tolerating my tendency for procrastination. I would like to thank him for understanding that when I say 10 minutes I really mean 40 minutes. I would also like to thank him for critical reading of this dissertation.

TABLE OF CONTENTS

List of Tables

List of Figures

Images in this dissertation are presented in color.

Chapter 1: Literature review

Introduction

Plants, as photosynthetic organisms, are essential for most other forms of life because they are able to harness energy from the sun and convert it into a form that can be used by other organisms. Since plants are so important, it is vital for us to understand how they work and what factors influence their yield. Being sessile, they cannot avoid unfavorable conditions, but must cope with environmental stresses. Plants are in constant contact with viruses, fungi, nematodes, insects, and bacteria. Some of these organisms have evolved the ability to cause disease on plants by evading or overcoming the plants' resistance mechanisms. Diseases caused by these pathogens result in significant crop losses each year (1).

The interaction between a plant and a pathogen is defined based on its outcome. In a compatible interaction, in which the host plant is susceptible and the pathogen is virulent, disease will occur. An incompatible interaction, involving a resistant host and an avirulent pathogen, leads to resistance.

Despite the rapidly accumulating knowledge of the components and mechanisms of resistance, the molecular basis of plant susceptibility to pathogen infection remains largely elusive. In order to study disease development, it is advantageous to have a model plant-pathogen system, such as *Arabidopsis thaliana* and the bacterial pathogen *Pst* DC3000. Both organisms have sequenced genomes and have many other attributes (discussed below) that make them good models for studying plant-pathogen interactions.

The pathogen: *Pseudomonas syringae* pv. *tomato* strain DC3000

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000) is an extracellular bacterial pathogen that causes bacterial speck disease in *A. thaliana* and tomato plants. Bacterial speck occurs throughout the world where conditions are cool and wet. The bacteria are spread by aerosols or splashed by rain and enter leaves through existing openings such as stomata or wounds. *Pst* DC3000 is an extracellular pathogen because it remains outside of the plant cell and multiplies in the leaf apoplastic space. In tomato, disease symptoms include small black or brown necrotic lesions (specks) that become surrounded by chlorotic halos caused by the bacterial toxin coronatine. Lesions also form on both unripe and ripe tomato fruit, causing decreased marketability of the fruit (2). The symptoms on *Arabidopsis* are similar to those on tomato; water soaking develops within two days and necrosis surronded by chlorosis occurs by three days after infection.

Pst DC3000 is a successful pathogen because of a variety of virulence mechanisms. The previously mentioned phytotoxin, coronatine, has been shown to be important for virulence because bacterial mutants that are unable to produce coronatine are less virulent on wild-type *Arabidopsis* (3-5). The most important virulence mechanism, however, is the type III protein secretion system. The type III protein secretion system is encoded by genes found in the 25 kb *hrp* (hypersensitive response and pathogenicity) gene cluster. The proteins encoded by these genes are required for both the HR and pathogenicity, since *hrp* mutant bacteria (e.g., *hrcC* and *hrpS*), which are defective in type III secretion, do not multiply or cause disease symptoms in host plants. Other genes in the *hrp* cluster encode proteins responsible for regulation of the type III secretion system, several effectors, and genes of unknown function (6).

The type III secretion system is only expressed *in planta* or in minimal media (which is thought to mimic *in planta* conditions) and its transcription is tightly regulated by *hrpR* and *hrpS*. These proteins are members of a two-component regulatory system that is required for the transcription of the *hrpL* gene. *hrpL* encodes an alternative sigma factor which is thought to bind a particular *cis*-element in the promoters of *hrpRS* regulated genes known as the *hrp* box. Most effector genes, as well as the *hrp* genes themselves, contain a *hrp* box in their promoter region (7).

The type III-secreted effectors are thought to be translocated directly to the host cell cytoplasm via the Hrp pilus, and a number of experiments substantiate this idea (6, 8-18). About 40 effectors have been identified in *Pst* DC3000, and a list of all the known effectors (Avr and virulence proteins) as well as a guide to commonly used terminology is available at the *Pseudomonas* database (http://www.pseudomonas-syringae.org/pst_home.html). Although effectors are known to be crucial for virulence, their mode of action in the plant cell is only beginning to be elucidated. Mutating or deleting individual effectors has little or no effect on virulence. This is likely due to either functional redundancy or the possibility that each effector has only a small quantitative effect on virulence. Currently, four approaches are most frequently used to study the functions of type III effectors. First, a search for sequence homology with known proteins can reveal possible effector functions that can be tested. Unfortunately, most effectors have no homology to other genes in the databases. Second, microarray analysis can be used to study effector function by analyzing transcriptional changes in the host induced by the wild-type pathogen or various effectors. Third, transgenic plants that express a single effector can be created and its affect on the host studied. Fourth, cross-kingdom yeast-two-hybrid screens to identify host proteins that physically interact with specific effectors can be conducted.

AvrPto

AvrPto is a well studied effector, mainly for its role in avirulence on tomato. Different races of *Pst* differ in their virulence on tomato plants. Resistance to Race 0 strains is controlled by a single resistance locus, called *Pto* (19). Ronald *et al.* (20) found that *avrPto* was responsible for limiting disease on resistant tomato plants carrying *Pto*. This gene is present in all the 14 Race 0 strains tested and in none of the 12 Race 1 strains. *P. syringae* pathogens of radish, bean, pea, and oat all have sequences homologous to *avrPto* (20). AvrPto has virulence activity in *Pst* race T1 when a functional *Pto* pathway is absent in the host (21, 22).

AvrPto was further analyzed by Salmeron and Staskawicz (23), who found that the protein is encoded by a single ORF whose predicted translation product is a 164 amino acid protein of 18.3 kD. Although the protein is mostly hydrophilic, its first 6 amino acids are hydrophobic. Salmeron and Staskawicz (23) also found that *avrPto* has a conserved *hrp* box in its promoter and its expression is coordinately regulated with the *hrp* genes. Induction of *avrPto* occurs within 1 hour after infiltration into either resistant or susceptible tomato plants (23). As with many gene-for-gene interactions, *Pto*-mediated resistance to *avrPto*-expressing *Pst* strains is associated with a localized HR.

Pto encodes a hydrophilic 321 amino acid protein that was identified as a serine-threonine protein kinase (24). There has been a considerable amount of research conducted on Pto (16, 25-46); for a review, see Pedley and Martin (2). By Southern analysis, *Pto*-like sequences are present in potato, tobacco, *Arabidopsis*, bean, soybean, pea, rice, maize, barley, wheat, and sugarcane (24). However, to date, none of these Pto-like proteins have been shown to have recognition specificity for AvrPto (2).

AvrPto acts within the plant cell, as demonstrated by *Agrobacterium*-mediated transient expression of *avrPto* in tobacco leaves (15, 47). The HR was observed in *Pto*-expressing leaves but not in leaves lacking Pto. This confirms that AvrPto acts alone, without additional *Pseudomonas* proteins, inside the plant cell to elicit HR in a Pto-specific fashion. In addition, AvrPto interacts directly with Pto in the yeast-two-hybrid system (15, 47, 48). Alterations of AvrPto or Pto that disrupt the interaction in yeast also abolish disease resistance in plants (15). Currently, the interaction of Pto and AvrPto has yet to be demonstrated *in vivo* (2). Several studies investigated the effect of point mutations (22, 49, 50) and deletions (15, 47) in *avrPto* on its ability to interact with Pto. Although the majority of mutations did not affect the interaction, alterations to several residues disrupted binding to Pto. In all of the cases where the mutation in AvrPto disrupted binding to Pto, HR was abolished as well. Only one study (22) evaluated the effect of *avrPto* mutations on virulence. Shan *et al.* (22) found three mutations that affected binding with Pto. However, these mutations did not decrease the virulence activity of AvrPto. In fact, there are no known mutations that code for stably expressed AvrPto proteins in *Pseudomonas* that have been shown to affect virulence.

AvrPto contains a putative myristylation site at the N-terminus (50, 51). AvrPto is associated exclusively with the plant plasma membrane (50). A G2A mutation of the myristylation motif abolished this localization (50). Although this mutation did not affect type III protein secretion in bacteria, the interaction with Pto in the yeast-two-hybrid system, or the stability in plant cells, the mutant protein failed to exhibit avirulence activity in tomato and tobacco. These findings suggest that association with the host plasma membrane is critical for recognition by Pto (50).

Bogdanove and Martin (48) screened a tomato cDNA library for proteins that interact with Pto in an AvrPto-dependent fashion. They found a catalase, two serine/threonine kinases, a large hydrophilic protein and Pti2 (a proteasome alpha subunit). They also looked for proteins that interacted with AvrPto and found a stress-related protein, an *N*-myristyltransferase and 2 small Ras-related GTP binding proteins. At the time of this publication, the authors had not established whether any of these proteins were actually involved in resistance or virulence. The functional significance of these interactions remains to be determined.

The host: *Arabidopsis thaliana*

A. thaliana has proven to be a valuable model for plant research. It has a short life cycle, many plants can be propagated in a limited space because of its small size, and it has a small, sequenced genome (115Mb, >25,500 genes). *A. thaliana* is easily transformable via *Agrobacterium*-mediated transformation. Many resources are available to the scientific community, including: the *Arabidopsis* Information Resource (*http://www.Arabidopsis.org/*), the TIGR *Arabidopsis thaliana* Database *http://www.tigr.org/tdb/e2k1/ath1/*, the Monsanto *Arabidopsis* polymorphism and Ler sequence collection (*http://www.Arabidopsis.org/Cereon/*), insertion knock-out collections (*http://signal.salk.edu/cgi-bin/tdnaexpress* and *http://nasc.nott.ac.uk/*), and the Massively Parallel Signature Sequencing site (*http://mpss.udel.edu/at/java.html*), which contains expression data for genes in different tissues under different experimental conditions. *Arabidopsis* and other plants exhibit similar defense responses; therefore, components of the plant-pathogen interaction that are identified in *Arabidopsis* will likely have similar counterparts in crop species (52).

Disease resistance mechanisms

In addition to pre-existing defenses, such as the plant cell wall, cutin, wax, and other structural components, which provide the first line of defense, there are many types of induced resistance responses that protect the plant against pathogenic microorganisms. The different classes of resistance to microbes include gene-for-gene resistance, systemic acquired resistance, non-host resistance and basal defenses. These forms of resistance often overlap and work cooperatively to prevent the growth of pathogens.

Flor's gene-for-gene hypothesis (53, 54) states that the genetic interaction between a pathogen avirulence gene product (Avr) and the corresponding host plant resistance gene product (R) leads to resistance. Gene-for-gene resistance is often accompanied by the hypersensitive response (HR) and up-regulation of local defenses. One example of a gene-for-gene interaction is described in this section. *avrRpt2* is an avirulence gene from *P. syringae* pv. *tomato* strain JL1065 that causes the HR in *Arabidopsis* cells that express *RPS2* (the corresponding *R* gene). RPS2 physically interacts with a protein called RIN4, whose presence is eliminated by AvrRpt2 in an RPS2-independent manner (55). AvrRpt2 is a cysteine protease (56). RPS2 initiates resistance signaling in response to the disappearance of RIN4, rather than by direct recognition of AvrRpt2 (55, 57).

Systemic acquired resistance (SAR) provides resistance throughout the plant against a wide range of pathogens for an extended period of time (58). Local infection leads to systemic resistance against subsequent challenge with potential pathogens. Necrogenic fungal, bacterial, and even viral pathogens or elicitors can all trigger this resistance mechanism (59). SAR is accompanied by elevated expression of pathogenesis-related (PR) genes.

Non-host resistance is the most durable and common form of plant resistance in nature (60). Very little is known about the molecular mechanism of non-host resistance, but there is one *Arabidopsis* gene, non-host resistance (*NHO1*), which plays significant role in non-host resistance to bacteria and fungi. It encodes a glycerol kinase and is required for wild-type *Arabidopsis* resistance to *Botrytis cinerea* and *P. syringae* isolates from bean or tobacco, which are normally not pathogenic on *Arabidopsis* (61, 62). The expression of *NHO1* is suppressed by virulent *P. syringae* (61). Interestingly, *hrp* mutants, saprophytes, and avirulent strains of bacteria are all able to multiply in *nho1* mutants (62). *nho1* plants are capable of responding with an HR to avirulent *P. syringae* strains. This result is interesting because HR usually signals up-regulation of a successful defense response.

Gene-for-gene resistance, SAR, and non-host resistance are all elicited by pathogens. There is another type of resistance, called basal defense, which is elicited by both pathogenic and non-pathogenic bacteria. Basal defenses involve up-regulation of several defense/stress genes (e.g. phenylalanine ammonia lyase, chalcone synthase and chitinase) and the production of phytoalexins (63, 64), and is elicited by molecules that are conserved in both plant pathogens and non-plant pathogens. Flg22 and flg15, two peptides corresponding to the most conserved domain of eubacterial flagellin, for example, elicit this basal defense (65). The receptor for these peptides is FLS-1/FLS2 (flagellin sensing 1 or 2). Treatment of *Arabidopsis* leaves with these peptides caused the rapid release of active oxygen species, papillae formation (to be discussed in the next section), and strongly inhibited bacterial multiplication (65). Papillae are elicited by flg22 and flg15, and are discussed in more detail in the next section.

Papillae

Deposition of papillae at the site of contact with bacteria or attempted penetration by fungal hyphae is an integral part of most forms of plant resistance to microbial pathogens and non-pathogens (66). Papillae form beneath infection sites between the cell wall and the plasma membrane and are composed of callose, phenolics, hydroxyproline-rich glycoproteins (HRGPs) (e.g., extensins), and other materials. Their formation involves the synthesis and directed deposition of these compounds to the site of the interaction (67). Callose is an exception in that it is synthesized at the site of infection. Callose is a β-1,3-glucan with some 1,6 branches (68). Callose is a convenient marker for papillae. It is easily stained with aniline blue and can be visualized by fluorescence microscopy. Although papillae normally contain callose, it is important to note that the formation of papillae lacking callose is possible (69).

Although the precise function of papillae during microbial attack has not been demonstrated unequivocally, it has been postulated that they act as physical barriers. According to this interpretation, papillae impede microbial penetration (69) or immobilize the invading microbe and potentially expose it to anti-microbial compounds (67), such as wall-degrading enzymes, phytoalexins, and active oxygen species. Callose may also contribute to host defenses by impeding nutrient transfer from the host to the pathogen or possibly by delaying pathogen growth long enough for other host defenses to become active (70). There are several studies that demonstrate the importance of callose and papillae (69, 71-73), as well as studies that demonstrate that callose is not important for defense against pathogens (69, 74, 75).

Papillae are deposited much more quickly in response to an avirulent strain than to a *hrp* mutant (76). The first response to avirulent bacteria is the apparent convolution of the plasma membrane adjacent to bacterial cells (67, 76, 77) and, within three to five hours after inoculation, lightly stained fibrillar materials accumulate between the convoluted membrane and the plant cell wall (76). The early stages of papilla formation are frequently associated with the presence of Golgi and ER in the underlying cytoplasm. Immunogold labeling revealed that callose is present at all stages of papillae development, but not in the cell wall before inoculation (67). Deposits increase in thickness and complexity between 3-8 h. The plasma membrane can be detached from developing deposits during plasmolysis (76). As papillae develop, distinct proliferation and swelling of the endoplasmic reticulum occurs in the majority of challenged cells. Smooth vesicles and multivesicular bodies (MVBs) become visible within the cytoplasm and near sites of deposition. In some cases, the MVBs appear to fuse with the plasma membrane, discharging vesicles out of the cell. As deposits increased in complexity, an electron-translucent material appears throughout the fibrillar matrix, which contains layers of irregularly shaped osmiophillic particles and vesicles. Histochemical studies indicate that the earliest deposits contain HRGPs and that the initial matrix becomes impregnated with phenolics and finally callose (76).

hrp mutant strains of *Xanthomonas campestris* pv. *vesicatoria* induce the formation of large papillae in pepper regardless of whether they are inactivated by antibiotic treatment before inoculation or not (77). In contrast, wild-type pathogenic strains do not elicit papillae formation unless they are inactivated by chloramphenicol or heat-killed before inoculation. However, if antibiotic treatment of the wild-type strain is delayed until 8 hr after inoculation, no large papillae are produced. These experiments show that the wild-type strain actively suppresses the deposition of papillae in a *hrp* gene-dependent manner (77).

Plant defense signaling hormones: salicylic acid, jasmonic acid and ethylene

The various forms of plant resistance just described require one or more defense hormones, including salicylic acid (SA), jasmonic acid (JA) and ethylene. Hormone signaling in response to pathogens is complex and depends on the plant-pathogen system. In many cases, antagonistic or synergistic cross-talk between the SA, JA and ethylene pathways has been described (78-85).

The *Arabidopsis* response to bacterial pathogens is strongly dependent on SA. Transgenic plants that constitutively express *nahG,* which encodes an enzyme, salicylate hydroxylase, that degrades SA to catechol, do not have detectable levels of SA (86), and are hyper-susceptible to a variety of pathogens, including *Pst* DC3000. The expression of PR genes has proven to be a good marker for SA-based defenses because SA is required for *PR* gene induction to occur; however, there is little evidence for their role in inhibiting bacterial growth.

Ethylene, traditionally known for its role in a wide variety of physiological processes including seed germination, cell elongation, epinasty and various forms of senescence, including fruit ripening, has been shown to mediate responses to pathogen infection as well (79, 80, 87-89). The *Arabidopsis ein2* (ethylene insensitive) mutant has reduced symptom development upon *Pst* DC3000 infection, without a reduction in bacterial growth (90). *ein2* is an integral membrane protein that acts downstream of the ethylene receptors and upstream of the gene transcription changes associated with the ethylene response (87).

JA is essential in flower development because JA biosynthetic and perception mutants are sterile (91). JA is structurally similar to the phytotoxin coronatine, produced by *Pst* DC3000 and several other strains of *P. syringae*. Besides causing chlorosis, coronatine causes stunted roots as well as other physiological changes in a broad variety of plants (92). The *coi1* (coronatine-insensitive) *Arabidopsis* mutant was isolated in a screen for mutants that exhibit normal root growth in the presence of coronatine (91, 93). *coi1* plants are also insensitive to JA, resistant to infection by bacterial pathogens (91, 94), and more susceptible than wild-type plants to some fungal pathogens (84, 95). COI1 encodes an F-box protein (93, 96-98), which regulates expression of JA-responsive genes, possibly by targeted ubiquitination of a histone deacetylase and other factors (96).

Rationale

Currently, one pathogen control is the use of expensive and toxic chemicals. An environmentally safer method of control involves genetic modification, such as breeding *R* genes into crops. The problem with the latter method is that monoculture and genetic uniformity create a significant selection pressure for pathogens to overcome host resistance mechanisms. For example, a mutation in the corresponding *avr* gene could be sufficient for the pathogen to evade recognition by a newly introduced *R* gene. One way for plant breeders to cope with this problem is to combine several *R* genes within a single cultivar so that multiple *avr* genes would have to be mutated in order to avoid detection. This process, called "pyramiding", along with crop rotation has helped reduce crop losses due to successful pathogens. However, there are a limited number of *R* genes available, and pathogens may evolve and eventually overcome all available *R* genes. Therefore, new methods other than *R* gene-mediated resistance and increased chemical use are needed to prevent the yield losses caused by plant pathogens.

The ultimate goal of this research is to determine the molecular basis of susceptibility of *Arabidopsis* to *Pst* DC3000. It is hoped that a basic knowledge of the mechanisms underlying disease progression can help the development of alternative disease control strategies. I focused my Ph.D. research on two specific questions.

1.) What is the virulence function of AvrPto in the compatible interaction between *Arabidopsis* and *Pst* DC3000?

2.) What is the molecular basis of symptom development during disease caused by *Pst* DC3000?

I attempted to achieve these objectives by two different approaches. I created transgenic plants that express *avrPto* and evaluated these *avrPto* plants to gain an understanding of the role of AvrPto in virulence. I also characterized an *Arabidopsis* mutant that has altered symptom development in response to pathogen infection.

References

1. Oerke, E. C. (1994) *Crop production and crop protection: estimated losses in major food and cash crops* (Amsterdam; New York, Elsevier).

2. Pedley, K. F. & Martin, G. B. (2003) Molecular basis of Pto-mediated resistance to bacterial speck disease in tomato. *Annu Rev Phytopathol*, 41, 215-243.

3. Bender, C. L., Stone, H. E., Sims, J. J. & Cooksey, D. A. (1987) Reduced pathogen fitness of *Pseudomonas-syringae* pv *tomato* Tn5 mutants defective In coronatine production. *Physiol Mol Plant Pathol*, 30, 273-283.

4. Brooks, D. M., Hernandez-Guzman, G., Kloek, A. P., Alarcon-Chaidez, F., Sreedharan, A., Rangaswamy, V., Penaloza-Vazquez, A., Bender, C. L. & Kunkel, B. N. (2004) Identification and characterization of a well-defined series of coronatine biosynthetic mutants of *Pseudomonas syringae* pv. *tomato* DC3000. *Mol Plant Microbe Interact*, 17, 162-174.

5. Mittal, S. & Davis, K. R. (1995) Role of the phytotoxin coronatine in the infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. *tomato. Mol Plant Microbe Interact*, 8, 165-171.

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

7. Zwiesler-Vollick, J., Plovanich-Jones, A. E., Nomura, K., Bandyopadhyay, S., Joardar, V., Kunkel, B. N. & He, S. Y. (2002) Identification of novel hrp-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv. *tomato* DC3000 genome. *Mol Microbiol*, 45, 1207-1218.

8. Chen, Z., Kloek, A. P., Boch, J., Katagiri, F. & Kunkel, B. N. (2000) The *Pseudomonas syringae avrRpt2* gene product promotes pathogen virulence from inside plant cells. *Mol Plant Microbe Interact*, 13, 1312-1321.

9. Gopalan, S., Bauer, D. W., Alfano, J. R., Loniello, A. O., He, S. Y. & 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, 1095-1105.

10. HE, S. Y. (1997) Hrp-controlled interkingdom protein transport: learning from flagellar assembly? *Trends Microbiol*, 5, 489-495.

11. JIN, Q. & HE, S. Y. (2001) Role of the Hrp pilus in type III protein secretion in *Pseudomonas syringae*. *Science*, 294, 2556-2558.

12. JIN, Q., THILMONY, R., ZWIESLER-VOLLICK, J. & HE, S. Y. (2003) Type III protein secretion in *Pseudomonas syringae*. *Microbes Infect*, 5, 301-310.

13. LI, C. M., BROWN, I., MANSFIELD, J., STEVENS, C., BOUREAU, T., ROMANTSCHUK, M. & TAIRA, S. (2002) The Hrp pilus of *Pseudomonas syringae* elongates from its tip and acts as a conduit for translocation of the effector protein HrpZ. *EMBO J*, 21, 1909-1915.

14. ROINE, E., WEI, W., YUAN, J., NURMIAHO-LASSILA, E. L., KALKKINEN, N., ROMANTSCHUK, M. & 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, 3459-3464.

15. TANG, X., FREDERICK, R. D., ZHOU, J., HALTERMAN, D. A., JIA, Y. & MARTIN, G. B. (1996) Initiation of plant disease resistance by physical interaction of AvrPto and Pto Kinase. *Science*, 274, 2060-2063.

16. TANG, X., XIE, M., KIM, Y. J., ZHOU, J., KLESSIG, D. F. & MARTIN, G. B. (1999) Overexpression of Pto activates defense responses and confers broad resistance. *Plant Cell*, 11, 15-29.

17. VAN DEN ACKERVEKEN, G., MAROIS, E. & BONAS, U. (1996) Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host plant cell. *Cell*, 87, 1307-1316.

18. YANG, B., ZHU, W., JOHNSON, L. B. & WHITE, F. F. (2000) The virulence factor AvrXa7 of *Xanthomonas oryzae* pv. *oryzae* is a type III secretion pathway-dependent nuclear-localized double-stranded DNA-binding protein. *Proc. Natl. Acad. Sci. U S A*, 97, 9807-9812.

19. PITBLADO, R. E. & MACNEILL, B. H. (1983) Genetic basis of resistance to *Pseudomonas syringae* pv. *tomato* in field tomatoes. *Can. J. Plant Pathol.*, 5, 251-255.

20. RONALD, P. C., SALMERON, J. M., CARLAND, F. M. & STASKAWICZ, B. J. (1992) The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. *J Bacteriol*, 174, 1604-1611.

21. CHANG, J. H., RATHJEN, J. P., BERNAL, A. J., STASKAWICZ, B. J. & MICHELMORE, R. W. (2000) avrPto enhances growth and necrosis caused by *Pseudomonas syringae* pv.*tomato* in tomato lines lacking either Pto or Prf. *Mol Plant Microbe Interact*, 13, 568-571.

22. SHAN, L., HE, P., ZHOU, J. M. & TANG, X. (2000) A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto. *Mol Plant Microbe Interact*, 13, 592-598.

23. SALMERON, J. M. & STASKAWICZ, B. J. (1993) Molecular characterization and hrp dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. *tomato*. *Mol Gen Genet*, 239, 6-16.

24. MARTIN, G. B., BROMMONSCHENKEL, S. H., CHUNWONGSE, J., FRARY, A., GANAL, M. W., SPIVEY, R., WU, T., EARLE, E. D. & TANKSLEY, S. D. (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science*, 262, 1432-1436.

25. CARLAND, F. M. & STASKAWICZ, B. J. (1993) Genetic characterization of the *Pto* locus of tomato: semi-dominance and cosegregation of resistance to *Pseudomonas syringae* pathovar *tomato* and sensitivity to the insecticide Fenthion. *Mol Gen Genet*, 239, 17-27.

26. CHANDRA, S., MARTIN, G. B. & LOW, P. S. (1996) The Pto kinase mediates a signaling pathway leading to the oxidative burst in tomato. *Proc. Natl. Acad. Sci. U S A*, 93, 13393-13397.

27. CHANG, J. H., TAI, Y. S., BERNAL, A. J., LAVELLE, D. T., STASKAWICZ, B. J. & MICHELMORE, R. W. (2002) Functional analyses of the *Pto* resistance gene family in tomato and the identification of a minor resistance determinant in a susceptible haplotype. *Mol Plant Microbe Interact*, 15, 281-291.

28. JIA, Y., LOH, Y. T., ZHOU, J. & MARTIN, G. B. (1997) Alleles of Pto and Fen occur in bacterial speck-susceptible and fenthion-insensitive tomato cultivars and encode active protein kinases. *Plant Cell*, 9, 61-73.

29. KIM, Y. J., LIN, N. C. & MARTIN, G. B. (2002) Two distinct *Pseudomonas* effector proteins interact with the Pto kinase and activate plant immunity. *Cell*, 109, 589-598.

30. LI, J., SHAN, L., ZHOU, J. M. & TANG, X. (2002) Overexpression of Pto induces a salicylate-independent cell death but inhibits necrotic lesions caused by salicylate-deficiency in tomato plants. *Mol Plant Microbe Interact*, 15, 654-661.

31. MARTIN, G. B., FRARY, A., WU, T., BROMMONSCHENKEL, S., CHUNWONGSE, J., EARLE, E. D. & TANKSLEY, S. D. (1994) A member of the tomato Pto gene family confers sensitivity to fenthion resulting in rapid cell death. *Plant Cell*, 6, 1543-1552.

32. MYSORE, K. S., CRASTA, O. R., TUORI, R. P., FOLKERTS, O., SWIRSKY, P. B. & MARTIN, G. B. (2002) Comprehensive transcript profiling of Pto- and Prf-mediated host defense responses to infection by *Pseudomonas syringae* pv. *tomato*. *Plant J*, 32, 299-315.

33. MYSORE, K. S., D'ASCENZO, M. D., HE, X. & MARTIN, G. B. (2003) Overexpression of the disease resistance gene *Pto* in tomato induces gene expression changes similar to immune responses in human and fruitfly. *Plant Physiol*, 132, 1901-1912.

34. RATHJEN, J. P., CHANG, J. H., STASKAWICZ, B. J. & MICHELMORE, R. W. (1999) Constitutively active Pto induces a Prf-dependent hypersensitive response in the absence of avrPto. *EMBO J*, 18, 3232-3240.

35. SESSA, G. & MARTIN, G. B. (2000) Signal recognition and transduction mediated by the tomato Pto kinase: a paradigm of innate immunity in plants. *Microbes Infect*, 2, 1591-1597.

36. THILMONY, R. L., CHEN, Z., BRESSAN, R. A. & MARTIN, G. B. (1995) Expression of the tomato *Pto* gene in tobacco enhances resistance to *Pseudomonas syringae* pv *tabaci* expressing *avrPto*. *Plant Cell*, 7, 1529-1536.

37. LOH, Y. T., ZHOU, J. M. & MARTIN, G. B. (1998) The myristylation motif of Pto is not required for disease resistance. *Mol Plant Microbe Interact*, 11, 572-576.

38. XIAO, F., LU, M., LI, J., ZHAO, T., YI, S. Y., THARA, V. K., TANG, X. & ZHOU, J. M. (2003) Pto mutants differentially activate Prf-dependent, avrPto-independent resistance and gene-for-gene resistance. *Plant Physiol*, 131, 1239-1249.

39. GU, Y. Q., WILDERMUTH, M. C., CHAKRAVARTHY, S., LOH, Y. T., YANG, C., HE, X., HAN, Y. & MARTIN, G. B. (2002) Tomato transcription factors pti4, pti5, and pti6 activate defense responses when expressed in *Arabidopsis*. *Plant Cell*, 14, 817-831.

40. GU, Y.-Q., YANG, C., THARA, V. K., ZHOU, J. & MARTIN, G. B. (2000) Pti4 is induced by ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase. *Plant Cell*, 12, 771.

41. HE, P., WARREN, R. F., ZHAO, T., SHAN, L., ZHU, L., TANG, X. & ZHOU, J. M. (2001) Overexpression of Pti5 in tomato potentiates pathogen-induced defense gene expression and enhances disease resistance to *Pseudomonas syringae* pv. *tomato*. *Mol Plant Microbe Interact*, 14, 1453-1457.

42. SESSA, G., D'ASCENZO, M. & MARTIN, G. B. (2000) The major site of the pti1 kinase phosphorylated by the pto kinase is located in the activation domain and is required for pto-pti1 physical interaction. *Eur J Biochem*, 267, 171-178.

43. THARA, V. K., TANG, X., GU, Y. Q., MARTIN, G. B. & ZHOU, J. M. (1999) *Pseudomonas syringae* pv *tomato* induces the expression of tomato EREBP-like genes *pti4* and *pti5* independent of ethylene, salicylate and jasmonate. *Plant J*, 20, 475-483.

44. WU, K., TIAN, L., HOLLINGWORTH, J., BROWN, D. C. & MIKI, B. (2002) Functional analysis of tomato Pti4 in *Arabidopsis*. *Plant Physiol*, 128, 30-37.

45. ZHOU, J., LOH, Y. T., BRESSAN, R. A. & MARTIN, G. B. (1995) The tomato gene *Pti1* encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. *Cell*, 83, 925-935.

46. ZHOU, J., TANG, X. & MARTIN, G. B. (1997) The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes. *EMBO J*, 16, 3207-3218.

47. SCOFIELD, S. R., TOBIAS, C. M., RATHJEN, J. P., CHANG, J. H., LAVELLE, D. T., MICHELMORE, R. W. & STASKAWICZ, B. J. (1996) Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science*, 274, 2063-2065.

48. BOGDANOVE, A. J. & MARTIN, G. B. (2000) AvrPto-dependent Pto-interacting proteins and AvrPto-interacting proteins in tomato. *Proc. Natl. Acad. Sci. U S A*, 97, 8836-8840.

49. CHANG, J. H., TOBIAS, C. M., STASKAWICZ, B. J. & MICHELMORE, R. W. (2001) Functional studies of the bacterial avirulence protein AvrPto by mutational analysis. *Mol Plant Microbe Interact*, 14, 451-459.

50. SHAN, L., THARA, V. K., MARTIN, G. B., ZHOU, J. M. & TANG, X. (2000) The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane. *Plant Cell*, 12, 2323-2338.

51. NIMCHUK, Z., MAROIS, E., KJEMTRUP, S., LEISTER, R. T., KATAGIRI, F. & DANGL, J. L. (2000) Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*. *Cell*, 101, 353-363.

52. BAKER, B., ZAMBRYSKI, P., STASKAWICZ, B. & DINESH-KUMAR, S. P. (1997) Signaling in plant-microbe interactions. *Science*, 276, 726-733.

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

54. KEEN, N. T. (1990) Gene-for-gene complementarity in plant-pathogen interactions. *Annu Rev Genet*, 24, 447-463.

55. AXTELL, M. J. & STASKAWICZ, B. J. (2003) Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. *Cell*, 112, 369-377.

56. AXTELL, M. J., CHISHOLM, S. T., DAHLBECK, D. & STASKAWICZ, B. J. (2003) Genetic and molecular evidence that the *Pseudomonas syringae* type III effector protein AvrRpt2 is a cysteine protease. *Mol Microbiol*, 49, 1537-1546.

57. MACKEY, D., BELKHADIR, Y., ALONSO, J. M., ECKER, J. R. & DANGL, J. L. (2003) *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell,* 112, 379-389.

58. RYALS, J. A., NEUENSCHWANDER, U. H., WILLITS, M. G., MOLINA, A., STEINER, H. Y. & HUNT, M. D. (1996) Systemic acquired resistance. *Plant Cell,* 8, 1809-1819.

59. DURRANT, W. E. & DONG, X. (2004) Systemic acquired resistance. *Annu Rev Phytopathol,* 42, 185-209.

60. MYSORE, K. S. & RYU, C. M. (2004) Nonhost resistance: how much do we know? *Trends Plant Sci,* 9, 97-104.

61. KANG, L., LI, J., ZHAO, T., XIAO, F., TANG, X., THILMONY, R., HE, S. & ZHOU, J. M. (2003) Interplay of the *Arabidopsis* nonhost resistance gene *NHO1* with bacterial virulence. *Proc. Natl. Acad. Sci. U S A,* 100, 3519-3524.

62. LU, M., TANG, X. & ZHOU, J. M. (2001) *Arabidopsis NHO1* is required for general resistance against *Pseudomonas* bacteria. *Plant Cell,* 13, 437-447.

63. JAKOBEK, J. L. & LINDGREN, P. B. (1993) Generalized induction of defense responses in bean is not correlated with the induction of the hypersensitive reaction. *Plant Cell,* 5, 49-56.

64. JAKOBEK, J. L., SMITH, J. A. & LINDGREN, P. B. (1993) Suppression of bean defense responses by *Pseudomonas syringae*. *Plant Cell,* 5, 57-63.

65. GOMEZ-GOMEZ, L., FELIX, G. & BOLLER, T. (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana. Plant J,* 18, 277-284.

66. AIST, J. R. (1976) Papillae and related wound plugs of plants cells. *Annu Rev Phytopathol,* 14, 145-163.

67. BROWN, I., TRETHOWAN, J., KERRY, M., MANSFIELD, J. & BOLWELL, G. P. (1998) Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains of *Xanthomonas campestris* and French bean mesophyll cells. *Plant J,* 15, 333-343.

68. VERMA, D. P. & HONG, Z. (2001) Plant callose synthase complexes. *Plant Mol Biol*, 47, 693-701.

69. JACOBS, A. K., LIPKA, V., BURTON, R. A., PANSTRUGA, R., STRIZHOV, N., SCHULZE-LEFERT, P. & FINCHER, G. B. (2003) An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation. *Plant Cell*, 15, 2503-2513.

70. DONOFRIO, N. M. & DELANEY, T. P. (2001) Abnormal callose response phenotype and hypersusceptibility to *Peronospoara parasitica* in defence-compromised *Arabidopsis nim1-1* and salicylate hydroxylase-expressing plants. *Mol Plant Microbe Interact*, 14, 439-450.

71. BAYLES, C. J., GHEMAWAT, M. S. & AIST, J. R. (1990) Inhibition by 2-deoxy-D-glucose of callose formation, papilla deposition, and resistance to powdery mildew In an *ml-O* Barley mutant. *Physiol Mol Plant Pathol*, 36, 63-72.

72. VANCE, C. P. & SHERWOOD, R. T. (1976) Cycloheximide treatments implicate papilla formation In resistance of Reed Canarygrass to fungi. *Phytopathology*, 66, 498-502.

73. ZIMMERLI, L., JAKAB, G., METRAUX, J. P. & MAUCH-MANI, B. (2000) Potentiation of pathogen-specific defense mechanisms in *Arabidopsis* by beta - aminobutyric acid. *Proc. Natl. Acad. Sci. U S A*, 97, 12920-12925.

74. NISHIMURA, M. T., STEIN, M., HOU, B. H., VOGEL, J. P., EDWARDS, H. & SOMERVILLE, S. C. (2003) Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science*, 301, 969-972.

75. SMART, M. G., AIST, J. R. & ISRAEL, H. W. (1986) Structure and function of wall appositions.2. Callose and the resistance of oversize papillae to penetration by *Erysiphe-graminis* F-Sp *Hordei*. *Can J Bot*, 64, 802-804.

76. BESTWICK, C. S., BENNETT, M. H. & MANSFIELD, J. W. (1995) Hrp mutant of *Pseudomonas syringae* pv *phaseolicola* induces cell wall alterations but not membrane damage leading to the hypersensitive reaction in lettuce. *Plant Physiol*, 108, 503-516.

77. Brown, I., Mansfield, J. & Bonas, U. (1995) Hrp genes in *Xanthomonas campestris* pv *vesicatoria* determine ability to suppress papilla deposition in pepper mesophyll cells. *Mol Plant Microbe Interact*, 8, 825-836.

78. Doares, S. H., Narvaez-Vasquez, J., Conconi, A. & Ryan, C. A. (1995) Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. *Plant Physiol*, 108, 1741-1746.

79. Feys, B. J. & Parker, J. E. (2000) Interplay of signaling pathways in plant disease resistance. *Trends Genet*, 16, 449-455.

80. O'Donnell, P. J., Schmelz, E., Block, A., Miersch, O., Wasternack, C., Jones, J. B. & Klee, H. J. (2003) Multiple hormones act sequentially to mediate a susceptible tomato pathogen defense response. *Plant Physiol*, 133, 1181-1189.

81. Penninckx, I. A., Eggermont, K., Terras, F. R., Thomma, B. P., De Samblanx, G. W., Buchala, A., Metraux, J. P., Manners, J. M. & Broekaert, W. F. (1996) Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *Plant Cell*, 8, 2309-2323.

82. Penninckx, I. A., Thomma, B. P., Buchala, A., Metraux, J. P. & Broekaert, W. F. (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. *Plant Cell*, 10, 2103-2113.

83. Rojo, E., Titarenko, E., Leon, J., Berger, S., Vancanneyt, G. & Sanchez-Serrano, J. J. (1998) Reversible protein phosphorylation regulates jasmonic acid-dependent and -independent wound signal transduction pathways in *Arabidopsis thaliana*. *Plant J*, 13, 153-165.

84. Thomma, B., Eggermont, K., Penninckx, I., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. A. & Broekaert, W. F. (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. U S A*, 95, 15107-15111.

85. Xu, Y., Chang, P., Liu, D., Narasimhan, M. L., Raghothama, K. G., Hasegawa, P. M. & Bressan, R. A. (1994) Plant defense genes are synergistically induced by ethylene and methyl jasmonate. *Plant Cell*, 6, 1077-1085.

86. GAFFNEY, T., FRIEDRICH, L., VERNOOIJ, B., NEGROTTO, D., NYE, G., UKNES, S., WARD, E., KESSMANN, H. & RYALS, J. (1993) Requirement of salicylic-acid for the induction of systemic acquired-resistance. *Science*, 261, 754-756.

87. ALONSO, J. M., HIRAYAMA, T., ROMAN, G., NOURIZADEH, S. & ECKER, J. R. (1999) EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science*, 284, 2148-2152.

88. LUND, S. T., STALL, R. E. & KLEE, H. J. (1998) Ethylene regulates the susceptible response to pathogen infection in tomato. *Plant Cell*, 10, 371-382.

89. O'DONNELL, P. J., CALVERT, C., ATZORN, R., WASTERNACK, C., LEYSER, H. M. O. & BOWLES, D. J. (1996) Ethylene as a signal mediating the wound response of tomato plants. *Science*, 274, 1914-1917.

90. BENT, A. F., INNES, R. W., ECKER, J. R. & STASKAWICZ, B. J. (1992) Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. *Mol Plant Microbe Interact*, 5, 372-378.

91. FEYS, B. J. F., BENEDETTI, C. E., PENFOLD, C. N. & TURNER, J. G. (1994) *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell*, 6, 751-759.

92. GNANAMANICKAM, S. S., STARRATT, A. N. & WARD, E. W. B. (1982) Coronatine production *invitro* and *invivo* and its relation to symptom development in bacterial-blight of soybean. *Can J Bot Rev*, 60, 645-650.

93. XIE, D. X., FEYS, B. F., JAMES, S., NIETO-ROSTRO, M. & TURNER, J. G. (1998) *COI1*: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science*, 280, 1091-1094.

94. KLOEK, A. P., VERBSKY, M. L., SHARMA, S. B., SCHOELZ, J. E., VOGEL, J., KLESSIG, D. F. & KUNKEL, B. N. (2001) Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (coi1) mutation occurs through two distinct mechanisms. *Plant J*, 26, 509-522.

95. VIJAYAN, P., SHOCKEY, J., LEVESQUE, C. A., COOK, R. J. & BROWSE, J. (1998) A role for jasmonate in pathogen defense of *Arabidopsis*. *Proc. Natl. Acad. Sci. U S A*, 95, 7209-7214.

96. DEVOTO, A., NIETO-ROSTRO, M., XIE, D., ELLIS, C., HARMSTON, R., PATRICK, E., DAVIS, J., SHERRATT, L., COLEMAN, M. & TURNER, J. G. (2002) COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in *Arabidopsis*. *Plant J*, 32, 457-466.

97. FENG, S., MA, L., WANG, X., XIE, D., DINESH-KUMAR, S. P., WEI, N. & DENG, X. W. (2003) The COP9 signalosome interacts physically with SCF COI1 and modulates jasmonate responses. *Plant Cell*, 15, 1083-1094.

98. XU, L., LIU, F., LECHNER, E., GENSCHIK, P., CROSBY, W. L., MA, H., PENG, W., HUANG, D. & XIE, D. (2002) The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in *Arabidopsis*. *Plant Cell*, 14, 1919-1935.

Chapter 2: A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants

This chapter has been previously published in Proceedings of the National Academy of Sciences USA.

HAUCK, P.*, THILMONY, R.* & HE, S. Y. (2003) A Pseudomonas syringae type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants, Proc Natl Acad Sci U S A, 100, 8577-82.

* These authors contributed equally to this work.

I would like to acknowledge Roger Thilmony for contribution of Figure 2-1, Table 2-1, Table 2-2 and Table 2-3.

Abstract

Bacterial effector proteins secreted through the type III secretion system (TTSS) play a crucial role in causing plant and human diseases. Although the ability of type III effectors to trigger defense responses in resistant plants is well understood, the disease-promoting functions of type III effectors in susceptible plants are largely enigmatic. Previous microscopic studies suggest that in susceptible plants the TTSS of plant-pathogenic bacteria transports suppressors of a cell wall-based plant defense activated by the TTSS-defective *hrp* mutant bacteria. However, the identity of such suppressors has remained elusive. We discovered that the *Pseudomonas syringae* TTSS down-regulated the expression of a set of *Arabidopsis* genes encoding putatively secreted cell wall and defense proteins in a salicylic acid-independent manner. Transgenic expression of AvrPto repressed a similar set of host genes, compromised defense-related callose deposition in the host cell wall, and permitted substantial multiplication of a *hrp* mutant. AvrPto is therefore one of the long postulated suppressors of a salicylic acid-independent, cell wall-based defense that is aimed at non-pathogenic bacteria.

Introduction

Many plant pathogenic bacteria, such as *Pseudomonas syringae*, carry a type III secretion system (TTSS), which delivers effector proteins into the plant cell (1-5). Translocation of these effectors is required for bacterial pathogenesis. The TTSS also plays a crucial virulence role in bacterial diseases of mammals (3, 4, 6, 7). However, mammalian and plant pathogenic bacteria appear to produce largely distinct sets of type III effectors, possibly reflecting their different lifestyles and unique host cellular structures (8-13). For intracellular mammalian pathogenic bacteria, such as *Salmonella* and *Shigella*, a key function of type III effectors is the regulation of host cytoskeleton dynamics, which aids the invasion of bacteria into the host cell (6). Most plant pathogenic bacteria, such as *P. syringae*, however, are noninvasive, extracellular pathogens; they colonize the host intercellular space outside the plant cell wall, a structure absent in animal cells. TTSS-defective bacteria do not usually multiply or cause disease symptoms in otherwise susceptible plants. The inability of TTSS mutants to multiply in the plant intercellular space is similar to that of saprophytic bacteria found in nature.

In plant pathogenic bacteria, the TTSS is encoded by *hrp* (*h*ypersensitive *r*eaction and *p*athogenicity) genes (1, 5). We are using *P. syringae* pv. tomato strain *Pst* DC3000 (*Pst* DC3000), which infects *Arabidopsis* and tomato (14, 15), to elucidate the virulence function of the TTSS in bacterial pathogenesis in plants. In *Arabidopsis*, *Pst* DC3000 multiplies aggressively for 2 days before the onset of disease symptoms, which is characterized by water soaking in the apoplast, followed by tissue necrosis and chlorosis (14, 15). We have shown (16, 17) that the ability of *Pst* DC3000 to infect

Arabidopsis depends on the TTSS because *hrp* mutants [e.g., *hrpS* and *hrcC* (formerly *hrpH*) mutants] of *Pst* DC3000 do not multiply or cause disease in *Arabidopsis*. The TTSS of *Pst* DC3000 is believed to secrete and/or translocate >30 effector proteins into the host cell (8-13). Cumulatively, these effectors alter host cellular processes and promote disease development through largely unknown mechanisms. Although the primary function of type III effectors is to promote plant susceptibility, some effectors may be recognized by the corresponding plant disease resistance proteins in resistant plants and trigger defense responses, including the hypersensitive response (HR) (18, 19). In fact, many type III effector genes in *P. syringae* were discovered based on their ability to trigger the HR in resistant plants and have been named *avr* (for avirulence) genes (20). For example, the type III effector, AvrPto, was identified based on its avirulence activity in plants (21-23). Although the ability of type III effectors to trigger defense responses in resistant plants is well understood, the mechanism by which type III effectors, as a group, enable plant pathogenic bacteria to proliferate in the intercellular space of a susceptible plant remains enigmatic. In addition to type III effectors, *Pst* DC3000 also produces the phytotoxin coronatine (COR), which is required for full virulence in *Arabidopsis* (24-26).

A decade ago, Jakobek and coworkers (27, 28) showed that in bean, general defense genes encoding phenylalanine ammonialyase, chalcone synthase, and chalcone isomerase, which are involved in the biosynthesis of antimicrobial phytoalexins, are induced by the *hrp* mutants of a non-host bacterium, *P. syringae* pv. *tabaci*, and saprophytic bacteria, but not by the wild-type virulent *P. syringae* pv. *phaseolicola*. Ultra-structural studies have illustrated that *hrp* mutants of *Xanthomonas campestris* pv.

vesicatoria and *P. syringae* pv. *phaseolicola,* as well as a saprophytic bacterium, cause the plant cell wall to thicken, forming a papilla (29-31). Papillae are cell wall appositions composed of callose, phenolics, hydroxyproline-rich glycoproteins (e.g., extensins), and other materials. The type III secretion-competent wild-type *X. campestris* pv. *vesicatoria*, on the other hand, does not induce papillae formation (30). These experiments led to the attractive hypothesis that TTSSs of plant pathogenic bacteria secrete one or more suppressors of this hallmark cell wall-based plant defense response elicited by nonpathogenic bacteria (e.g., *hrp* mutants and saprophytic bacteria). However, the identity of such a suppressor has remained elusive. Similarly, the plant defense response that is aimed at *hrp* mutant bacteria, but is overcome by the TTSS, is also poorly defined at the molecular level.

In this chapter, we used a combination of large-scale host gene expression profiling, transgenic expression of a *Pst* DC3000 effector, and cytological examination to identify AvrPto as a suppressor of the papilla-associated cell wall defense. Furthermore, we show that the TTSS of *Pst* DC3000 is involved in highly biased suppression of a set of *Arabidopsis* genes that encode putatively secreted cell wall and defense proteins in a salicylic acid (SA)-independent manner. This research provides a much needed guide for further progress on the elucidation of the virulence functions of type III effectors in susceptible plants.

Materials and methods

Plant growth and bacteria enumeration.

Arabidopsis thaliana accession Col-0 *gl1* plants were grown in soil in growth chambers with a day/night cycle of 12 h/12 h, a light intensity of 100 μE, and a constant temperature of 20°C. Four- to 5-week-old plants were used for experiments. Bacteria were grown in low-salt Luria–Bertani broth (14, 32) to the mid- to late-logarithmic phase at 30°C. Bacterial cultures were centrifuged to recover bacteria, which were resuspended in sterile water to a final OD_{600} of 0.002 [equivalent to 1×10^6 colony-forming units (CFU)/ml]. Fully expanded leaves were infiltrated with bacterial suspensions, and bacteria were enumerated as described by Katagiri *et al.* (14). The mean values of the bacterial populations are plotted with the SD displayed as error. Plants analyzed in Figure 2-4 were sprayed daily with a 30-μM dexamethasone solution containing 0.02% Silwet L-77 (Osi Specialties, Friendship, WV). Bacterial suspensions were infiltrated into leaves 1 day after the first dexamethasone treatment. The regulation-defective *hrpS* mutant and the secretion-defective *hrcC* mutant used in this article were described (17).

Construction of the COR$^-$ *hrpS* double mutant.

The COR$^-$ *hrpS* double mutant was generated by introducing a reported (25) Tn*5Sp*-disrupted *hrpS* gene into the chromosome of DC3118 (COR$^-$ mutant) through marker exchange mutagenesis. The COR$^-$ mutant causes a normal HR in tobacco, but slightly reduced and delayed disease symptoms in *Arabidopsis*, suggesting a virulence role of COR in *Pst* DC3000–*Arabidopsis* interaction. The COR$^-$ *hrpS* mutant does not elicit an HR in tobacco or cause disease in *Arabidopsis*. The wild-type *hrpS* gene carried on pHRPRS2 (33) restored the ability of the COR$^-$ *hrpS* mutant to elicit an HR in tobacco and cause disease symptoms in *Arabidopsis*.

Production of *AvrPto* transgenic plants.

avrPto was amplified by PCR from *Pst* DC3000 (not strain JL1065) genomic DNA using the following primers: sense primer 5'-CCGCTCGAGACCATGGGAAATATATGTGTC-3' and anti-sense primer 5'-GACTAGTTCATTGCCAGTTACGGTACG-3'. The *avrPto* fragment was cloned into pTA7002 under the control of the dexamethasone-inducible promoter (34, 35) and confirmed by sequencing. AvrPto transgenic plants were produced after a protocol that was described (36). Seven independent *avrPto* transformants were analyzed and all exhibited characteristics similar to those of lines 76 and 129 reported here.

Microarray experiments.

Four- to 5-week-old *A. thaliana* accession Col-0 *gl1* leaves were vacuum-infiltrated with bacterial suspensions containing 1 x 10^6 CFU/ml bacteria (14). For microarray analysis, infiltrated leaves were collected at 12, 24, and 36 h post-inoculation, before the appearance of water-soaking symptoms (at ≈48 h) and necrosis and chlorosis (at ≈72 h). Total RNA was isolated from each leaf sample and equal amounts of RNA from different time points were pooled for DNA microarray analysis according to the protocol described (37). The first two microarray experiments were performed by using the *Arabidopsis* Functional Genomic Consortium's (Michigan State University) microarray slides, each containing ~7,200 unique genes (37). Subsequent experiments were performed by using a subarray enriched for *Pst* DC3000-regulated genes (R.T. and S.Y.H., unpublished data).

Genes with a ≥2-fold expression difference (a ratio of ≤0.5 for repressed genes or a ratio of ≥2.0 for induced genes) in at least two of the three biological replicates of the *Pst* DC3000/*hrpS* mutant comparison in Col-0 *Arabidopsis* plants (I-A, I-B, and I-C) are described in Table 2-1.

Gene clustering analysis shown in Figure 2-2B was performed by using the CLUSTER and TREEVIEW programs (38). The predicted protein locations were determined using TARGETP analysis conducted on the *Arabidopsis* genome by the Munich Information Center for Protein Sequences (Neuherberg, Germany), which can be accessed at http://mips.gsf.de/proj/thal/db/tables/tables_menu.html (39).

Callose staining.

Arabidopsis leaves were sprayed with 30 μM dexamethasone and then infiltrated 24 h later with a bacterial suspension of $OD_{600} = 0.2$ (1×10^8 CFU/ml). Leaves were harvested 12 h after bacterial infiltration, cleared, and stained with aniline blue for callose as described (40). Leaves were examined with a Leica DM RA2 microscope with an A4 fluorescence cube. The number of callose depositions was determined with QUANTITY ONE software (Bio-Rad). More than 10 adjacent fields of view along the length of the leaf (not including the mid-vein or leaf edge) were analyzed and averaged. The values in Figure 2-3*B* are the average and SD of more than five independent leaves for each treatment.

Results

Roles of SA- and ethylene-mediated defense pathways in resistance to *hrp* mutants.

Recently, several *P. syringae* type III effectors, most notably AvrPtoB, VirPphA, VirPphF, AvrRpt2, and AvrRpm1 have been shown or suggested to modulate the HR or SA defense (41-45). To test the hypothesis that it is host defense that prevents efficient multiplication of the TTSS-defective mutants in the intercellular space, we examined the multiplication of the *Pst* DC3000 *hrcC* mutant in *nahG* (46) and *ein2* (47) plants, which are defective in two major defense pathways effective against avirulent and/or virulent strains of *P. syringae*: the SA-mediated pathway and the ethylene-mediated pathway, respectively (48). We found that the *hrcC* mutant reached a slightly higher population in *nahG* plants, compared with wild-type control plants (Figure 2-1). However, the 5-fold population increase was small compared with the >10,000-fold increase of the *Pst* DC3000 population in wild-type leaves (Figure 2-1). No significant increase in multiplication was observed for the *hrcC* mutant population in the *ein2* plants, compared with that in wild-type plants (Figure 2-1). Thus, abrogation of the SA- or ethylene-mediated defense pathway is not sufficient for a TTSS-defective mutant to multiply efficiently in the *Arabidopsis* intercellular space. These observations argue against a primary role of the SA- or ethylene-mediated resistance in preventing the growth of the nonpathogenic *hrp* mutants in *Arabidopsis*.

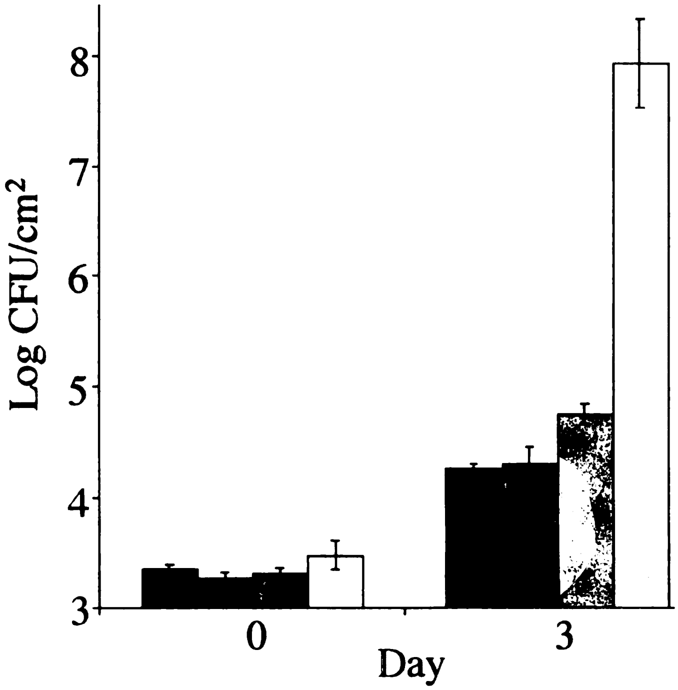


Figure 2-1. Bacterial populations in wild-type Col-0, *ein2*, and *nahG* transgenic plants. *hrcC* mutant growth in Col-0 (black bars), *ein2* (dark gray bars) and *nahG* (light gray bars) leaves. *Pst* DC3000 growth in Col-0 (white bars) is shown for comparison. Graph was contributed by Roger Thilmony.

Biased suppression of *Arabidopsis* genes encoding putatively secreted cell wall and defense proteins.

To date, a host gene expression signature that marks the virulence function of the TTSS has not been identified in any plant pathogenic bacterium. To gain molecular insight into the enigmatic virulence functions of *Pst* DC3000 type III effectors, we used a cDNA microarray to examine the expression of ≈7,200 randomly chosen *Arabidopsis* genes in pre-symptomatic leaves inoculated with *Pst* DC3000 or *hrp* mutants (Table 2-1).

In initial experiments we compared the gene expression profiles in leaf tissues inoculated with DC3000, the *hrpS* regulatory mutant, or the *hrcC* secretory mutant (17). Comparison of gene expression profiles using DC3000 and the *hrpS* mutant enabled us to identify 385 genes that are differentially regulated at >2-fold (a ratio <0.5 for repressed genes or >2.0 for induced genes in at least two of the three biological replicates; R.T., E. Bray-Speth, and S.Y.H., unpublished results). A similar profile was obtained using DC3000 and the *hrcC* mutant. Surprisingly, we found many jasmonic acid (JA)-response genes among DC3000-regulated genes. The TTSS was recently found to influence the production of the phytotoxin coronatine (COR), a molecular mimic of JA, in DC3000 (9, 49). Further analysis using the DC3118 COR$^-$ mutant ((25); defective in the production of COR) led to identification of a large number of COR-responsive genes. In order to identify TTSS-regulated host genes, we compared leaf tissues inoculated with the COR$^-$ mutant (defective in only COR production) to that of tissues inoculated with the COR$^-$ *hrpS* double mutant (defective in both COR production and type III secretion). Using this comparison, we selected genes that were differentially expressed at >2.0-fold in two biological replicates of experiment I and at >1.8-fold in both biological replicates of experiment II. That analysis identified the 117 genes contained in Table 2-1. The differential expression patterns in experiments I and II are globally similar. The quantitative difference between experiments I and II may suggest an additive contribution of COR toxin to the regulation of at least some of these host genes. To examine the reproducibility of our microarray results, we also conducted RNA blot analysis of 10 selected genes (At2g38540, At1g72610, At1g12090, At2g10940, At1g03870, At1g29670, At3g16240, At2g17500, At5g26340, and At4g02380), all independently confirming their TTSS-dependent expression (R.T., E. Bray-Speth, and S.Y.H., unpublished results). Of the 117 genes whose expression was associated with the functions of the *Pst* DC3000 TTSS (Table 2-1), 53 were repressed and 64 were induced.

Examination of the *Arabidopsis* genes repressed by the *Pst* DC3000 TTSS revealed that a surprisingly large percentage of the genes encode putatively secreted proteins. In fact, 42% of repressed genes are predicted to encode proteins that enter the plant secretory pathway, compared with only 17% of the whole genome and 16% of genes on the microarray used in this article (Table 2-2). On the other hand, the proteins encoded by the TTSS-induced genes exhibited no obvious bias toward secreted proteins. This result is in contrast to the moderately enriched chloroplast-targeted proteins in both TTSS-repressed and TTSS-induced gene sets (Table 2-2). Interestingly, we observed relatively little type III effector-mediated repression of genes involved in primary metabolic pathways in the cytoplasm, nucleus, or mitochondria, suggesting that in the first 36 h post-infection, host cells had not yet undergone global, nonspecific deterioration. This result is expected because we used pre-symptomatic tissues for RNA isolation.

The strong bias of TTSS-repressed genes toward those encoding secreted proteins can best be explained by suppression of extracellular plant defense. Indeed, we found that the majority of TTSS-repressed genes are apparently associated with plant cell wall functions including hydroxyproline-rich proteins or extensins, which are known components of papillae; and at least four genes which share sequence similarities with genes encoding known extracellular defense-associated proteins: a germin-like protein (50, 51), a nonspecific lipid transfer protein (52, 53), and two acid phosphatases ((54); see Table 2-3). Interestingly, germin-like proteins have also been shown to be associated with papillae (50, 51). Overall, the biased repression of genes encoding secreted proteins appears to provide a molecular explanation for the type III secretion-dependent suppression of papillae formation observed using microscopic analysis (29, 30) as well as additional extracellular host responses that are not microscopically visible.

The TTSS of *Pst* DC3000 induced the expression of several SA-dependent putative defense genes, including *PR1* (Table 2-1). This finding supports earlier observations (55, 56) that virulent *P. syringae* strains induce these genes in susceptible *Arabidopsis* plants, albeit with slower kinetics and at lower levels compared with those in resistant plants. Because we compared a bacterial strain that was able to secrete type III effector proteins to a strain that was type III secretion deficient, we can now conclude that type III effectors are responsible for the induction of these genes in *Arabidopsis*. Yet, *Pst* DC3000 multiplies aggressively under these conditions, suggesting that this level of SA-dependent defense is not effective at limiting *Pst* DC3000 multiplication or symptom development.

Table 2-1. ***Arabidopsis*** **genes repressed or induced by** ***Pseudomonas syringae*** **pv. tomato DC3000 in a type III secretion-dependent manner**

Locus	I-A[*]	±SD[¶]	I-B	±SD	I-C	±SD	II-A	±SD	II-B	±SD	III-A	±SD	III-B	±SD	IV	±SD	V	±SD	VI-A	±SD	VI-B	±SD	Predicted location[‡]
Repressed by the TTSS of DC3000																							
At2g38540	**0.31**	0.1	**0.23**	0.0	**0.25**	0.0	**0.43**	0.2	**0.53**	0.1	**3.34**	0.9	**1.52**	0.1	**0.07**	0.0	**0.18**	0.1	**0.29**	0.0	**0.42**	0.2	sP
At1g72610	**0.23**	0.0	**0.33**	0.0	**0.16**	0.1	**0.36**	0.1	**0.42**	0.2	**0.42**	0.1	**0.57**	0.1	**0.54**	0.1	**0.59**	0.0	**0.18**	0.0	**0.35**	0.1	sP
At1g12090	**0.37**	0.1	**0.28**	0.1	**0.19**	0.1	**0.38**	0.0	**0.40**	0.1	**1.33**	0.3	**1.11**	0.1	**0.14**	0.0	**0.26**	0.1	**0.35**	0.0	**0.37**	0.0	sP
At2g10940	**0.25**	0.0	**0.23**	0.0	**0.10**	0.1	**0.28**	0.1	**0.33**	0.1	**1.52**	0.2	**1.40**	0.2	**0.04**	0.0	**0.09**	0.0	**0.27**	0.0	**0.26**	0.0	sP
At1g03870	**0.24**	0.0	**0.29**	0.0	**0.19**	0.0	**0.54**	0.0	**0.36**	0.0	**1.11**	0.0	**1.12**	0.0	**0.25**	0.1	**0.27**	0.1	**0.31**	0.0	**0.39**	0.0	sP
At3g45970	**0.29**	0.1	**0.41**	0.1	**0.07**	0.0	**0.50**	0.0	**0.42**	0.0	**0.87**	0.3	**1.31**	0.1	**0.18**	0.0	**0.17**	0.0	**0.49**	0.1	**0.42**	0.1	sP
At1g29660	**0.50**	0.2	**0.32**	0.0	**0.24**	0.0	**0.35**	0.0	**0.51**	0.0	**0.59**	ND	**0.94**	0.0	**0.08**	ND	**0.13**	0.0	**0.25**	0.0	**0.29**	0.0	sP
At3g16370	**0.19**	0.1	**0.23**	0.0	**0.13**	0.0	**0.35**	0.1	**0.51**	0.1	**1.01**	0.1	**0.91**	0.1	**0.08**	0.0	**0.14**	0.1	**0.17**	0.0	**0.23**	0.0	sP
At5g45950	**0.33**	0.0	**0.42**	0.0	**0.35**	0.0	**0.52**	0.1	**0.55**	0.0	**0.83**	0.1	**1.01**	0.1	**0.28**	0.0	**0.31**	0.1	**0.37**	0.0	**0.46**	0.0	sP
At1g29670	**0.11**	0.0	**0.19**	0.0	**0.09**	0.0	**0.30**	0.0	**0.43**	0.0	**1.23**	0.2	**0.74**	0.0	**0.04**	0.0	**0.05**	0.0	**0.15**	0.0	**0.24**	0.0	sP
At1g68560	**0.47**	ND[§]	**0.37**	0.0	**0.32**	0.1	**0.54**	0.1	**0.50**	0.0	**1.32**	ND	**1.12**	0.0	**0.31**	0.0	**0.67**	0.0	**0.43**	0.0	**0.51**	0.0	sP
At4g23820	**0.26**	0.0	**0.28**	0.0	**0.15**	0.0	**0.43**	0.0	**0.47**	0.0	**1.25**	0.3	**1.15**	0.0	**0.35**	0.0	**0.92**	0.1	**0.30**	0.0	**0.31**	0.1	sP
At1g04040	**0.42**	0.2	**0.23**	0.0	**0.16**	0.1	**0.44**	0.1	**0.36**	0.0	**0.81**	0.3	**0.92**	0.0	**0.14**	0.0	**0.23**	0.0	**0.24**	0.0	**0.28**	0.1	sP
At5g44020	**0.34**	0.1	**0.27**	0.0	**0.21**	0.0	**0.42**	0.0	**0.45**	0.0	**1.50**	ND	**1.07**	0.0	**0.13**	ND	**0.23**	0.0	**0.25**	0.0	**0.32**	0.0	sP
At2g37450	**0.45**	ND	**0.34**	0.1	**0.35**	0.1	**0.53**	0.1	**0.55**	0.1	**1.12**	0.2	**0.88**	0.1	**0.46**	0.1	**0.25**	0.0	**0.34**	0.0	**0.49**	0.0	sP
At4g08950	**0.30**	ND	**0.43**	0.0	**0.22**	0.0	**0.42**	0.0	**0.41**	0.0	**0.69**	ND	**0.80**	0.1	**0.40**	0.1	**0.28**	0.1	**0.29**	0.0	**0.42**	0.1	sP
At4g34260	**0.26**	0.1	**0.25**	0.0	**0.24**	0.0	**0.42**	0.1	**0.53**	0.1	**ND**	ND	**ND**	ND	**0.42**	0.0	**0.61**	0.1	**0.37**	0.0	**0.37**	0.1	sP
At5g15350	**0.27**	0.1	**0.26**	0.0	**0.25**	0.1	**0.44**	0.2	**0.54**	0.1	**0.65**	0.1	**0.99**	0.0	**0.61**	0.0	**0.78**	0.3	**0.33**	0.0	**0.35**	0.0	sP
At3g07460	**0.35**	0.2	**0.34**	0.0	**0.24**	0.1	**0.44**	0.0	**0.49**	0.0	**0.82**	0.3	**0.96**	0.1	**0.34**	0.0	**0.53**	0.0	**0.31**	0.0	**0.45**	0.1	sP
At4g17340	**0.25**	0.1	**0.29**	0.0	**0.18**	0.0	**0.41**	0.0	**0.40**	0.0	**1.03**	0.0	**1.15**	0.0	**0.28**	0.1	**0.22**	0.0	**0.29**	0.0	**0.35**	0.0	sP
At3g16240	**0.26**	0.1	**0.20**	0.0	**0.13**	0.1	**0.36**	0.1	**0.29**	0.0	**1.61**	0.0	**1.39**	0.1	**0.10**	0.0	**0.11**	0.0	**0.25**	0.0	**0.26**	0.0	sP
At2g19860	**0.19**	0.0	**0.25**	0.0	**0.21**	0.0	**0.38**	0.0	**0.36**	0.0	**0.95**	0.2	**0.77**	0.0	**0.25**	0.0	**0.37**	0.0	**0.22**	0.0	**0.30**	0.0	sP
At5g02160	**0.38**	0.2	**0.33**	0.0	**0.18**	0.0	**0.40**	0.0	**0.32**	0.0	**1.61**	0.7	**1.29**	0.1	**0.18**	0.0	**0.16**	0.0	**0.33**	0.1	**0.42**	0.1	cTP
At5g56500	**0.22**	0.1	**0.33**	0.0	**0.16**	0.0	**0.34**	0.1	**0.46**	0.0	**0.46**	0.1	**0.73**	0.0	**0.36**	0.0	**0.48**	0.1	**0.19**	0.0	**0.34**	0.0	cTP
At4g01310	**0.48**	0.2	**0.34**	0.0	**0.35**	0.0	**0.48**	0.1	**0.50**	0.1	**2.06**	0.7	**1.06**	0.1	**0.27**	0.0	**0.43**	0.0	**0.38**	0.1	**0.47**	0.1	cTP
At1g33290	**0.43**	0.2	**0.25**	0.0	**0.46**	0.0	**0.49**	0.1	**0.50**	0.0	**0.95**	0.1	**0.97**	0.1	**0.34**	0.1	**0.36**	0.1	**0.31**	0.0	**0.45**	0.1	cTP
At1g14150	**0.37**	0.1	**0.29**	0.0	**0.33**	0.0	**0.32**	0.0	**0.46**	0.1	**1.37**	0.4	**1.02**	0.0	**0.22**	0.0	**0.14**	0.0	**0.25**	0.0	**0.38**	0.0	cTP
At3g26060	**0.49**	0.0	**0.35**	0.0	**0.32**	0.0	**0.37**	0.1	**0.54**	0.0	**3.01**	0.1	**1.19**	0.1	**0.17**	0.0	**0.21**	0.0	**0.31**	0.0	**0.44**	0.0	cTP

Table 2-1 (cont'd)

At5g58260	**0.37**	0.1	**0.29**	0.0	**0.55**	0.0	**0.40**	0.0	**0.55**	0.0	**2.48**	0.3	**0.95**	0.0	**0.21**	0.1	**0.11**	0.0	**0.26**	0.0	**0.46**	0.1	cTP
At1g20020	**0.30**	0.0	**0.32**	0.0	**0.30**	0.0	**0.28**	0.0	**0.54**	0.1	**2.41**	0.0	**0.97**	0.1	**0.16**	0.0	**0.18**	0.0	**0.33**	0.0	**0.50**	0.0	cTP
At3g63140	**0.30**	0.0	**0.28**	0.0	**0.32**	0.1	**0.35**	0.1	**0.54**	0.1	**2.25**	0.2	**0.82**	0.1	**0.29**	0.0	**0.35**	0.1	**0.26**	0.0	**0.39**	0.0	cTP
At5g23060	**0.22**	0.0	**0.26**	0.0	**0.28**	0.1	**0.45**	0.1	**0.51**	0.0	**1.91**	0.1	**0.83**	0.0	**0.43**	0.0	**0.38**	0.1	**0.25**	0.0	**0.35**	0.0	cTP
At2g29630	**0.28**	0.0	**0.32**	0.0	**0.24**	0.1	**0.35**	0.0	**0.54**	0.0	**1.80**	0.4	**1.32**	0.1	**0.15**	0.0	**0.15**	0.0	**0.40**	0.0	**0.45**	0.0	cTP
At3g61870	**0.43**	0.0	**0.34**	0.0	**0.42**	0.0	**0.54**	0.0	**0.53**	0.0	**2.64**	0.3	**0.96**	0.0	**0.36**	0.0	**0.39**	0.0	**0.32**	0.0	**0.44**	0.1	cTP
At5g13630	**0.44**	ND	**0.39**	0.0	**0.40**	0.0	**0.52**	0.1	**0.54**	0.0	**1.95**	ND	**0.70**	0.0	**1.32**	0.0	**1.26**	0.2	**0.37**	0.0	**0.57**	0.0	cTP
At2g36290	**0.37**	0.1	**0.31**	0.0	**0.38**	0.0	**0.54**	0.0	**0.44**	0.0	**1.12**	0.2	**1.21**	0.1	**0.54**	0.1	**0.39**	0.0	**0.40**	0.0	**0.46**	0.0	cTP
At5g44680	**0.35**	0.2	**0.22**	0.0	**0.14**	0.0	**0.51**	0.1	**0.43**	0.0	**0.69**	0.1	**0.73**	0.0	**0.09**	0.0	**0.11**	0.0	**0.23**	0.0	**0.32**	0.0	cTP
At1g67700	**0.44**	0.1	**0.34**	0.0	**0.40**	0.0	**0.55**	0.1	**0.43**	0.1	**1.33**	0.6	**0.91**	0.0	**0.50**	0.0	**0.40**	0.0	**0.35**	0.0	**0.51**	0.1	mTP
At3g62550	**0.32**	0.1	**0.18**	0.0	**0.26**	0.1	**0.44**	0.1	**0.33**	0.1	**0.51**	0.2	**0.79**	0.1	**0.34**	0.0	**0.07**	0.0	**0.22**	0.0	**0.37**	0.1	other
At1g09310	**0.27**	0.1	**0.19**	0.0	**0.22**	0.0	**0.35**	0.1	**0.43**	0.1	**0.93**	0.5	**0.91**	0.1	**0.08**	0.0	**0.33**	0.0	**0.19**	0.0	**0.39**	0.1	other
At1g49210	**0.26**	ND	**0.23**	0.0	**0.16**	0.1	**0.50**	0.1	**0.40**	0.1	**0.94**	ND	**0.92**	0.0	**0.27**	0.1	**0.30**	0.1	**0.25**	0.0	**0.33**	0.0	other
At5g48250	**0.23**	0.0	**0.29**	0.0	**0.21**	0.0	**0.46**	0.0	**0.41**	0.0	**0.93**	0.1	**1.09**	0.0	**0.24**	0.1	**0.19**	0.0	**0.31**	0.0	**0.39**	0.0	other
At5g35480	**0.32**	0.0	**0.25**	0.0	**0.33**	0.1	**0.50**	0.0	**0.42**	0.1	**0.56**	ND	**0.79**	0.0	**0.36**	ND	**0.21**	0.0	**0.19**	0.0	**0.40**	0.1	other
At2g15970	**0.24**	0.1	**0.28**	0.0	**0.15**	0.0	**0.38**	0.1	**0.46**	0.0	**0.85**	0.3	**0.96**	0.0	**0.09**	0.0	**0.08**	0.0	**0.30**	0.0	**0.44**	0.1	other
At5g43270	**0.28**	0.1	**0.35**	0.0	**0.22**	0.1	**0.48**	0.0	**0.49**	0.0	**0.92**	0.1	**0.87**	0.1	**0.37**	0.0	**0.51**	0.0	**0.33**	0.0	**0.43**	0.0	other
At4g16985	**0.28**	0.0	**0.31**	0.0	**0.16**	0.0	**0.44**	0.0	**0.46**	0.1	**0.41**	0.1	**0.73**	0.0	**0.34**	0.0	**0.53**	0.1	**0.20**	0.0	**0.36**	0.1	other
At1g01430	**0.45**	0.2	**0.35**	0.0	**0.31**	0.0	**0.49**	0.1	**0.46**	0.1	**1.20**	0.2	**0.94**	0.1	**0.39**	0.0	**0.56**	0.1	**0.30**	0.0	**0.47**	0.1	other
At5g16030	**0.25**	0.0	**0.21**	0.0	**0.13**	0.0	**0.53**	0.1	**0.31**	0.1	**0.83**	0.1	**0.82**	0.0	**0.42**	0.1	**0.40**	0.1	**0.22**	0.0	**0.35**	0.0	other
At1g18620	**0.28**	0.0	**0.22**	0.0	**0.18**	0.0	**0.53**	0.1	**0.36**	0.1	**0.92**	0.0	**0.64**	0.0	**0.87**	0.0	**1.03**	0.2	**0.26**	0.0	**0.34**	0.0	other
At5g06690	**0.42**	0.0	**0.35**	0.0	**0.40**	0.1	**0.48**	0.0	**0.53**	0.0	**1.58**	0.5	**1.13**	0.1	**0.16**	0.0	**0.11**	0.0	**0.41**	0.0	**0.55**	0.0	other
At1g09340	**0.24**	ND	**0.25**	0.0	**0.33**	0.0	**0.37**	0.0	**0.54**	0.2	**2.56**	ND	**0.82**	0.1	**0.40**	0.0	**0.36**	0.0	**0.22**	0.0	**0.42**	0.1	other
At2g28900	**0.34**	ND	**0.34**	0.0	**0.30**	0.1	**0.31**	0.1	**0.55**	0.1	**1.51**	ND	**1.20**	0.1	**0.06**	0.0	**0.06**	0.0	**0.35**	0.0	**0.49**	0.1	other
At5g09660	**0.26**	0.0	**0.26**	0.0	**0.22**	0.0	**0.42**	0.1	**0.55**	0.1	**1.11**	0.2	**0.65**	0.0	**0.69**	0.0	**0.56**	0.2	**0.30**	0.0	**0.50**	ND	other

Table 2-1 (cont'd)

Induced by the TTSS of DC3000																			
At3g11340	**2.79**	ND	**4.75**	0.1	**15.32**	0.9	**8.57**	0.8	**12.23**	0.9	**0.04**	ND	**0.15**	0.0	**9.65**	2.2	**8.36**	0.3	**2.93**	0.4	**2.56**	0.4	sP
At3g57260	**2.53**	0.1	**3.47**	0.1	**8.24**	1.5	**2.00**	0.3	**2.13**	0.3	**0.05**	0.0	**0.06**	0.0	**5.75**	1.0	**3.63**	0.4	**2.68**	0.1	**1.91**	0.1	sP
At5g19230	**3.20**	1.5	**3.23**	0.1	**6.02**	1.6	**2.72**	0.2	**3.40**	0.4	**0.40**	0.1	**0.61**	0.0	**2.46**	0.2	**1.81**	0.2	**3.30**	0.3	**1.99**	0.4	sP
At3g50930	**2.68**	0.5	**2.57**	0.0	**3.84**	0.4	**4.48**	1.8	**3.93**	0.8	**0.59**	0.1	**1.30**	0.0	**3.01**	0.0	**1.67**	0.4	**2.84**	0.5	**1.88**	0.2	sP
At5g39670	**3.09**	1.0	**3.13**	0.2	**4.55**	1.6	**6.53**	3.6	**4.42**	1.2	**0.16**	0.0	**0.35**	0.0	**9.74**	0.6	**1.73**	0.4	**3.32**	0.6	**1.82**	0.3	sP
At2g43590	**5.45**	0.2	**4.13**	0.4	**7.71**	0.2	**3.23**	0.3	**4.97**	0.2	**0.16**	0.0	**0.27**	0.0	**28.30**	4.9	**21.51**	1.0	**2.34**	0.1	**1.57**	0.1	sP
At1g63840	**2.29**	0.1	**2.58**	0.1	**5.55**	0.2	**2.50**	0.8	**2.26**	0.3	**1.16**	0.0	**1.33**	0.1	**4.21**	ND	**4.73**	0.0	**2.51**	0.4	**1.55**	0.3	sP
At3g49120	**2.01**	ND	**3.46**	0.6	**6.95**	0.7	**2.02**	0.0	**3.46**	0.5	**0.17**	ND	**0.45**	0.0	**3.44**	0.6	**5.17**	0.6	**1.26**	0.1	**1.49**	0.3	sP
At3g29030	**2.26**	0.8	**1.13**	0.1	**3.35**	0.0	**3.44**	1.5	**4.74**	0.9	**0.06**	0.0	**0.24**	0.0	**8.64**	0.8	**4.47**	0.1	**0.99**	0.0	**1.14**	0.2	sP
At2g14610	**2.52**	1.4	**2.38**	0.2	**11.71**	3.8	**7.16**	2.3	**4.94**	0.2	**0.01**	ND	**0.08**	0.0	**6.54**	ND	**6.69**	0.6	**0.64**	0.1	**0.88**	0.3	sP
At1g29330	**3.66**	0.7	**3.91**	0.3	**4.78**	0.0	**2.44**	0.2	**1.93**	0.1	**2.09**	0.0	**2.15**	0.1	**2.18**	0.4	**1.99**	0.2	**4.16**	0.7	**2.73**	0.3	sP
At5g60900	**1.85**	ND	**1.80**	0.1	**2.78**	1.1	**2.99**	0.7	**2.82**	0.8	**0.03**	ND	**0.16**	0.0	**6.29**	0.2	**1.04**	0.2	**1.29**	0.2	**1.45**	0.3	sP
At2g17500	**8.56**	4.1	**8.18**	0.4	**10.50**	3.8	**1.96**	0.1	**3.87**	1.0	**1.59**	0.1	**2.37**	0.3	**2.36**	0.3	**5.00**	1.0	**9.66**	1.4	**4.50**	0.6	sP
At5g02020	**2.59**	0.1	**1.58**	0.1	**1.77**	0.2	**3.47**	0.6	**2.02**	0.1	**2.59**	0.1	**3.23**	0.1	**4.97**	0.2	**1.29**	0.1	**1.75**	0.1	**1.14**	0.1	cTP
At4g34200	**2.22**	0.5	**2.20**	0.1	**3.55**	0.5	**3.91**	0.9	**3.20**	1.3	**0.96**	0.1	**0.97**	0.1	**2.26**	0.3	**5.23**	2.1	**1.68**	0.4	**1.39**	0.7	cTP
At1g17745	**2.57**	0.5	**1.49**	0.1	**5.02**	0.4	**3.12**	0.4	**3.58**	0.6	**0.20**	0.0	**0.43**	0.0	**10.07**	0.6	**12.40**	2.9	**1.09**	0.3	**1.42**	0.2	cTP
At1g72890	**2.62**	0.5	**2.55**	0.0	**2.96**	0.5	**2.12**	0.3	**2.17**	0.4	**0.35**	0.1	**0.55**	0.0	**1.14**	0.2	**1.51**	0.2	**1.88**	0.1	**1.42**	0.2	cTP
At3g22231	**3.10**	1.4	**1.74**	0.1	**2.34**	0.4	**2.14**	0.0	**5.14**	1.2	**0.01**	0.0	**0.02**	0.0	**5.24**	0.0	**1.27**	0.1	**1.30**	0.2	**1.43**	0.1	cTP
At2g42890	**1.70**	ND	**2.19**	0.1	**2.01**	0.1	**2.05**	0.4	**2.15**	0.4	**1.07**	ND	**1.04**	0.1	**2.22**	0.2	**1.64**	0.2	**2.23**	0.3	**1.57**	0.1	cTP
At5g05730	**2.57**	1.1	**3.18**	0.1	**2.78**	1.0	**2.36**	1.1	**3.66**	0.5	**0.42**	0.2	**0.49**	0.0	**3.07**	1.2	**5.62**	2.2	**1.93**	0.2	**1.85**	0.7	cTP
At3g44720	**2.67**	1.3	**3.98**	0.3	**4.00**	2.9	**3.22**	0.0	**2.95**	0.7	**0.35**	0.1	**0.58**	0.1	**4.02**	0.0	**1.48**	0.1	**2.61**	0.1	**1.86**	0.6	cTP
At1g74710	**4.25**	0.7	**4.60**	0.5	**5.55**	0.6	**4.16**	0.7	**5.57**	0.9	**0.25**	0.0	**0.25**	0.0	**16.69**	1.5	**2.49**	0.2	**3.94**	0.1	**2.29**	0.1	cTP
At3g26840	**3.59**	0.2	**3.53**	0.3	**4.95**	1.9	**2.69**	0.6	**2.77**	0.4	**0.43**	0.1	**0.94**	0.0	**7.36**	0.3	**9.99**	0.1	**2.54**	0.2	**2.46**	0.1	cTP
At5g10630	**6.63**	0.7	**7.94**	0.5	**17.62**	6.0	**2.27**	0.3	**2.52**	0.4	**0.82**	0.0	**1.27**	0.1	**3.51**	0.8	**5.01**	1.8	**7.79**	0.4	**7.39**	0.7	cTP
At1g61820	**5.71**	1.9	**13.74**	2.7	**24.82**	8.3	**1.86**	0.4	**5.32**	0.7	**0.19**	0.0	**0.22**	0.0	**7.54**	2.6	**30.29**	1.2	**6.31**	0.6	**4.06**	0.1	cTP
At5g54810	**1.75**	0.1	**2.08**	0.2	**2.84**	0.5	**2.53**	0.0	**3.10**	0.4	**0.66**	0.5	**0.68**	0.0	**2.09**	0.0	**4.44**	0.2	**1.64**	0.1	**1.52**	0.1	cTP
At5g40530	**3.96**	1.1	**2.56**	0.0	**2.88**	0.2	**1.98**	0.2	**2.31**	0.2	**1.20**	0.2	**1.57**	0.1	**1.36**	0.2	**2.65**	0.2	**1.93**	0.1	**1.88**	0.1	cTP
At3g14050	**2.60**	0.7	**2.87**	0.1	**2.46**	0.1	**1.95**	0.1	**1.99**	0.3	**0.51**	0.0	**0.84**	0.0	**4.93**	0.2	**3.28**	0.4	**3.34**	0.2	**1.86**	0.0	cTP
At2g30770	**2.19**	0.2	**6.93**	0.4	**11.45**	1.8	**5.46**	0.1	**5.93**	0.1	**0.05**	0.0	**0.09**	0.0	**4.54**	0.9	**5.42**	0.2	**0.95**	0.0	**1.43**	0.2	mTP
At4g37010	**3.01**	0.8	**4.16**	0.4	**7.05**	1.3	**2.23**	0.9	**2.79**	0.4	**0.12**	0.0	**0.30**	0.0	**16.59**	0.9	**32.32**	5.2	**2.14**	0.4	**2.31**	0.5	mTP
At5g17760	**5.49**	1.7	**2.89**	0.0	**7.58**	0.9	**4.55**	0.3	**5.04**	0.9	**0.38**	0.1	**0.74**	0.0	**5.85**	0.5	**2.58**	0.1	**4.52**	0.1	**2.28**	0.2	mTP
At1g13340	**7.06**	3.2	**5.83**	0.1	**3.40**	0.8	**3.70**	0.9	**4.36**	0.3	**0.63**	0.5	**0.92**	0.1	**5.84**	0.2	**5.23**	0.3	**4.57**	0.2	**2.06**	0.2	mTP

Table 2-1 (cont'd)

At5g07440	**6.79**	1.6	**8.26**	0.3	**7.92**	0.5	**2.30**	0.1	**2.70**	0.2	**1.05**	0.2	**0.97**	0.1	**2.30**	0.3	**6.61**	0.4	**4.61**	0.4	**4.45**	0.4	mTP
At5g57900	**4.82**	2.2	**4.89**	0.2	**5.92**	2.8	**2.82**	0.5	**3.23**	0.6	**1.11**	0.4	**1.68**	0.1	**2.37**	0.6	**3.19**	0.5	**3.11**	0.5	**2.49**	0.4	mTP
At2g47130	**2.70**	0.1	**2.00**	0.1	**1.97**	0.5	**3.83**	0.2	**3.42**	0.5	**0.05**	0.0	**0.10**	0.0	**10.26**	2.1	**2.65**	0.4	**1.09**	0.1	**1.19**	0.0	other
At3g60450	**2.56**	0.8	**2.05**	0.1	**1.94**	0.5	**3.55**	0.4	**3.04**	0.4	**0.36**	0.0	**0.53**	0.0	**3.38**	0.7	**4.05**	0.5	**1.64**	0.1	**1.24**	0.1	other
At5g25250	**1.92**	0.1	**2.12**	0.2	**3.22**	0.8	**4.31**	0.1	**5.02**	0.3	**0.12**	0.0	**0.18**	0.0	**8.02**	0.4	**3.75**	0.5	**1.47**	0.1	**1.35**	0.1	other
At4g36990	**2.42**	0.2	**2.25**	0.1	**2.57**	0.5	**2.68**	0.2	**3.23**	0.2	**0.24**	0.1	**0.43**	0.0	**6.26**	0.4	**6.42**	1.1	**1.35**	0.0	**1.45**	0.0	other
At4g12720	**4.10**	0.9	**2.84**	0.0	**2.73**	0.3	**4.68**	0.5	**3.47**	1.0	**1.04**	0.2	**1.37**	0.1	**9.03**	0.4	**2.93**	0.2	**2.71**	0.3	**1.50**	0.1	other
At3g60440	**4.23**	2.0	**3.44**	0.4	**3.75**	0.9	**4.83**	0.1	**5.07**	0.8	**0.34**	0.0	**0.47**	0.0	**9.11**	0.6	**8.48**	0.4	**2.85**	0.0	**1.58**	0.0	other
At1g27730	**1.03**	ND	**2.58**	0.3	**2.70**	0.4	**4.09**	0.9	**2.38**	0.2	**0.67**	ND	**0.76**	0.2	**1.19**	0.0	**1.10**	0.1	**2.86**	0.6	**1.64**	0.2	other
At2g04430	**3.15**	0.2	**2.59**	0.4	**2.71**	0.4	**3.71**	0.0	**3.69**	0.1	**0.35**	0.1	**0.68**	0.0	**3.41**	1.3	**2.72**	0.1	**1.86**	0.3	**1.65**	0.1	other
At4g39670	**1.99**	ND	**3.22**	0.0	**7.07**	0.4	**2.61**	0.9	**3.59**	0.4	**0.15**	ND	**0.33**	0.0	**4.85**	0.1	**4.09**	0.4	**2.18**	0.1	**1.66**	0.1	other
At3g52430	**5.15**	0.3	**3.82**	0.6	**3.85**	1.3	**4.39**	0.9	**4.23**	0.0	**0.14**	0.1	**0.13**	0.0	**4.99**	0.2	**1.33**	0.1	**2.62**	0.2	**1.66**	0.1	other
At1g68620	**3.44**	0.2	**5.54**	0.2	**9.05**	2.1	**2.22**	0.2	**3.40**	0.2	**0.37**	0.1	**0.69**	0.0	**5.47**	0.9	**5.80**	0.7	**1.69**	0.1	**1.70**	0.0	other
At2g35940	**2.40**	0.3	**2.37**	0.1	**3.96**	0.8	**2.18**	0.6	**2.72**	0.0	**1.63**	ND	**1.65**	0.1	**2.64**	ND	**3.14**	1.1	**2.53**	0.0	**1.78**	0.1	other
At4g17500	**3.09**	1.2	**3.42**	0.0	**4.24**	0.6	**3.84**	0.1	**2.71**	0.0	**0.47**	0.2	**0.66**	0.0	**1.09**	0.0	**1.68**	0.3	**2.88**	0.1	**2.42**	0.2	other
At4g39950	**2.82**	0.9	**3.64**	0.2	**4.63**	0.9	**2.27**	0.1	**2.72**	0.2	**1.07**	0.0	**1.91**	0.4	**3.17**	0.3	**5.36**	0.1	**4.08**	0.2	**2.85**	0.5	other
At5g13330	**3.65**	0.3	**5.52**	0.3	**7.52**	1.6	**3.17**	0.6	**2.16**	0.2	**1.46**	0.0	**1.32**	0.1	**8.66**	0.9	**3.87**	1.7	**4.35**	0.3	**2.93**	0.1	other
At1g23850	**6.80**	1.8	**5.98**	0.8	**18.02**	2.9	**2.30**	0.6	**2.74**	0.5	**0.75**	0.1	**0.98**	0.1	**2.21**	ND	**2.13**	0.6	**5.32**	0.2	**4.05**	0.3	other
At3g06500	**6.26**	0.6	**6.91**	0.5	**10.27**	2.5	**3.02**	0.1	**2.25**	0.3	**2.19**	0.4	**2.16**	0.0	**1.85**	0.6	**4.25**	0.0	**5.61**	0.1	**4.52**	0.6	other
At4g21830	**13.94**	11.2	**18.89**	2.1	**92.03**	30.6	**2.71**	0.4	**2.19**	0.1	**0.18**	0.0	**0.98**	0.1	**1.63**	0.4	**0.91**	0.0	**14.82**	1.4	**5.21**	1.3	other
At1g52890	**11.38**	2.6	**16.48**	0.3	**21.98**	9.8	**3.59**	0.5	**3.26**	0.4	**0.89**	0.2	**1.18**	0.1	**0.80**	0.0	**2.09**	0.1	**6.98**	0.0	**6.07**	0.2	other
At1g19180	**6.34**	1.7	**9.52**	1.0	**9.06**	1.8	**4.33**	1.9	**3.42**	0.7	**0.91**	0.2	**1.17**	0.1	**2.42**	0.2	**3.72**	0.7	**6.80**	1.6	**7.00**	1.2	other
At3g44870	**12.09**	1.1	**21.38**	0.0	**39.32**	2.5	**4.06**	1.7	**3.61**	0.9	**0.32**	0.1	**0.51**	0.0	**3.70**	ND	**2.10**	0.2	**11.24**	0.8	**12.54**	0.2	other
At1g02450	**3.08**	1.0	**2.57**	0.2	**4.47**	1.7	**6.79**	0.9	**12.20**	2.0	**0.07**	0.0	**0.27**	0.0	**3.51**	0.7	**1.90**	ND	**2.46**	0.4	**2.08**	0.2	other
At5g26340	**3.60**	0.9	**5.16**	0.1	**3.17**	0.6	**6.90**	0.2	**4.99**	0.1	**0.90**	0.1	**1.20**	0.1	**3.46**	0.3	**2.58**	0.1	**2.96**	0.1	**2.28**	0.1	other
At5g60800	**13.00**	7.2	**6.43**	0.0	**9.70**	3.8	**8.74**	2.2	**6.30**	0.5	**0.41**	0.1	**0.57**	0.0	**2.51**	0.2	**2.06**	0.2	**5.26**	0.4	**2.88**	0.6	other
At1g33960	**6.28**	2.0	**8.22**	0.3	**16.90**	1.4	**11.86**	6.0	**25.77**	3.4	**0.12**	0.0	**0.09**	0.0	**31.15**	9.9	**11.54**	2.1	**5.85**	0.4	**2.99**	0.2	other
At3g60420	**9.15**	1.1	**8.40**	0.5	**19.90**	3.2	**13.10**	0.1	**15.36**	1.2	**0.39**	0.0	**0.49**	0.1	**19.91**	1.6	**11.71**	1.4	**7.15**	0.8	**3.03**	0.2	other
At4g02380	**4.48**	1.9	**3.93**	0.3	**5.86**	0.3	**13.97**	0.4	**6.49**	1.8	**0.15**	0.1	**0.24**	0.0	**5.38**	0.1	**3.83**	0.4	**2.29**	0.3	**2.25**	0.3	other
At2g24850	**24.19**	5.6	**28.06**	1.1	**107.5**	15.8	**16.32**	1.5	**12.05**	2.0	**0.06**	0.0	**0.13**	0.0	**2.41**	0.1	**2.10**	0.2	**6.97**	0.5	**7.34**	0.5	other
At1g77760	**5.15**	1.6	**3.53**	0.1	**11.95**	3.9	**1.97**	0.4	**3.18**	0.1	**1.83**	0.5	**2.47**	0.2	**2.13**	0.2	**3.38**	1.8	**7.53**	0.6	**2.72**	0.6	other
At5g08790	**2.12**	0.2	**2.04**	0.1	**3.05**	0.5	**3.96**	0.6	**3.56**	0.3	**0.67**	0.0	**0.92**	0.0	**2.94**	0.2	**3.10**	0.4	**1.72**	0.1	**1.44**	0.1	other

Table 2-1 (cont'd)

*Each data column in bold lists the average ratios from two technical replicates of the same biological sample (A, B, or C).
Each experimental comparison consists of at least two biological replicates and a total of four microarray experiments.
Experiment I: DC3000/hrpS mutant (17) in Col-0 plants, 12-, 24-, and 36-h pooled;
experiment II: DC3118 COR- mutant (25)/DC3118 COR- hrpS mutant in Col-0 plants, 24- and 36-h pooled;
experiment III: NahG (47)/Col-0 plants inoculated with DC3000, 12-, 24-, and 36-h pooled;
experiment IV: AvrPto-129 plants/Col-0 plants 24 h after spraying with 30 μM dexamethasone;
experiment V: AvrPto-76 plants/Col-0 plants 24 h after spray with 30 μM dexamethasone;
and experiment VI: DC3000 avrPto mutant (59)/DC3000 hrpS mutant in Col-0 plants 12-, 24-, and 36-h pooled.

†SD of the expression ratio.

‡TARGETP-predicted location.
sP, secreted protein; cTP, chloroplast-targeted protein; mTP, mitochondrion-targeted protein; and other, location unknown.

§No value was determined because of flagged spots on the microarray.

Table 2-2 was contributed by Roger Thilmony.

Table 2-2. Predicted locations of proteins encoded by TTSS-regulated *Arabidopsis* genes

Predicted location	Repressed genes	Induced genes	Microarray	Genome-wide
Secreted	42%	20%	16%	17%
Chloroplast	28%	23%	18%	14%
Mitochondria	2%	9%	10%	11%
Others	28%	47%	56%	58%

Predicted locations of proteins encoded by the 53 TTSS-repressed and 64 TTSS-induced *Arabidopsis* genes (Table 2-1) were analyzed by TargetP (http://www.cbs.dtu.dk/services/TargetP/) and compared to those of the 25,534 genes in the *Arabidopsis* genome and the 7,155 genes present on the microarray used in this study. This table was contributed by Roger Thilmony.

Table 2-3. TTSS-repressed genes that encode proteins predicted to enter the secretory pathway.

Locus	Gene homology, species, accession number	BLASTP *E* value	TargetP score
At2g38540	Cell wall-localized nonspecific lipid transfer protein 1 (LTP1), *Arabidopsis thaliana*, Q42589	4e-43	0.98
At1g72610	Germin-like protein 1 (AtGLP1), cell wall localized, *Arabidopsis thaliana*, P94040	3e-94	0.64
At1g12090	Extensin-like protein, *Arabidopsis thaliana*, T51717	4e-35	0.89
At2g10940	Proline-rich protein, extensin-like, *Pinus taeda*, AAF75825	1e-15	0.73
At1g03870	Arabinogalactan-protein 9, fasciclin-like, *Arabidopsis thaliana*, AAK20861	1e-112	0.91
At3g45970	Expansin-like protein 1, AtEXPL1, *Arabidopsis thaliana*, Q9LZT4	1e-143	0.85
At5g15350	Cell wall-localized phytocyanin, *Pinus taeda*, AAF75824	1e-13	0.93
At1g29660	Proline-rich protein APG-like, extracellular GDSL-motif lipase/hydrolase-like, *Arabidopsis thaliana*, AAK30016	1e-44	0.93
At1g29670	Proline-rich protein APG-like, extracellular GDSL-motif lipase/hydrolase-like, *Arabidopsis thaliana*, AAK30016	4e-52	0.99
At5g45950	Proline-rich protein APG-like, extracellular GDSL-motif lipase/hydrolase-like, *Arabidopsis thaliana*, AAK30018	2e-56	0.93
At4g23820	Polygalacturonase PG1, *Glycine max*, AAD46483	2e-23	0.99
At1g68560	Alpha-xylosidase XYL1, *Arabidopsis thaliana*, AAD05539	0.0	0.98
At3g16370	Proline-rich protein APG-like, extracellular GDSL-motif lipase/hydrolase-like, *Arabidopsis thaliana*, AAK30016	4e-70	0.98
At5g44020	Acid phosphatase 1, *Lycopersicon esculentum*, T06587	2e-47	0.84
At1g04040	Acid phosphatase 1, *Lycopersicon esculentum*, T06587	2e-45	0.94
At2g37450	Nodulin MtN21, *Medicago truncatula*, CAA75575	6e-41	0.90
At4g08950	Phosphate-induced protein 1 (*phi*-1), *Nicotiana tabacum*, BAA33810	1e-121	0.87
At4g34260	Putative large secreted protein, *Streptomyces coelicolor*, NP_624665	1e-136	0.90
At3g07460	Hypothetical protein	5e-94	0.99
At4g17340	Aquaporin water channel protein, *Helianthus annuus*, T14000	1e-88	0.71
At3g16240	Delta tonoplast integral water channel protein, *Arabidopsis thaliana*, AAC49281	1e-102	0.81
At2g19860	Hexokinase 2 (AtHXK2), *Arabidopsis thaliana*, P93834	0.0	0.78

This table was contributed by Roger Thilmony.

Transgenic expression of a single effector, AvrPto, regulates host genes in a manner similar to that of the *Pst* DC3000 TTSS.

To provide further evidence that the regulation of TTSS-associated genes was caused by the action of type III effectors, we decided to examine host gene expression in response to type III effectors expressed in plants. We expressed AvrPto, a type III effector well known for its avirulence activity in plants (21-23), in susceptible *Arabidopsis* under the control of the glucocorticoid-inducible promoter (34, 35). In these transgenic plants, the expression of AvrPto was induced to a level detectable by western blotting 24 h after spraying with 30 μM dexamethasone (Figure 2-2A). Leaves became chlorotic after 4 days of daily induction with dexamethasone. However, no disease-associated water soaking or necrosis developed. Two independent lines of AvrPto transgenic *Arabidopsis* plants, AvrPto-76 and AvrPto-129, were further analyzed by microarray. Remarkably, AvrPto alone regulated ≈80% of the TTSS-regulated genes, including those that encode putatively secreted cell wall and defense protein genes, in the same manner as *Pst* DC3000 (Figure 2-2B). These results confirm that type III effector-associated genes are indeed regulated directly by at least the type III effector AvrPto. The striking similarity between the TTSS- and AvrPto-regulated host gene expression profiles demonstrates that AvrPto expression in transgenic *Arabidopsis* globally mimicked the *Pst* DC3000 TTSS functions at the molecular level.

We also found that the repression of TTSS/AvrPto-regulated *Arabidopsis* secreted cell wall and defense protein genes in *nahG* plants was not reproducibly different from that in wild-type plants (see columns III-A and III-B in Table 2-1). Thus, the TTSS- and AvrPto-targeted cell wall-based defense is largely SA-independent. This result suggests that the AvrPto-suppressed cell wall-based defense is fundamentally different from that suppressed by AvrPtoB, VirPphA, AvrPphF, AvrRpt2, or AvrRpm1, which target HR cell death or SA-mediated defenses (41-45). Consistent with this conclusion, AvrPto-expressing plants still responded to *Pst* DC3000 (*avrRpt2*) with an HR (data not shown).

The TTSS of *Pst* DC3000 secretes >30 effector proteins (10, 11). Because mutations in individual effector genes often give only a subtle virulence phenotype or none at all, it is widely believed that the virulence functions of individual effector proteins, at the concentrations delivered by bacteria, are redundant or additive (57, 58). Consistent with this hypothesis, we show that AvrPto is only one of the *Pst* DC3000 effectors that modulate TTSS-associated *Arabidopsis* genes because an AvrPto deletion mutant (59) still regulated *Arabidopsis* gene expression (see columns VI-A and VI-B in Table 2-1) in a manner similar to *Pst* DC3000. This result provides molecular evidence from the host side for the functional redundancy of *Pst* DC3000 type III effectors.

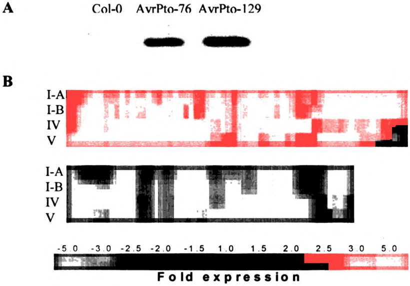


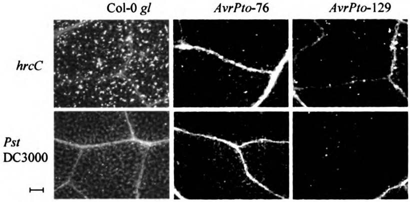
Figure 2-2. Phenotype of *avrPto* transgenic plants. (*A*) Western blot analysis of AvrPto expression in leaves of wild-type and AvrPto transgenic plants 24 h after spraying with 30 µM dexamethasone. (*B*) Cluster analysis of the expression profiles of 117 TTSS-regulated genes (colored bars) following *Pst* DC3000 infection and transgenic expression of AvrPto. Rows I-A and I-B represent *Pst* DC3000 TTSS-regulated genes from two independent biological replicates (Columns I-A and I-B; Supporting Information Table A-1). Rows IV and V represent gene expression in AvrPto-129 and AvrPto-76 transgenic plants, respectively, 24 h after dexamethasone induction (Columns IV and V; Supporting Information Table A-1).

The cell wall-based extracellular defense is compromised in *Pst* DC3000-infected and *AvrPto* transgenic plants.

Because the germin-like proteins and hydroxyproline-rich cell wall proteins repressed by the *Pst* DC3000 TTSS and AvrPto are associated with the papilla-associated cell wall defense, we suspected that AvrPto is one of the long postulated suppressors of extracellular defense elicited by *hrp* mutant bacteria (30). We examined this possibility by treating leaves with aniline blue to stain callose, a major component of papillae (29, 30). Indeed, we found that the *hrcC* mutant (positive control) induced a large number of highly localized callose deposits in leaves of wild-type plants (Figure 2-3). A significantly lower level of callose deposition was found in *Pst* DC3000-infected wild-type leaves (Figure 2-3), demonstrating that the TTSS of *Pst* DC3000 is involved in the suppression of callose-associated cell wall modifications in *Arabidopsis*. This result establishes that the *Arabidopsis*- *Pst* DC3000 system can be used to identify the suppressor of the papilla-associated plant defense.

We next examined the ability of the *hrcC* mutant to induce callose deposition in AvrPto-expressing plants. We found that AvrPto-expressing plants were compromised in mounting an active papilla-based response to the *hrcC* mutant (Figure 2-3). The number of callose deposits in *hrcC*-inoculated AvrPto leaves was only ~5% of that in *hrcC*-inoculated wild-type leaves. As expected, *Pst* DC3000 also did not induce a significant level of callose deposition in AvrPto-expressing leaves (Figure 2-3). Thus, transgenic expression of AvrPto functionally mimicked the TTSS of *Pst* DC3000 not only in regulating *Arabidopsis* gene expression, but also in effectively suppressing the papilla-associated plant cell wall defense.

A



B

Number of callose deposits per 0.58 mm^2

	Col-0 *gl*	AvrPto-76	AvrPto-129
hrcC mutant	459.4 ± 46.9	15.9 ± 6.5	21.6 ± 0.4
Pst DC3000	35.2 ± 8.1	51.2 ± 28.4	46.3 ± 25.1

Figure 2-3. Callose deposits in wild-type and *avrPto* leaves. (*A*) Portions of wild-type and AvrPto transgenic leaves stained with aniline blue for callose (white dots in these images) after inoculation with the *hrcC* mutant or *Pst* DC3000. Scale bar, 100 µm. (*B*) Average number of callose depositions per field of view (0.58 mm^2) with standard deviation displayed as error.

Enhanced growth of the *hrcC* mutant in *avrPto* transgenic plants.

The ability of AvrPto to mimic *Pst* DC3000 in the regulation of host gene expression and the suppression of callose deposition prompted us to examine the susceptibility of the AvrPto transgenic plants to the *hrcC* mutant. We found that expression of AvrPto alone was sufficient to allow substantial multiplication of the *hrcC* mutant in the transgenic plants (up to 500-fold, which was ≈10-fold lower than the levels reached by *Pst* DC3000 in these experiments; Figure 2-4). Unlike *Pst* DC3000, however, *hrcC*-inoculated leaves did not exhibit typical water soaking or extensive necrosis, suggesting a requirement of other effectors for wild-type levels of bacterial multiplication and symptom production. Transgenic expression of AvrPto did not significantly affect *Pst* DC3000 multiplication because *Pst* DC3000 multiplied similarly in the AvrPto plants and wild-type Columbia plants (Figure 2-4).

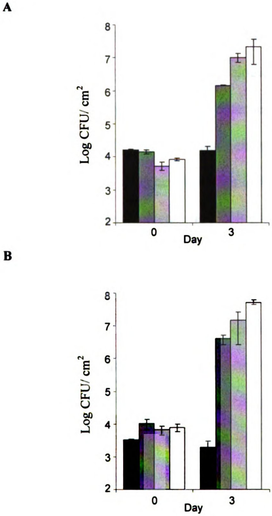


Figure 2-4. Bacterial populations in wild-type and avrPto transgenic plants. (*A*) *hrcC* mutant growth in wild-type (black bars) and AvrPto-76 (dark gray bars) plants. *Pst* DC3000 growth in wild-type (light gray bars) and AvrPto-76 (white bars) plants. (*B*) *hrcC* mutant growth in wild-type (black bars) and AvrPto-129 (dark gray bars) plants. *Pst* DC3000 growth in wild-type (light gray bars) and AvrPto-129 (white bars) plants.

Discussion

The hypothesis of a suppressor of a HR-independent cell wall-based plant defense was formulated almost a decade ago (28). However, the identity of such a suppressor remained elusive. Using a combination of large-scale host gene expression profiling, transgenic expression of AvrPto, and cytological examination, we have now demonstrated that AvrPto is a suppressor of this defense response in susceptible *Arabidopsis*. In addition, our TTSS-specific host gene expression analysis provided global insight into the collective virulence functions of *Pst* DC3000 type III effectors in *Arabidopsis*, revealing an SA-independent, plant cell wall-based extracellular defense as a major target for *Pst* DC3000 type III effectors.

The ability of AvrPto to globally mimic the TTSS modulation of host gene expression, to effectively suppress the papilla-associated cell wall response, and to substantially enhance multiplication of non-pathogenic *hrp* mutant bacteria provides important insights into two long-standing questions in plant-microbe interactions: First, why do the vast majority of nonpathogenic microbes (e.g., saprophytic bacteria) in nature fail to colonize plants? Second, what is the role of the TTSS in the evolution of bacterial pathogenicity? One possibility is that the SA-independent papilla-associated cell wall defense is a critical part of the still poorly defined plant basal defense system that prevents multiplication of saprophytic bacteria. In this scenario, acquisition of the TTSS and the AvrPto class of type III effectors, which may vary in different bacteria, by a saprophytic ancestor may have enabled it to down regulate this cell wall-based defense, allowing it to multiply substantially in the plant intercellular space. The acquisition of suppressors could therefore represent a milestone in the evolution of *P. syringae* as a virulent pathogen of higher plants. Effector interference with the plant cell wall-based defense also provides a possible explanation for the production of a largely distinct set of type III effectors by extracellular plant pathogenic bacteria, compared with intracellular mammalian pathogenic bacteria (10-12). Down-regulation of the coordinated extracellular host defenses may be especially important for plant pathogenic bacteria (such as *P. syringae*) and reflects the need for this group of bacteria to overcome the unique host cell wall-based defense of plants. Future research is needed to further define the exact extracellular defense compounds and structures that are modulated by *P. syringae* type III effectors to overcome plant resistance. Such research will provide critical information for comparative studies of the common and unique functions of type III effectors produced by plant pathogenic bacteria and mammalian pathogenic bacteria, some of which also inhibit host defense (60, 61).

Our identification of AvrPto as a suppressor of papilla-associated extracellular responses is intriguing because in tomato, AvrPto interacts with two Ras-related small G proteins, Rab proteins, which are involved in vesicular trafficking (62). Previous ultra-structural studies (29, 30) showed that papilla formation was accompanied by accelerated extracellular trafficking, as illustrated by an increased abundance of host endoplasmic reticulum and membrane vesicles. One of the AvrPto-interacting Rab proteins shows sequence similarity with Rab8, which in mammalian systems is involved specifically in extracellular secretion (63). Therefore, one mechanism by which AvrPto could act to suppress cell wall-based plant defense would be to inhibit an extracellular vesicle trafficking pathway (Figure 2-2). This inhibition may indirectly lead to feedback repression of genes encoding secreted proteins that are transported through this particular trafficking pathway. It is also possible that AvrPto interacts with a component of a signal transduction pathway to inhibit the expression of the cell wall-based extracellular defense. A recent proposal hypothesizes that the tomato Pto kinase, with which AvrPto interacts to trigger resistance responses in tomato, may be a virulence target of AvrPto guarded by the resistance protein Prf (48, 64). If this hypothesis is true, AvrPto could interact with a Pto-like kinase in *Arabidopsis* to directly down-regulate a signal transduction pathway leading to the activation of a SA-independent, host cell wall-based defense and other associated genes (Figure 2-5).

Whereas the AvrPto class of effectors appears to play a key role in overcoming a largely SA-independent cell wall-based extracellular defense, we hypothesize that an additional class of effectors in *Pst* DC3000 could have evolved to optimize bacterial virulence in specific plant genotypes by blocking gene-for-gene resistance, HR-type programmed cell death, and/or SA-dependent responses. The gene-for-gene resistance and/or SA-dependent responses could result either from plant recognition of certain effectors as Avr proteins, or from cellular perturbation caused by the virulence action of other effectors. Effectors modulating these particular defense responses are exemplified by AvrPtoB, VirPphA, AvrPphF, AvrRpt2, and AvrRpm1 (41-45, 65, 66). This class of effectors would be especially relevant to battling the ever-evolving host recognition system and may account for the presence of a large number of effector genes in the *P. syringae* genome. It is apparent that plants use type III effectors as a main source of recognition to activate innate defense and turn virulence-intended effector proteins into avirulence proteins. To remain a successful pathogen, *P. syringae* must evade

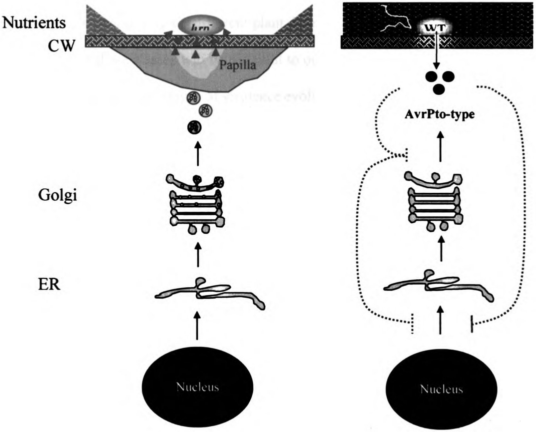


Figure 2-5. A hypothetical model of the function of the AvrPto class of type III effectors. (*A*) The plant cell responds to a *hrp* mutant bacterium with a papilla-based extracellular defense by production and transport of cell wall and defense proteins through the secretory pathway. Extracellular defense compounds (blue triangles) and a large papilla beneath the *hrp* mutant bacterium inhibit the bacterial metabolism and/or produce a 'desolate zone' that isolates the *hrp* mutant bacterium from access to nutrients/water. Wavy lines above the cell wall (CW) indicate nutrients/water. Golgi: Golgi apparatus. ER: Endoplasmic reticulum. (*B*) Wild-type (WT) DC3000 delivers the AvrPto class of type III effectors (red circles) into the plant cell. Mechanism 1: Effectors suppress the extracellular secretory pathway, which could lead to feedback repression of the genes encoding secreted cell wall and defense proteins that enter this particular secretory pathway. Mechanism 2: Effectors directly inhibit the transcription of the genes encoding cell wall and defense proteins that are components of the papilla-based defense.

recognition by mutating these *avr* genes or evolve additional effector genes that mask *avr* gene recognition. It is possible that various defense mechanisms, as well as the actions of various effectors, may be interconnected at some level. However, the two classes of effectors appear to target different plant defenses. Therefore, elucidating the functions of both of these classes will be essential to our understanding of *P. syringae* pathogenesis and the different stages of virulence evolution in *P. syringae*.

The study of the functions of the 30 or more *Pst* DC3000 effectors has been thwarted by the typically weak contributions they individually make to virulence. Deletion of a single effector gene does not often lead to a noticeable loss of virulence. In most cases, the virulence contribution, as measured by attenuation of symptoms and bacterial growth, is subtle, which supports the concept that type III effectors, at the concentrations delivered by bacteria, contribute to virulence in a subtle or partially redundant manner (57, 58). Therefore, to efficiently study the functions of most type III effectors in *P. syringae* and other plant pathogenic bacteria, methods other than the traditional ones that measure bacterial populations or assess disease symptoms must be developed. Despite the apparent functional subtlety and redundancy of type III effectors when delivered by bacteria, we show here that transgenic expression of AvrPto alone, which likely results in a higher level of AvrPto in the plant cell than that delivered by bacteria during infection, could effectively substitute for the redundant/additive functions of a class of effectors in *Pst* DC3000 to modulate host gene expression, to effectively suppress the papilla-associated cell wall response, and to substantially enhance multiplication of nonpathogenic *hrp* mutant bacteria. Because TTSS suppression of cell wall-based defense is likely to be a common feature in plant pathogenic bacteria (29, 30), we believe that the global host gene expression, cytological examination, and transgenic expression methods used to identify AvrPto as a suppressor of this host defense will facilitate the functional study of type III effectors not only in *P. syringae* but also in other plant pathogenic bacteria.

Acknowledgements

We thank J. Walton and members of our laboratory for critical reading of the manuscript; R. Schaffer, J. Landgraf, M. Larson, C. Wilkerson, and E. Wisman for help with microarray and bioinformatic analyses; K. Osteryoung and T. Sanderfoot for assistance with callose analysis; and K. Bird for assistance in preparation of this paper. This work was supported by grants from the U.S. Department of Energy (to S.Y.H.), the U.S. National Science Foundation (to S.Y.H.), and the U.S. Department of Agriculture (to R.T.).

References

1. ALFANO, J. R. & COLLMER, A. (1997) The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. *J Bacteriol*, 179, 5655-5662.

2. BUTTNER, D. & BONAS, U. (2002) Getting across--bacterial type III effector proteins on their way to the plant cell. *EMBO J*, 21, 5313-5322.

3. CORNELIS, G. R. & VAN GIJSEGEM, F. (2000) Assembly and function of type III secretory systems. *Annu Rev Microbiol*, 54, 735-774.

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

5. LINDGREN, P. B. (1997) The role of hrp genes during plant-bacterial interactions. *Annu Rev Phytopathol*, 35, 129-152.

6. GALAN, J. E. & COLLMER, A. (1999) Type III secretion machines: bacterial devices for protein delivery into host cells. *Science*, 284, 1322-1328.

7. STASKAWICZ, B. J., MUDGETT, M. B., DANGL, J. L. & GALAN, J. E. (2001) Common and contrasting themes of plant and animal diseases. *Science*, 292, 2285-2289.

8. BOCH, J., JOARDAR, V., GAO, L., ROBERTSON, T. L., LIM, M. & KUNKEL, B. N. (2002) Identification of *Pseudomonas syringae* pv. *tomato* genes induced during infection of *Arabidopsis thaliana*. *Mol Microbiol*, 44, 73-88.

9. FOUTS, D. E., ABRAMOVITCH, R. B., ALFANO, J. R., BALDO, A. M., BUELL, C. R., CARTINHOUR, S., CHATTERJEE, A. K., D'ASCENZO, M., GWINN, M. L., LAZAROWITZ, S. G., LIN, N. C., MARTIN, G. B., REHM, A. H., SCHNEIDER, D. J., VAN DIJK, K., TANG, X. & COLLMER, A. (2002) Genomewide identification of *Pseudomonas syringae* pv. *tomato* DC3000 promoters controlled by the HrpL alternative sigma factor. *Proc. Natl. Acad. Sci. U S A*, 99, 2275-2280.

10. GUTTMAN, D. S., VINATZER, B. A., SARKAR, S. F., RANALL, M. V., KETTLER, G. & GREENBERG, J. T. (2002) A functional screen for the type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. *Science*, 295, 1722-1726.

11. PETNICKI-OCWIEJA, T., SCHNEIDER, D. J., TAM, V. C., CHANCEY, S. T., SHAN, L., JAMIR, Y., SCHECHTER, L. M., JANES, M. D., BUELL, C. R., TANG, X., COLLMER, A. & ALFANO, J. R. (2002) Genomewide identification of proteins secreted by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. U S A*, 99, 7652-7657.

12. SALANOUBAT, M., GENIN, S., ARTIGUENAVE, F., GOUZY, J., MANGENOT, S., ARLAT, M., BILLAULT, A., BROTTIER, P., CAMUS, J. C., CATTOLICO, L., CHANDLER, M., CHOISNE, N., CLAUDEL-RENARD, C., CUNNAC, S., DEMANGE, N., GASPIN, C., LAVIE, M., MOISAN, A., ROBERT, C., SAURIN, W., SCHIEX, T., SIGUIER, P., THEBAULT, P., WHALEN, M., WINCKER, P., LEVY, M., WEISSENBACH, J. & BOUCHER, C. A. (2002) Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature*, 415, 497-502.

13. ZWIESLER-VOLLICK, J., PLOVANICH-JONES, A. E., NOMURA, K., BANDYOPADHYAY, S., JOARDAR, V., KUNKEL, B. N. & HE, S. Y. (2002) Identification of novel hrp-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv. *tomato* DC3000 genome. *Mol Microbiol*, 45, 1207-1218.

14. KATAGIRI, F., THILMONY, R. & HE, S. Y. (2002) The *Arabidopsis thaliana-Pseudomonas syringae* interaction. *The Arabidopsis Book*, 1.

15. WHALEN, M. C., INNES, R. W., BENT, A. F. & STASKAWICZ, B. J. (1991) Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell*, 3, 49-59.

16. ROINE, E., WEI, W., YUAN, J., NURMIAHO-LASSILA, E. L., KALKKINEN, N., ROMANTSCHUK, M. & 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, 3459-3464.

17. YUAN, J. & HE, S. Y. (1996) The *Pseudomonas syringae* Hrp regulation and secretion system controls the production and secretion of multiple extracellular proteins. *J Bacteriol*, 178, 6399-6402.

18. GOODMAN, R. N. & NOVACKY, A. J. (1994) *The hypersensitive reaction in plants to pathogens: a resistance phenomenon* (St. Paul, Minn., APS Press).

19. GREENBERG, J. T. (1997) Programmed cell death in plant-pathogen interactions. *Annu Rev Plant Physiol Plant Mol Biol*, 48, 525-545.

20. LEACH, J. E. & WHITE, F. F. (1996) Bacterial avirulence genes. *Annu Rev Phytopathol*, 34, 153-179.

21. RONALD, P. C., SALMERON, J. M., CARLAND, F. M. & STASKAWICZ, B. J. (1992) The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. *J Bacteriol*, 174, 1604-1611.

22. SCOFIELD, S. R., TOBIAS, C. M., RATHJEN, J. P., CHANG, J. H., LAVELLE, D. T., MICHELMORE, R. W. & STASKAWICZ, B. J. (1996) Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science*, 274, 2063-2065.

23. TANG, X., XIE, M., KIM, Y. J., ZHOU, J., KLESSIG, D. F. & MARTIN, G. B. (1999) Overexpression of Pto activates defense responses and confers broad resistance. *Plant Cell*, 11, 15-29.

24. BENDER, C. L., ALARCON-CHAIDEZ, F. & GROSS, D. C. (1999) *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol Mol Biol Rev*, 63, 266-292.

25. MA, S. W., MORRIS, V. L. & CUPPELS, D. A. (1991) Characterization of a DNA region required for production of the phytotoxin coronatine by *Pseudomonas syringae pv tomato. Mol Plant Microbe Interact*, 4, 69-74.

26. MITTAL, S. & DAVIS, K. R. (1995) Role of the phytotoxin coronatine in the infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. *tomato*. *Mol Plant Microbe Interact*, 8, 165-171.

27. JAKOBEK, J. L. & LINDGREN, P. B. (1993) Generalized induction of defense responses in bean is not correlated with the induction of the hypersensitive reaction. *Plant Cell*, 5, 49-56.

28. JAKOBEK, J. L., SMITH, J. A. & LINDGREN, P. B. (1993) Suppression of bean defense responses by *Pseudomonas syringae*. *Plant Cell*, 5, 57-63.

29. BESTWICK, C. S., BENNETT, M. H. & MANSFIELD, J. W. (1995) Hrp mutant of *Pseudomonas syringae* pv *phaseolicola* induces cell wall alterations but not membrane damage leading to the hypersensitive reaction in lettuce. *Plant Physiol*, 108, 503-516.

30. BROWN, I., MANSFIELD, J. & BONAS, U. (1995) Hrp genes in *Xanthomonas campestris* pv *vesicatoria* determine ability to suppress papilla deposition in pepper mesophyll cells. *Mol Plant Microbe Interact*, 8, 825-836.

31. BROWN, I., TRETHOWAN, J., KERRY, M., MANSFIELD, J. & BOLWELL, G. P. (1998) Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains of *Xanthomonas campestris* and French bean mesophyll cells. *Plant J*, 15, 333-343.

32. SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989) *Molecular cloning: a laboratory manual* (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press).

33. WEI, W., PLOVANICH-JONES, A., DENG, W. L., JIN, Q. L., COLLMER, A., HUANG, H. C. & HE, S. Y. (2000) The gene coding for the Hrp pilus structural protein is required for type III secretion of Hrp and Avr proteins in *Pseudomonas syringae* pv. *tomato*. *Proc. Natl. Acad. Sci. U S A*, 97, 2247-2252.

34. AOYAMA, T. & CHUA, N. H. (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J*, 11, 605-612.

35. MCNELLIS, T. W., MUDGETT, M. B., LI, K., AOYAMA, T., HORVATH, D., CHUA, N. H. & STASKAWICZ, B. J. (1998) Glucocorticoid-inducible expression of a bacterial avirulence gene in transgenic *Arabidopsis* induces hypersensitive cell death. *Plant J*, 14, 247-257.

36. GOPALAN, S., BAUER, D. W., ALFANO, J. R., LONIELLO, A. O., HE, S. Y. & 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, 1095-1105.

37. SCHAFFER, R., LANDGRAF, J., ACCERBI, M., SIMON, V., LARSON, M. & WISMAN, E. (2001) Microarray analysis of diurnal and circadian-regulated genes in *Arabidopsis*. *Plant Cell*, 13, 113-123.

38. EISEN, M. B., SPELLMAN, P. T., BROWN, P. O. & BOTSTEIN, D. (1998) Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. U S A*, 95, 14863-14868.

39. EMANUELSSON, O., NIELSEN, H., BRUNAK, S. & VON HEIJNE, G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol*, 300, 1005-1016.

40. ADAM, L. & SOMERVILLE, S. C. (1996) Genetic characterization of five powdery mildew disease resistance loci in *Arabidopsis thaliana. Plant J.*, 9, 341-356.

41. ABRAMOVITCH, R. B., KIM, Y. J., CHEN, S., DICKMAN, M. B. & MARTIN, G. B. (2003) *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. *EMBO J*, 22, 60-69.

42. CHEN, Z., KLOEK, A. P., BOCH, J., KATAGIRI, F. & KUNKEL, B. N. (2000) The *Pseudomonas syringae avrRpt2* gene product promotes pathogen virulence from inside plant cells. *Mol Plant Microbe Interact*, 13, 1312-1321.

43. JACKSON, R. W., ATHANASSOPOULOS, E., TSIAMIS, G., MANSFIELD, J. W., SESMA, A., ARNOLD, D. L., GIBBON, M. J., MURILLO, J., TAYLOR, J. D. & VIVIAN, A. (1999) Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar *phaseolicola. Proc. Natl. Acad. Sci. U S A*, 96, 10875-10880.

44. MACKEY, D., HOLT, B. F., WIIG, A. & DANGL, J. L. (2002) RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in *Arabidopsis*. *Cell*, 108, 743-754.

45. TSIAMIS, G., MANSFIELD, J. W., HOCKENHULL, R., JACKSON, R. W., SESMA, A., ATHANASSOPOULOS, E., BENNETT, M. A., STEVENS, C., VIVIAN, A., TAYLOR, J. D. & MURILLO, J. (2000) Cultivar-specific avirulence and virulence functions assigned to avrPphF in *Pseudomonas syringae* pv. *phaseolicola*, the cause of bean halo-blight disease. *EMBO J*, 19, 3204-3214.

46. DELANEY, T. P., UKNES, S., VERNOOIJ, B., FRIEDRICH, L., WEYMANN, K., NEGROTTO, D., GAFFNEY, T., GUTRELLA, M., KESSMANN, H., WARD, E. & RYALS, J. (1994) A central role of salicylic-acid in plant-disease resistance. *Science*, 266, 1247-1250.

47. GUZMAN, P. & ECKER, J. R. (1990) Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. *Plant Cell*, 2, 513-523.

48. DANGL, J. L. & JONES, J. D. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, 411, 826-833.

49. PENALOZA-VAZQUEZ, A., PRESTON, G. M., COLLMER, A. & BENDER, C. L. (2000) Regulatory interactions between the Hrp type III protein secretion system and coronatine biosynthesis in *Pseudomonas syringae* pv. *tomato* DC3000. *Microbiol*, 146 (Pt 10), 2447-2456.

50. SCHWEIZER, P., CHRISTOFFEL, A. & DUDLER, R. (1999) Transient expression of members of the germin-like gene family in epidermal cells of wheat confers disease resistance. *Plant J*, 20, 541-552.

51. WEI, Y., ZHANG, Z., ANDERSEN, C. H., SCHMELZER, E., GREGERSEN, P. L., COLLINGE, D. B., SMEDEGAARD-PETERSEN, V. & THORDAL-CHRISTENSEN, H. (1998) An epidermis/papilla-specific oxalate oxidase-like protein in the defence response of barley attacked by the powdery mildew fungus. *Plant Mol Biol*, 36, 101-112.

52. MALDONADO, A. M., DOERNER, P., DIXON, R. A., LAMB, C. J. & CAMERON, R. K. (2002) A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. *Nature*, 419, 399-403.

53. MOLINA, A. & GARCIA-OLMEDO, F. (1997) Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. *Plant J*, 12, 669-675.

54. JAKOBEK, J. L. & LINDGREN, P. B. (2002) Expression of a bean acid phosphatase cDNA is correlated with disease resistance. *J Exp Bot*, 53, 387-389.

55. NAWRATH, C., HECK, S., PARINTHAWONG, N. & METRAUX, J. P. (2002) EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in *Arabidopsis*, is a member of the MATE transporter family. *Plant Cell*, 14, 275-286.

56. TAO, Y., XIE, Z., CHEN, W., GLAZEBROOK, J., CHANG, H. S., HAN, B., ZHU, T., ZOU, G. & KATAGIRI, F. (2003) Quantitative nature of *Arabidopsis* responses

during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell*, 15, 317-330.

57. COLLMER, A., LINDEBERG, M., PETNICKI-OCWIEJA, T., SCHNEIDER, D. J. & ALFANO, J. R. (2002) Genomic mining type III secretion system effectors in *Pseudomonas syringae* yields new picks for all TTSS prospectors. *Trends Microbiol*, 10, 462-469.

58. KJEMTRUP, S., NIMCHUK, Z. & DANGL, J. L. (2000) Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition. *Curr Opin Microbiol*, 3, 73-78.

59. JIN, Q. & HE, S. Y. (2001) Role of the Hrp pilus in type III protein secretion in *Pseudomonas syringae*. *Science*, 294, 2556-2558.

60. FANG, F. & VAZQUEZ-TORRES, A. (2002) Salmonella selectively stops traffic. *Trends Microbiol*, 10, 391-392.

61. JURIS, S. J., SHAO, F. & DIXON, J. E. (2002) Yersinia effectors target mammalian signalling pathways. *Cell Microbiol*, 4, 201-211.

62. BOGDANOVE, A. J. & MARTIN, G. B. (2000) AvrPto-dependent Pto-interacting proteins and AvrPto-interacting proteins in tomato. *Proc. Natl. Acad. Sci. U S A*, 97, 8836-8840.

63. PERANEN, J., AUVINEN, P., VIRTA, H., WEPF, R. & SIMONS, K. (1996) Rab8 promotes polarized membrane transport through reorganization of actin and microtubules in fibroblasts. *J Cell Biol*, 135, 153-167.

64. VAN DER BIEZEN, E. A. & JONES, J. D. (1998) Plant disease-resistance proteins and the gene-for-gene concept. *Trends Biochem Sci*, 23, 454-456.

65. AXTELL, M. J. & STASKAWICZ, B. J. (2003) Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. *Cell*, 112, 369-377.

66. MACKEY, D., BELKHADIR, Y., ALONSO, J. M., ECKER, J. R. & DANGL, J. L. (2003) *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell*, 112, 379-389.

Chapter 3. Further characterization of *avrPto* plants.

Abstract

Over-expression of AvrPto, an effector from *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), in *Arabidopsis* compromises defense-related callose deposition in the host cell wall. In this chapter, I describe results that show that over-expression of AvrPto enhances multiplication of several other bacterial strains, including *Pst* DC3000 (*avrRpt2*). *Pst* DC3000 (*avrRpt2*) normally triggers the hypersensitive response (HR) and resistance and induces the appearance of several proteins in the apoplast of *Arabidopsis* ecotype Col-0 plants. *avrPto*-expressing plants still undergo the HR similary to wild-type plants when inoculated with *Pst* DC3000 (*avrRpt2*). They do not, however, have the same proteins in their apoplastic space as Col-0 plants. These results suggest that HR is not sufficient to confer resistance to avirulent *P. syringae* in *avrPto* plants and that AvrPto interferes with secretion/leakage of host proteins induced by avirulent *P. syringae*.

Introduction

Formation of papillae, localized production of reactive oxygen species, and increased synthesis of compounds such as phytoalexins and extracellular pathogenesis-related (PR) proteins are known defense responses, but their efficacy in limiting pathogen multiplication is currently unknown. For a pathogen to be successful, it must be able to suppress or evade these defenses and to release nutrients from host cells. The process by which *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) overcomes plant defenses and obtains nutrients is not well understood. *Pst* DC3000 transfers a large number of effector proteins, via the type III secretion system, into host cells. In an incompatible host, some of these effectors are recognized by cognate *R* genes and this recognition leads to up-regulation of plant defenses and the hypersensitive response (HR). The HR is defined as a rapid and localized host cell death and is thought to restrict pathogen growth.

Several lines of evidence suggest that effectors are essential for virulence in compatible hosts. First, *hrp* mutants, which do not secrete type III effectors, do not multiply or cause disease symptoms in host plants. Second, involvement of several effectors in virulence has been demonstrated by various methods (1-4). However, the exact mechanisms by which effectors enable plant pathogenic bacteria to proliferate in the intercellular space of plant leaves and cause disease remains enigmatic.

The microarray analyses described in Chapter 2 showed that repression of host genes by type III effectors was biased towards host genes that encode secreted proteins. In fact, 42% of the repressed genes were predicted to be targeted for secretion. This biased repression suggests that host extracellular secretion is a target of *Pst* DC3000 type III effectors. Furthermore, expression of a single effector, AvrPto, in transgenic *Arabidopsis* plants, resulted in repression of 85% of those same genes. This result suggests that AvrPto may be involved in disrupting host secretion. In this chapter, I describe further characterization of *avrPto* plants to gain insights into the mechanism by which *avrPto* over-expression leads to increased bacterial growth.

Materials and Methods

Plant growth and bacterial enumeration

Arabidopsis thaliana accession Col-0 *gl1* plants and Col-0 *gl avrPto* transgenic plants (Chapter 2) were grown in soil in growth chambers with a day/night cycle of 12 h/12 h, a light intensity of 100 µE, and a constant temperature of 20°C. Four- to five-week-old plants were used for experiments. Bacteria were grown in low-salt Luria–Bertani broth (5, 6) to the mid- to late-logarithmic phase at 30°C. Bacterial cultures were centrifuged to recover bacteria, and the pellets were re-suspended in sterile water to a final OD_{600} of 0.002 [equivalent to 1×10^6 colony-forming units (CFU)/ml]. Fully expanded leaves were infiltrated with bacterial suspensions, and bacteria were enumerated as described by Katagiri *et al.* (5). The mean values of the bacterial populations are plotted with the standard deviation displayed as error. Plants analyzed in Figure 3-1 were sprayed daily with a 30-µM dexamethasone solution (7, 8) containing 0.02% Silwet L-77 (Osi Specialties, Friendship, WV). Bacterial suspensions were infiltrated into leaves 24 h after the first dexamethasone treatment.

Callose staining

Arabidopsis leaves were sprayed with 30 µM dexamethasone (7, 8) and then infiltrated 24 h later with a bacterial suspension of $OD_{600} = 0.2$ (1×10^8 CFU/ml). Leaves were harvested 6 h after bacterial infiltration, cleared, and stained with aniline blue for callose as previously described (9). Leaves were examined with a Leica DM RA2 microscope with an A4 fluorescence cube. The number of callose deposits was determined with QUANTITY ONE software (Bio-Rad). More than 10 adjacent fields of view along the length of the leaf (not including the mid-vein or leaf edge) were analyzed and averaged. The values in Table 1 are the average and standard deviation of more than five independent leaves for each treatment.

Immunoblotting

Approximately 9 mg of tissue was homogenized in 90 ul 2 x loading buffer (0.125M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol) and denatured at 100°C for 10 minutes. An equal volume of each sample was separated on a 15 % SDS-polyacrylamide gel (6) and proteins were transferred onto Immobilon-P membrane (Millipore #IPVH00010 Bedford, MA) using a semi-dry apparatus (SEMI PHOR, Hoefer Scientific Instruments, San Francisco, CA). Immunoblotting was carried out using PR1 and PR5 antisera and anti-rabbit alkaline phosphatase conjugate. Protein bands were visualized by SIGMA FAST (Sigma B5655 St. Louis, MO)

Secretion assays

Arabidopsis leaves were sprayed with 30 µM dexamethasone (7, 8) and then infiltrated 24 h later with a bacterial suspension of $OD_{600} = 0.2$ (1×10^8 CFU/ml). Plants were then incubated for 6 h under low humidity, excised from pots, and vacuum-infiltrated with water containing 0.004% Silwet L-77 (Osi Specialties, Friendship, WV). Excess water was removed from leaves and the tissue was centrifuged at 400 g for 20 minutes. Intercellular wash fluid (IWF) was collected and stored at -20°C. Samples were mixed with 2 x loading buffer, denatured at 100°C for 10 minutes. Protein samples were then separated on a 15% SDS-PAGE gel, which was then stained with Coomassie Blue.

Results

Enhanced growth of *Pseudomonas fluorescens* 55 and *Pst* DC3000 (*avrRpt2*) in *avrPto* plants

It was previously shown that over-expression of AvrPto in *Arabidopsis* plants allowed the growth of the *hrcC* mutant (Chapter 2). *hrc/hrp* mutants behave similarly to the vast majority of non-plant pathogenic bacteria found in nature. To determine if a similar result would be obtained with a non-phytopathogenic bacterium, the growth of a saprophyte, *Pseudomonas fluorescens* 55 (*Pf* 55), was assayed in *avrPto* plants. Figure 3-1a shows that this strain was able to multiply almost 600-fold in *avrPto* plants, whereas the population decreased in wild-type plants. Next, I examined whether an avirulent strain of *Pst* DC3000 would be able to multiply to higher levels in *avrPto* plants than in wild-type plants. Indeed, *avrPto* plants allowed over 900-fold more growth of *Pst* DC3000 (*avrRpt2*) than did wild-type plants (Figure 3-1b).

AvrPto does not inhibit the HR triggered by AvrRpt2

The resistance of *Arabidopsis* to *Pst* DC3000 (*avrRpt2*) is mediated by the corresponding *R* gene *RPS2,* and is associated with the HR. I investigated whether the HR was altered in *avrPto* plants, which have a functional *RPS2* gene. There was little difference in the HR of *avrPto* and wild-type plants to *Pst* DC3000 (*avrRpt2*) (Figure 3-2), although *avrPto* plants collapsed slightly earlier than wild-type plants.

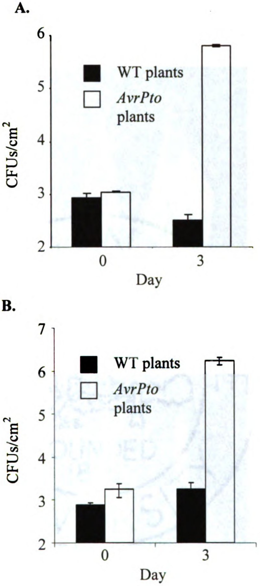


Figure 3-1. Growth of *Pf* 55 and *Pst* DC3000 (*avrRpt2*) in *avrPto*-expressing plants. Multiplication of *Pf* 55 (**A**) and *Pst* DC3000 (*avrRpt2*) (**B**) in wild-type (WT) plants and *avrPto*-expressing plants. The growth of *Pf* 55 and *Pst* DC3000 (*avrRpt2*) was significantly greater (approximately 500-fold and 900-fold, respectively) in *avrPto*-expressing lines than in wild-type (WT) plants.

Wild-type leaves

avrPto leaves

Figure 3-2. *avrPto* leaves after infiltration of *Pst* DC3000 (*avrRpt2*). Leaves from wild-type and *avrPto* plants 7 hours after infiltration with 2×10^6 CFU/ml of *Pst* DC3000 (*avrRpt2*). HR is evident in both the wild-type and *avrPto* leaves.

Callose deposition upon *Pf* 55 and *Pst* DC3000 (*avrRpt2*) infection is compromised in *avrPto* transgenic plants

In Chapter 2, it was shown that *avrPto* plants are unable to deposit callose in response to the *hrcC* mutant. Here I wanted to examine whether *avrPto* plants would be compromised in the callose response to *Pf* 55 and *Pst* DC3000 (*avrRpt2*). As shown in Table 3-1, the *hrpA* mutant, *Pf* 55, and *Pst* DC3000 (*avrRpt2*), elicited 180-fold, 34.2-fold 74.3-fold, respectively, more callose deposits in wild-type plants than in *avrPto* plants. As expected, *Pst* DC3000 elicited very low numbers of callose deposits in either wild-type or *avrPto* plants.

Pst DC3000 (*avrRpt2*) triggers the appearance of several proteins in the IWF of wild-type plants, but not in *avrPto* plants

As described in Chapter 2, a large percentage of genes that were expressed at lower levels in *avrPto* plants compared to wild-type plants encode proteins predicted to be secreted. The proteins in the IWF of wild-type and *avrPto* plants were examined by separation on SDS-PAGE gels and staining with Coomassie Blue. It was discovered that *Pst* DC3000 (*avrRpt2*), but not *Pst* DC3000, elicited the appearance of several proteins in the IWF of wild-type plants (Figure 3-3a). Furthermore, these proteins were not detected in the IWF of *avrPto* plants (Figure 3-3a). To determine whether these apoplastic proteins were PR proteins, immunoblot analyses using PR1 and PR5 antibodies were conducted. There was no difference in the amount of PR-1 or PR-5 in the apoplastic fluid from wild-type and *avrPto* plants (Figure 3b and c).

Table 3-1. Average callose deposition in wild-type and *avrPto* plants 6 hours after inoculation with *Pst* DC3000, *hrpA* mutants, *Pf 55*, or *Pst* DC3000 (*avrRpt2*).

	Wild-type [1]	*avrPto* plants [1]
Pst DC3000	9.9 ± 3.4	2.3 ± 0.8
hrpA mutant	253.3 ± 35.5	1.4 ± 0.7
Pf 55	273.4 ± 40.1	8.0 ± 5.5
Pst DC3000 (*avrRpt2*)	207.9 ± 91.6	2.8 ± 1.6

[1] Average callose depositions per field of view with standard deviation displayed as error.

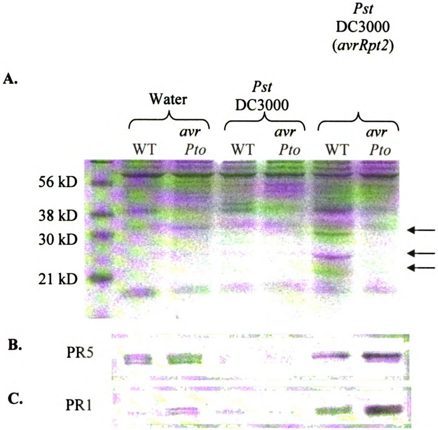


Figure 3-3. Protein profiles of the IWFs from *avrPto* plants inoculated with water, *Pst* DC3000 or *Pst* DC3000 (*avrRpt2*). **A.** Coomassie stained SDS-PAGE gel showing that *Pst* DC3000 (*avrRpt2*), but not *Pst* DC3000, induces the appearance of host proteins (indicated by arrows) in the IWF of wild-type (WT) plants. Neither strain triggers the appearance of these proteins in *avrPto* plants. Western blots of **B.** PR5 and **C.** PR1 show that PR expression in wild-type and *avrPto* plants is similar and thus, cannot be the proteins whose appearance is triggered by *Pst* DC3000 (*avrRpt2*). The level of expression induced by different bacterial strains is variable between different experiments.

The IWF of *avrPto* plants does not support more bacterial growth than that of wild-type plants.

To determine whether the IWF of *avrPto* plants contains more nutrients and can, therefore, support bacterial growth, the IWFs from these plants were collected and inoculated with the *hrcC* mutant. The *hrcC* mutant grew over 1,600-fold more in the IWF from wild-type plants than in water. This level of growth is comparable, only 6-fold less than the amount of growth observed in LB (Figure 3-4a). The growth of *hrcC* was similar in the IWFs from wild-type and *avrPto* plants (Figure 3-4b).

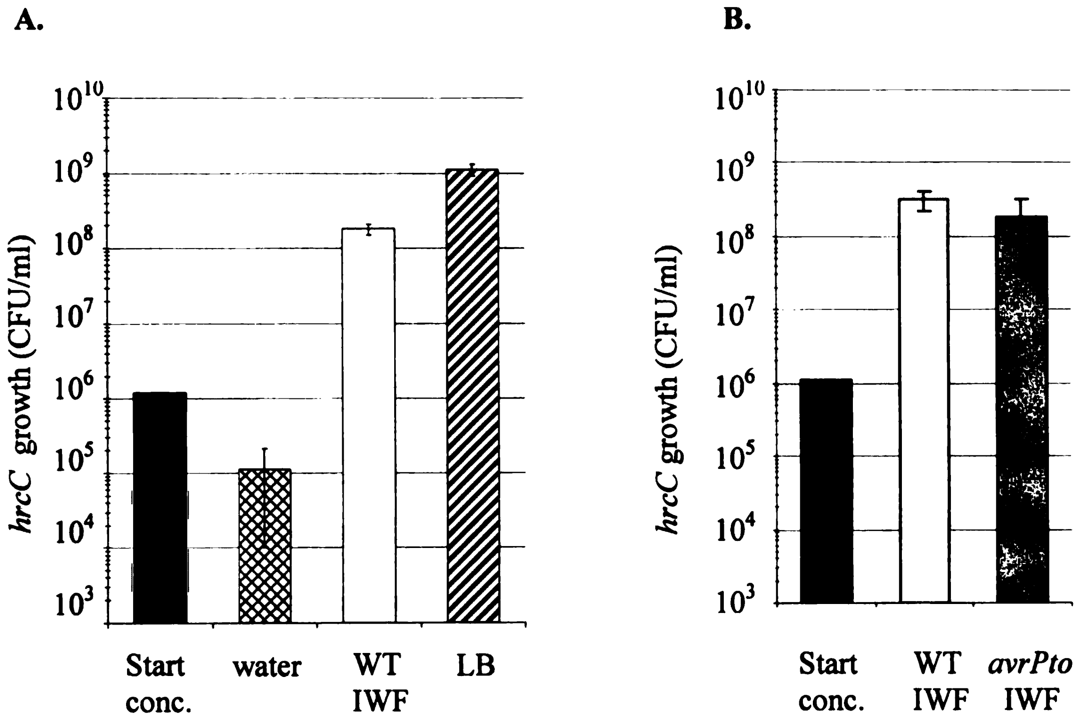


Figure 3-4. Growth of the *hrcC* mutant in the IWF from wild-type (WT) and *avrPto* plants. Growth was assayed after ~ 24 hr incubation. **A.** The growth of the *hrcC* mutant in the IWF from WT plants was significantly greater than in water and comparable to that in LB media. **B.** The growth of the *hrcC* mutant in the IWF from WT plants and *avrPto* plants was not significantly different.

Discussion

In Chapter 2 I demonstrated that the *hrcC* mutant is able to multiply significantly higher in *avrPto* plants than in wild-type plants. In this study, I wanted to determine if other non-virulent bacteria would multiply in *avrPto*-expressing plants as well. I show that, besides *hrcC*, *avrPto* plants also allowed a significant increase in growth of the non-phytopathogenic bacterium *Pf* 55, and the normally avirulent strain *Pst* DC3000 (*avrRpt2*). I examined several possible mechanisms for the increased growth of *Pst* DC3000 (*avrRpt2*) in *avrPto* plants. *avrPto* plants may be compromised in the HR, cell-wall based defenses, or the secretion of defense proteins, and/or *avrPto* plants may be leaking nutrients into the apoplastic space.

To test the hypothesis that AvrPto may suppress the HR, inoculated *avrPto* plants with *Pst* DC3000 (*avrRpt2*). *Pst* DC3000 (*avrRpt2*) caused a normal HR when inoculated into *avrPto* plants. Thus HR was uncoupled from resistance in *avrPto* plants. My finding adds to a growing list of studies that show uncoupling of HR from disease resistance in different pathosystems (2, 10-12). I conclude that the increased susceptibility of *avrPto* plants to *Pst* DC3000 (*avrRpt2*) is not correlated with loss of the HR.

In contrast, I found a correlation of increased growth with suppression of callose deposition and the absence of several extracellular proteins in the apoplast of *avrPto* plants. I observed that the *hrpA* mutant, *Pf* 55 and *Pst* DC3000 (*avrRpt2*) elicited high levels of callose deposition in wild-type plants, but not in *avrPto* plants. Callose deposition is a marker for papilla formation, which is believed to require an intact host secretion system. In addition, I found that four protein bands (between 21 and 30 kD) were present in the IWF of wild-type plants infected with *Pst* DC3000 (*avrRpt2*). These proteins were neither detected in the IWF from *avrPto* plants after treatment with *Pst* DC3000 (*avrRpt2*), nor from wild-type plants treated with *Pst* DC3000. These results support the hypothesis that AvrPto disrupts extracellular secretion in the host.

A previous study showed that the apoplastic fluid of plants treated with a salicylic acid analog, 2, 6-dichlorolisonicotinic acid (INA), contains 3 proteins: PR-1 (16 kD), PR-5 (26 kD), and PR-2 (37 kD). These bands were absent in the apoplastic fluid from control plants treated with water (13). Since the sizes of PR proteins are similar to those proteins found in the apoplast of *Pst* DC3000 (*avrRpt2*) inoculated wild-type plants, western blot analyses were conducted to assay for the presence of PR proteins. There were no differences in the abundance of PR-1 or PR-5 in the IWF of wild-type plants compared to that of *avrPto* plants. It is possible that there are multiple protein secretion pathways in the *Arabidopsis* cell and the one involved in PR protein secretion may be different from the one responsible for secreting the proteins elicited by *Pst* DC3000 (*avrRpt2*). The PR secretion pathway may not be affected by AvrPto.

The identity of the proteins found in the IWF of wild-type plants inoculated with *Pst* DC3000 (*avrRpt2*) is being determined. Preliminary results indicate that these proteins include plastocyanin, calmodulin, and an oxygen-evolving enhancer protein 3 (data not shown). It is unknown, yet, if these apparently chloroplastic proteins are involved in the resistance response. In can be concluded, however, that the appearance of these proteins in the apoplast is not necessary for the HR.

Alternatively, it is possible that *Pst* DC3000 (*avrRpt2*) causes non-specific leakage in both wild-type and *avrPto* plants, but the expression of these proteins could be lower in *avrPto* plants than in wild-type plants. In this case, the proteins were detected only in the *Pst* DC3000 (*avrRpt2*) inoculated wild-type plants because they were more abundant there. This possibility is being explored using antibodies against plastocyanin (kindly provided by R. B. Klösgen). However, the background bands in all treatments appeared to be similar, arguing against a general non-specific leakage. Furthermore, RabE, an intracellular protein, was not detected in our IWF by immunoblotting (E. Bray Speth, unpublished data).

Besides blocking secretion of potential defense compounds, AvrPto could cause leakage of nutrients into the apoplast, thus promoting bacterial multiplication. To test this hypothesis, I inoculated the IWF from non-inoculated plants with the *hrcC* mutant. This strain was able to grow equally well in the IWF from *avrPto* and wild-type plants. Therefore, I can conclude that there are abundant water-extractable nutrients available in the apoplast of *Arabidopsis* leaves and that the level is the same in both wild-type and *avrPto* plants. It is important to note that nutrients in the leaf could be inaccessible to the bacteria under natural conditions, but are released to the fluid during our experimental procedure. In addition, the apoplastic fluid assay does not address whether water is limiting in the leaf. *avrPto* plants may cause leakage of water and this may be sufficient to allow growth of non-virulent strains of bacteria. However, I did not observe any water soaking in uninoculated *avrPto* plants. Lastly, these IWF experiments suggest that there is no difference in effective and stable, water-extractable, antimicrobial compounds in the IWF from *avrPto* plants or wild-type plants.

In summary, my microarray, callose staining, and IWF experiments conducted with *avrPto* plants support the hypothesis that AvrPto contributes to virulence by interfering with a host's extracellular secretion system. In addition, yeast-two-hybrid results (Appendix A) revealed that AvrPto interacts with RabE family members. Rabs are small GTPases that are putatively involved in extracellular protein secretion. Future experiments using transgenic plants that over-express constitutively active, dominant-negative, and wild-type versions of RabE will further test the hypothesis that AvrPto contributes to virulence by disrupting a secretion pathway necessary for successful plant resistance.

Acknowledgements:

I thank Ralph Bernd Klösgen (Martin-Luther-Universität Halle-Wittenberg, Germany for providing plastocyanin antibody.

References

1. Chang, J. H., Rathjen, J. P., Bernal, A. J., Staskawicz, B. J. & Michelmore, R. W. (2000) avrPto enhances growth and necrosis caused by *Pseudomonas syringae* pv.*tomato* in tomato lines lacking either Pto or Prf. *Mol Plant Microbe Interact*, 13, 568-571.

2. Chen, Z., Kloek, A. P., Boch, J., Katagiri, F. & Kunkel, B. N. (2000) The *Pseudomonas syringae avrRpt2* gene product promotes pathogen virulence from inside plant cells. *Mol Plant Microbe Interact*, 13, 1312-1321.

3. Chen, Z., Kloek, A. P., Cuzick, A., Moeder, W., Tang, D., Innes, R. W., Klessig, D. F., McDowell, J. M. & Kunkel, B. N. (2004) The *Pseudomonas syringae* type III effector AvrRpt2 functions downstream or independently of SA to promote virulence on *Arabidopsis thaliana. Plant J*, 37, 494-504.

4. Shan, L., He, P., Zhou, J. M. & Tang, X. (2000) A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto. *Mol Plant Microbe Interact*, 13, 592-598.

5. Katagiri, F., Thilmony, R. & He, S. Y. (2002) The *Arabidopsis thaliana-Pseudomonas syringae* interaction. *The Arabidopsis Book*, 1.

6. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual* (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press).

7. Aoyama, T. & Chua, N. H. (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J*, 11, 605-612.

8. McNellis, T. W., Mudgett, M. B., Li, K., Aoyama, T., Horvath, D., Chua, N. H. & Staskawicz, B. J. (1998) Glucocorticoid-inducible expression of a bacterial avirulence gene in transgenic *Arabidopsis* induces hypersensitive cell death. *Plant J*, 14, 247-257.

9. Adam, L. & Somerville, S. C. (1996) Genetic characterization of five powdery mildew disease resistance loci in *Arabidopsis thaliana. Plant J.*, 9, 341-356.

10. YU, I. C., PARKER, J. & BENT, A. F. (1998) Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis dnd1* mutant. *Proc. Natl. Acad. Sci. U S A*, 95, 7819-7824.

11. CENTURY, K. S., HOLUB, E. B. & STASKAWICZ, B. J. (1995) *NDR1*, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. *Proc. Natl. Acad. Sci. U S A*, 92, 6597-6601.

12. LU, M., TANG, X. & ZHOU, J. M. (2001) *Arabidopsis NHO1* is required for general resistance against *Pseudomonas* bacteria. *Plant Cell*, 13, 437-447.

13. UKNES, S., MAUCH-MANI, B., MOYER, M., POTTER, S., WILLIAMS, S., DINCHER, S., CHANDLER, D., SLUSARENKO, A., WARD, E. & RYALS, J. (1992) Acquired resistance in *Arabidopsis*. *Plant Cell*, 4, 645-656.

Chapter 4: Characterization of an *Arabidopsis thaliana* mutant, *noc1*, with altered symptom development in response to *Pseudomonas syringae* pv. *tomato* DC3000 infection

Abstract

Very little is known about the molecular basis of the development of specific disease-associated symptoms in plants. In this study, approximately 10,000 ethylmethane sulfonate-mutagenized *A. thaliana* ecotype Columbia *gl1* plants were screened for reduced disease symptom development in response to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) infection. A recessive mutation, *noc1* (no chlorosis), caused a defect specifically in chlorosis development, while *Pst* DC3000 multiplication and necrosis development remained normal. In wild-type plants, the abundance of chlorophyll *a* and *b* decreased after infection with *Pst* DC3000. The total amount of chlorophyll in the *noc1* mutant, however, remained relatively unchanged after infection with *Pst* DC3000. Although jasmonic acid (JA) and ethylene have been implicated in chlorosis, the *noc1* mutant was not impaired in its response to JA or ethylene. Linkage mapping revealed that the mutation was located in a 619-kb region between At4g22340 and At4g24050 on the long arm of chromosome 4.

Introduction

Understanding plant-pathogen interactions is vital for our future ability to improve resistance in crop plants. *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) causes speck disease in *Arabidopsis* and tomato, characterized by bacterial multiplication and the progressive appearance of symptoms in the infected leaves. Typically, the appearance of water-soaking is followed by chlorosis (yellowing of the tissue) and ultimately necrosis (cell death) in the infected leaves. The molecular basis for these symptoms is unknown.

Chlorosis is caused by chlorophyll breakdown, one of the events accompanying plant senescence. Chlorophyll can be degraded via two pathways: an oxygen-dependent (or oxidative bleaching) pathway and an oxygen-independent pathway (1). The existence of the oxygen-dependent pathway is controversial (2) and thus will not be discussed here. The oxygen-independent pathway (Figure 4-1) is the generally accepted pathway and is also known as the "chlorophyllase pathway" because the first step is catalyzed by chlorophyllase, which converts chlorophyll into chlorophyllide. Mg-dechelatase converts chlorophyllide into pheophorbide, which is then either converted into pyropheophorbide by pheophorbidase or into a red chlorophyll catabolite by pheophorbide oxygenase. Cleavage of the chlorophyll tetrapyrrolic ring by pheophorbide oxygenase causes the loss of green color in downstream products. Only one gene encoding an enzyme upstream of pheophorbide oxygenase has been cloned from *Arabidopsis*. Expression of this gene, *AtCLH1* (chlorophyll-chlorophyllido hydroxylase) (3), is up-regulated by ethylene (4), wounding, methyl jasmonate (MeJA) or jasmonic acid (JA), and coronatine, which is a toxin produced by *Pst* DC3000 (5).

"Stay-green" plants are traditionally considered delayed-senescence variants, in which degradation of chlorophyll and the photosynthetic apparatus is partially or completely prevented (6). There are at least four different categories of stay-green plants (6, 7). In one type, senescence is initiated late, but then proceeds at a normal rate. In the second type, senescence is initiated on time, but proceeds at a slower rate. In the third class, senescence proceeds normally, but chlorophyll is retained indefinitely. In the fourth class, plants stay green when they are killed rapidly by freezing, boiling, or drying (6, 7). Some stay-green plants are a result of a combination of these types. Stay-green cereals are economically important because they carry out photosynthesis for a longer period of time, which results in an increase in yield (7).

For unknown reasons, most stay-green mutants impaired in chlorophyll catabolism are deficient at the ring-opening step catalyzed by pheophorbide oxygenase [i.e. *sid* from *Festuca pratensis* (8) and the stay-green mutant of *Lolium temulentum* (9)]. Stay-green mutants not affected in chlorophyll catabolism have been described as well. The cause of one soybean stay-green phenotype is a maternally inherited *cytG* mutation, which renders chlorophyll *b* more stable than chlorophyll *a* (10).

Oh *et al.* (11) conducted a screen to find *Arabidopsis* mutants with delayed senescence. They found eleven mutants that exhibited delayed loss of chlorophyll content (*ore1-11*). Loss of photochemical efficiency, however, was only delayed in *ore1, 2, 3* and *9,* but not in *ore10* or *11*. *ore2* and *ore3* were found to be allelic to a previously isolated mutant, *ein2* (ethylene-insensitive). EIN2 is an integral membrane protein that acts downstream of the ethylene receptors and upstream of the gene transcription changes associated with the ethylene response (12). *ORE9* encodes an F-box protein that has been suggested to target negative regulators of senescence for ubiquitin-mediated degradation (13). In *ore10* and *ore11* mutants, all chlorophyll-containing protein complexes [LHCI (light harvesting complex I), PSI (photosystem I) reaction center, PSII (photosystem II) reaction center] except for LHCII are degraded. This result suggests that chlorophyll stability in these non-photosynthetic stay-green mutants is due to defects in their proteolytic pathways (14). The response of the stay-green and *ore* mutants to *Pst* DC3000 infection is not known.

In this study, an *Arabidopsis* mutant, no-chlorosis 1 (*noc1*), showing altered chlorosis development following inoculation with *Pst* DC3000, was isolated and characterized. Infected *noc1* leaves do not develop chlorosis; they do, however, develop normal disease-associated necrosis and permit normal levels of *Pst* DC3000 multiplication. Uncoupling of chlorosis and necrosis has not previously been described. Characterization of this mutation should lend insight into the molecular basis of disease-related chlorosis, which is common to several plant diseases.

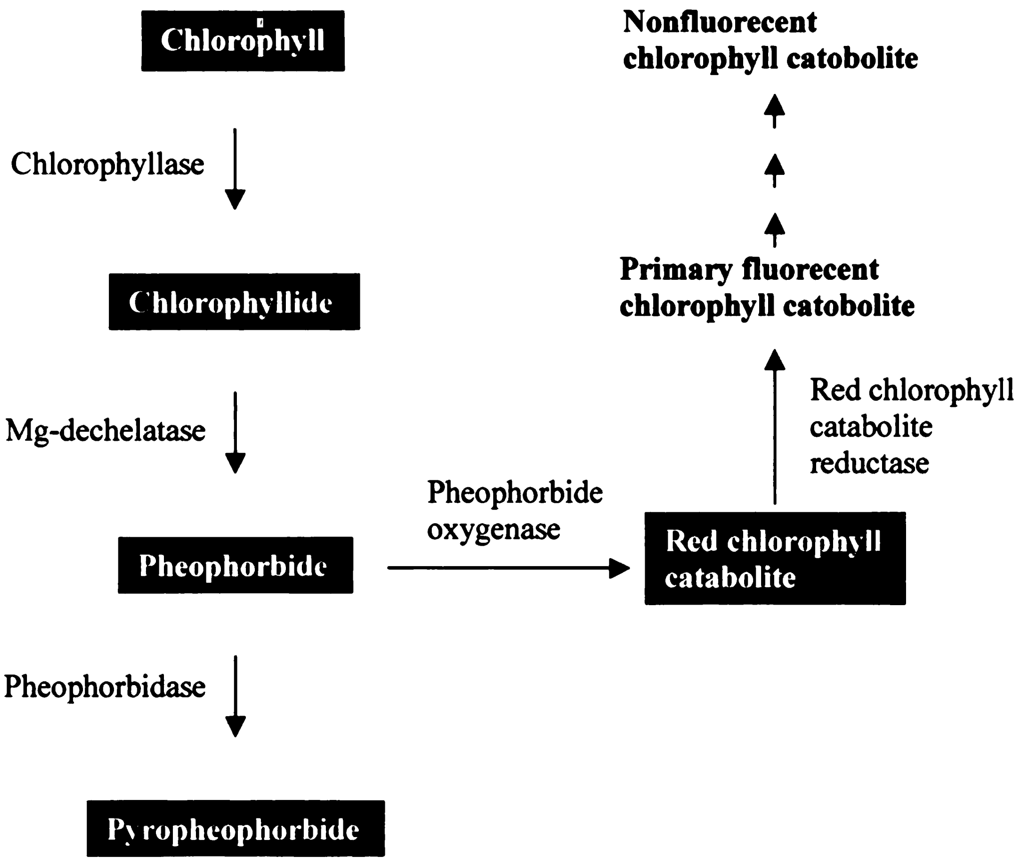


Figure 4-1. The chlorophyll degradation pathway. Simplified model of the steps involved in chlorophyll catabolism in higher plants. Products upstream of the ring cleavage step catalyzed Pheophorbide oxygenase are green and indicated as green boxes.

Methods

Plant material, mutagenesis, and growth conditions

Approximately 1g of *Arabidopsis thaliana* ecotype Columbia *gl1* seeds was mixed with 100 ml of distilled water and 250 µl of ethylmethane sulfonate (EMS). The mixture was incubated overnight at room temperature in the dark with gentle agitation. The seeds were washed six times with 500 ml of distilled water, resuspended in 300 ml of 0.1% agarose and sown onto a soil mixture (equal portions of Bacto high porosity professional plant mix, perlite, and vermiculite, covered with a thin layer of fine vermiculite). The flats were covered with lids and incubated in the dark at 4°C for three days. The flats were then transferred to a growth chamber [20°C with 12 hours of fluorescent light (100 µEinsteins/m^2/sec) and 12 hours of darkness] until the end of the life cycle. The plants were self-fertilized for two generations to create a population of M2 plants.

Screening and isolation of *Arabidopsis* mutants

Four to six-week-old M2 plants were dipped in a 1x10^8 CFU/ml suspension of *Pst* DC3000 and 0.05% Silwet L-77 (Osi Specialties, Friendship, WV) for two to three seconds. The inoculated plants were incubated in high (80-90%) humidity conditions for 96 hours and screened for a lack of symptom development.

Bacteria enumeration in infiltrated leaves of *noc1* mutants and wild-type plants

Four- to five-week-old plants were used for bacteria enumeration. *Pst* DC3000 was grown in low-salt Luria–Bertani broth (15, 16) to the mid- to late-logarithmic phase at 30°C. Bacterial cultures were pelleted and resuspended in sterile water to a final OD_{600} of 0.002 [equivalent to 1 x 10^6 colony-forming units (CFU)/ml]. Fully expanded leaves were vacuum-infiltrated with bacterial suspensions, and bacteria were enumerated as described by Katagiri *et al.* (15).

RNA isolation and northern blotting

RNA isolation from leaf tissue and Northern blotting were conducted as described by DebRoy *et al.* (17). Tissue was collected at 0, 24, and 48 hours-post-infiltration with *Pst* DC3000 and frozen in liquid nitrogen. RNA was isolated using the Promega RNAgents kit (Cat#Z5110) according to manufacturer's instructions. About 20 µg of RNA was denatured with two volumes of loading buffer (500 µl formamide, 170 µl formaldehyde, 100 µl 10X MOPS buffer, and 10 µl of 1 mg/ml ethidium bromide) for 10 minutes at 65°C. The RNA was separated on a formaldehyde agarose gel and transferred onto a nylon membrane (Hybond N+; Amersham Pharmacia Biotech #RPN303B) via capillary transfer (16). The membrane was hybridized overnight at 60°C in 20 ml Church's buffer [1% crystalline BSA (fraction V), 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, 7% SDS] and 600 µl denatured salmon sperm DNA. Approximately 100 ng of *AtCLH1* DNA was labeled with ^{32}P CTP and purified using a BIORAD column (Cat #732-6223) according to the manufacturer's instructions. The radiolabeled DNA probe was then added to the Church's buffer and the membrane was incubated with the probe for 16 hours at 60°C. Membranes were washed to a stringency of 0.5X

SSC (10 minutes at 60°C) and exposed to X-ray film (Kodak Scientific Imaging film X-OMAT AR#1651454).

Microarray analysis

Microarray analysis was conducted as described by Hauck *et al.* (18). RNA from 5-week-old wild-type and *noc1* plants, taken at 24 and 48 hours post infiltration with 2×10^6 cells/mL, were pooled. The custom microarray slides used were printed with approximately 600 non-redundant *A. thaliana* ESTs, found to be reproducibly differentially regulated during the compatible *A. thaliana-Pst* DC3000 interaction (Thilmony and He, unpublished data), were used. The subarray was derived from the *Arabidopsis* Functional Genomic Consortium's microarray slides, which contain about 7,200 unique genes (19).

Chlorophyll extraction

Chlorophyll abundance assays were performed using leaf tissue infiltrated with 2×10^6 CFU/mL *Pst* DC3000 and samples were collected at 0, 24, 48, 72 and 96 hours post-inoculation. All chlorophyll extraction steps were conducted in near darkness. Leaf disks from four separate leaves at each time point were frozen in liquid nitrogen and stored at –80°C. The frozen tissue was homogenized in 600 µl of 80% acetone. The tubes were centrifuged at 500 x g for three minutes at 4°C and the supernatant was transferred to a new tube and kept on ice. The absorbance of four dilutions (1:10, 1:5, 1:3, and 1:2.5) of each sample was determined using a spectrophotometer. The amount of chlorophyll was calculated as previously described (20).

MeJA sensitivity assay

Seeds were vapor-sterilized by incubation in a sealed container with a beaker containing 100 ml of bleach (6.15% sodium hypochlorite) and 3 ml of concentrated HCl for 16 hours. The seeds were then sown on *Arabidopsis* germination media [4.3 g/L Murashige-Skoog salts (Invitrogen), 30 g/L sucrose (J.T. Baker), 0.5 g/L MES (Sigma), pH 5.7] containing 50 µM MeJA (21). Seeds were placed in the dark at 4°C for three days and then transferred to the growth chamber. After one week, the seedlings were analyzed for their response to MeJA.

Ethylene sensitivity assay

Seeds were sown on 3 mm Whatman paper (Whatman International Ltd. Maidstone, England) moistened with distilled water, and incubated in the dark at 4°C for three days. The seedlings were then placed in a desiccator with 10 µl/L of ethylene and incubated in continuous darkness at 20 °C for four days (22).

Gene mapping

Mapping of the *noc1* gene was conducted as described by Lukowitz, Gillmor and Scheible (23). Initial genome-wide screening was conducted using an array of primers (Invitrogen Carlsbad, CA) to detect simple sequence length polymorphisms (SSLPs) from each chromosome. Additional SSLPs identified in the Monsanto

Arabidopsis Polymorphism and Ler sequence collection (St. Louis, MO) were used to further define the region containing the *noc1* mutation (24).

Results

Identification of the *noc1* mutant

Approximately 10,000 EMS-mutagenized *A. thaliana* ecotype Columbia *gl1* plants were screened for altered symptom development after dipping the plants in a suspension of *Pst* DC3000. One mutant isolated from this screen, *noc1* (no-chlorosis), was found to be defective in chlorosis development. *noc1* leaves remained green while wild-type leaves began to show chlorosis between 48 and 72 hours after inoculation (Figure 4-2). The severity and timing of water soaking and necrosis (24 hours and 96 hours post inoculation, respectively) were similar in both *noc1* and wild-type plants. There were no noticeable differences between wild-type and *noc1* plants in size, morphology, growth, or development, and the initiation or rate of senescence. Although *noc1* has altered symptom development, bacterial multiplication in *noc1* plants was not statistically different from that in wild-type plants (Figure 4-3). This result indicates that in the *noc1* plants, bacterial growth and chlorosis symptom production were uncoupled.

The decrease in total chlorophyll level is greater in wild-type plants than in *noc1* plants after infection with *Pst* DC3000

To determine whether the chlorotic response to *Pst* DC3000 infection was due to chlorophyll degradation, a chlorophyll abundance assay was conducted using tissue infiltrated with $2x10^6$ CFU/mL of *Pst* DC3000 and collected at 0, 24, 48, 72 and 96 hours post-inoculation. The results from one representative experiment are shown in Figure 4-4. Prior to inoculation with *Pst* DC3000, *noc1* and wild-type plants had approximately equal amounts of total chlorophyll (27.1 mg/cm^2 and 28.5 mg/cm^2, respectively). Wild-type plants began to lose chlorophyll by 24 hours post-inoculation and continued through 96 hours post-inoculation. *noc1* plants, on the other hand, did not begin to lose chlorophyll until after 48 hours post-inoculation. At 72 hours, *noc1* plants contained more than twice as much total chlorophyll as wild-type plants (26.8 mg/cm^2 in *noc1* plants *vs.* 12.4 mg/cm^2 in wild-type plants). This experiment demonstrates that wild-type plants lose chlorophyll faster than *noc1* plants after *Pst* DC3000 infection.

The expression of one gene, *AtCLH1*, in the chlorophyll degradation pathway is slightly reduced in *noc1* plants

One possible explanation for the greater amount of chlorophyll in *noc1* is a block in the chlorophyll degradation pathway. The first enzyme in the oxygen-independent chlorophyll degradation pathway is AtCLH1 (3). *AtCLH1*, also called *ATHCOR1* (*A. thaliana* coronatine-induced) was shown to be induced by the *Pst* DC3000-produced phytotoxin coronatine, which causes chlorosis in tomato (25). Since this gene encodes an enzyme in the chlorophyll degradation pathway, a mutation in *AtCLH1* could explain the absence of chlorosis in *noc1* upon infection with *Pst* DC3000. Alternatively, a difference in expression could indicate altered flux through the chlorophyll degradation pathway. Northern blot analysis was performed to determine whether *AtCLH1* gene expression upon infection with *Pst* DC3000 was altered in the *noc1* mutant. The transcript level of *AtCLH1* was only slightly reduced in *noc1* plants compared to that in wild-type plants (Figure 4-5).

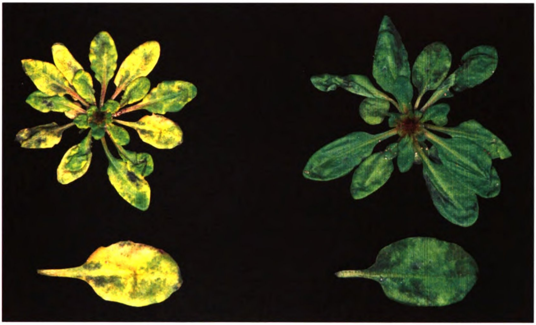


Figure 4-2. Phenotype of the *noc1* mutant after *Pst* DC3000 inoculation. *noc1* mutant (right), and wild-type (left) developed symptoms three days after vacuum infiltration of *Pst* DC3000 at 1 x 10^6 CFU/ml.

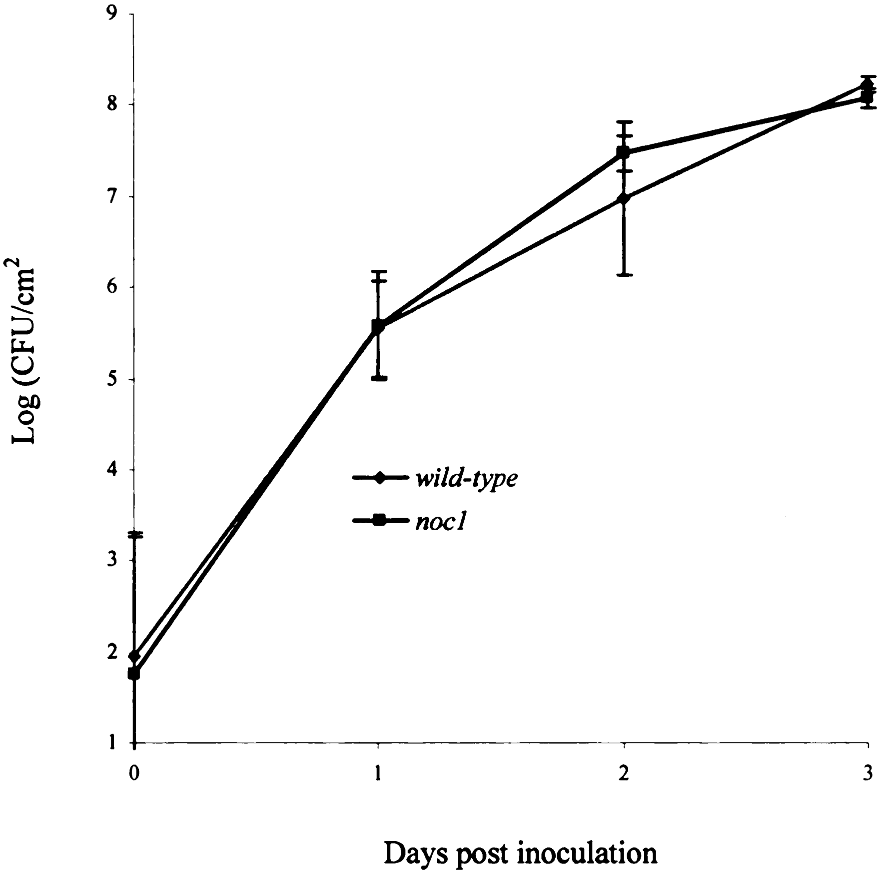


Figure 4-3. Growth of *Pst* DC3000 in *noc1* plants. Plants were inoculated with 1 x 10^6 CFU/ml of *Pst* DC3000. The mean values of the bacterial populations in wild-type (red) and *noc1* (green) plants are plotted with the standard deviation displayed as error.

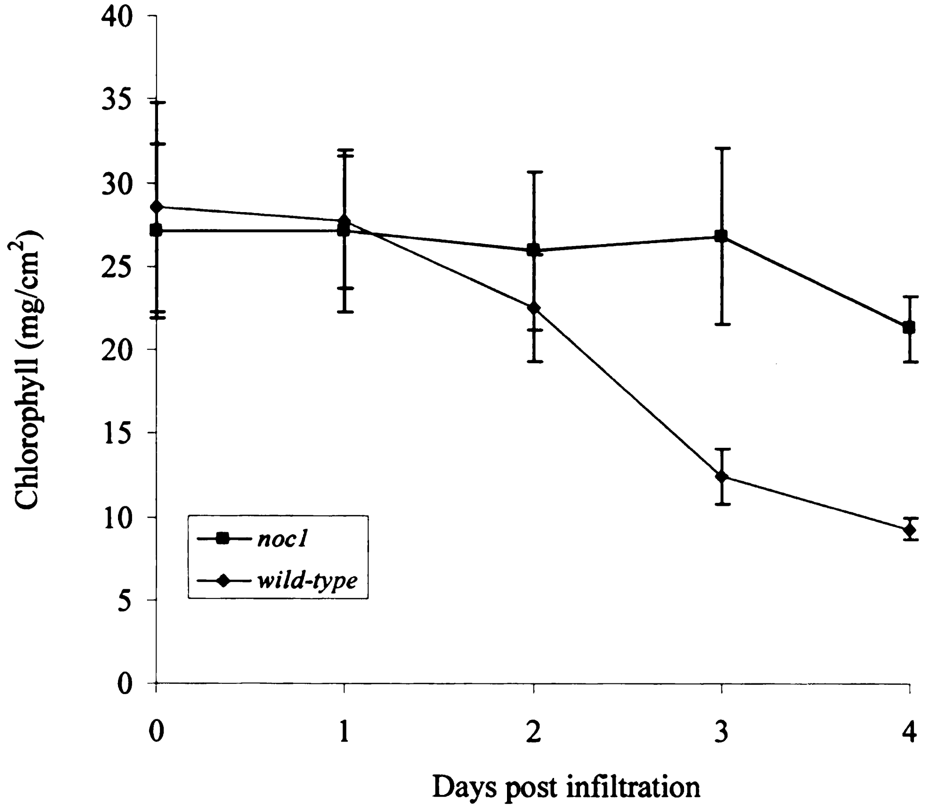


Figure 4-4. Total amount of chlorophylls (a and b) in wild-type and *noc1* leaves during the course of *Pst* DC3000 infection. Wild-type (red) and *noc1* (green) leaves were inoculated with *Pst* DC3000 at a concentration of 1 x 10^6 CFU/ml.

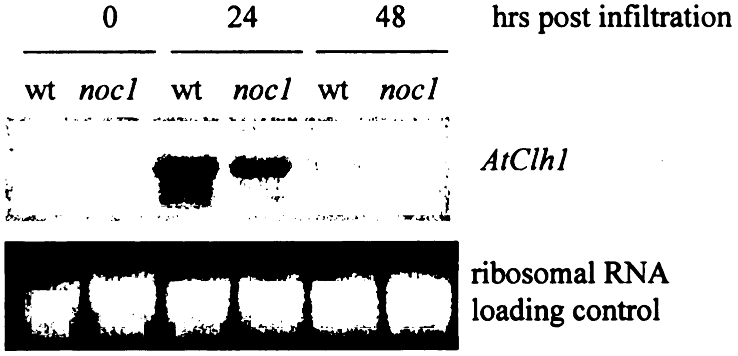


Figure 4-5. Northern blot analysis of *AtClh1* transcript. *AtClh1* transcript abundance in wild-type (WT) was compared to *noc1* leaves during the course of *Pst* DC3000 infection.

Microarray

Microarray analysis was performed to examine *Arabidopsis* gene expression differences between *noc1* and wild-type plants during *Pst* DC3000 infection. *AtClh1* is represented on the microarray slides used in these experiments. We found that transcript abundance was 1.37-fold higher in wild-type plants than in the *noc1* mutant. This result independently confirms the Northern result. Genes for which there was greater than a two-fold difference between *noc1* and wild-type were considered to be differentially regulated (Table 4-1). No JA/coronatine, salicylic acid, or ethylene-responsive genes were differentially regulated. The only gene that showed more than a 2-fold (2.5-fold) higher level of expression in *noc1* than in wild-type plants was alanine: glyoxylate aminotransferase 2 homologue (At2g38400). Only 12 genes had more than a 2-fold lower level of expression in *noc1* compared to wild-type plants. The *Arabidopsis* ATP-dependent Clp protease (At3g48870), which is associated with chloroplasts and may play a role in protein degradation in the chloroplast (26), was expressed at a 2.5-fold lower level in *noc1* than in wild-type plants.

Table 4-1. Differentially regulation of genes in the *noc1* mutant compared to wild-type plants.

Ratio	At Locus	Description
0.41	At2g38400	AGT2 alanine:glyoxylate aminotransferase 2 homolog
2.07	At4g23820	put. polygalacturonase, similar to genes induced by nematodes and senescence
2.08	At3g14210	lipase acylhydrolase, myrosinase assoc
2.12	At4g01310	L5P family of plastid ribosomal proteins
2.20	At1g52400	Beta glucosidase, suppressed by salt, ER localized
2.22	At3g54050	Fructose-1,6-Bisphosphatase
2.26	At1g72610	germin-like protein
2.36	At1g32060	phosphoribulokinase/Ribulose-5-phosphate kinase, Phosphopentokinase, chloroplast, Calvin cycle, light regulated via thioredoxin by reversible oxidation/reduction of sulfhydryl/disulfide groups
2.31	At2g02950	PKS1 phytochrome kinase substrate 1, modulates light signaling
2.38	At5g13550	sulfate transporter Sultr1, high affinity sulfate transporter, root H+/Sulfate cotransporter for sulfate uptake
2.42	At4g15440	HPL hyperoxide lyase
2.47	At3g28290	At14a, similarity to integrins
2.55	At3g48870	AtClpC *Arabidopsis* ATP-dependent Clp protease, ATP-binding subunit, degrades proteins in the chloroplast

Arabidopsis genes that were differential expressed (at least 2-fold difference) upon *Pst* DC3000 infection. Ratio represents wild-type/*noc1*.
RNA was isolated from plants 24 and 48 hours post infiltration with *Pst* DC3000.

Sensitivity to JA is similar in *noc1* and wild-type plants

The bacterial toxin coronatine has been shown to induce chlorosis in tomato plants (27). Coronatine is structurally and functionally similar to MeJA. In addition, both coronatine and MeJA induce similar biological responses in *Arabidopsis* seedlings, including inhibition of root elongation and stimulation of ethylene production (21). Coronatine insensitive (*coi1*) plants are resistant to *Pst* DC3000 (28). To determine whether the *noc1* mutant is affected in its sensitivity to MeJA, *noc1*, *coi1*, and wild-type seeds were grown on *Arabidopsis* germination media containing MeJA. As shown in Figure 4-6, both the wild-type and *noc1* seedlings had short roots and stunted growth. *coi1* seedlings, on the other hand, had long roots and normal growth. This result shows that the root response to JA was not altered in the *noc1* mutant.

Sensitivity to ethylene is similar in *noc1* and wild-type plants

ein2 mutants show decreased symptom development after infection with *Pst* DC3000 without a reduction in bacterial growth (29). To determine whether *noc1* was deficient in ethylene perception, dark-grown seedlings were treated with ethylene and observed for the triple response. Wild-type and *noc1* seedlings both had tight apical hooks and short hypocotyls. *ein2* seedlings, however, had elongated hypocotyls (Figure 4-7). Based on these results, the *noc1* mutation does not affect perception and response to ethylene.

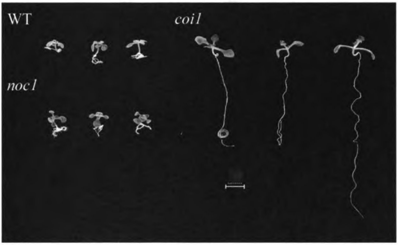


Figure 4-6. Wild-type, *noc1,* and *coi1* seedlings on 50μM MeJA medium. Wild-type (WT), *noc1* and *coi1* seedlings were germinated on medium containing 50 μM MeJA (Scale bar, 3 mm).

Figure 4-7. Wild-type (WT), *noc1,* and *ein2* seedlings germinated in the presence of ethylene. Seedlings were germinated in complete darkness with 10 µl/L of ethylene (Scale bar, 3 mm).

The NOC1 gene is located on the long arm of chromosome 4

The *noc1* mutation shows normal Mendelian genetics and is recessive. *noc1* was crossed with Ler plants and the F1 progeny were selfed to create an F2 population for mapping. Bulked segregant analysis was used to analyze a pool of approximately 100 F2 individuals that showed the mutant phenotype (homozygous for the *noc1* mutation). One marker, NGA1107, showed linkage to the mutation. This marker is located on the long arm of chromosome 4.

F2 individuals were tested using additional SSLP markers from the Monsanto *Arabidopsis* Polymorphism and Ler sequence collection (St. Louis, MO) to further pinpoint the mutation on chromosome 4. Currently, the mutation is mapped between At4g22340 and At4g24050, a region that includes 193 genes. A mutation in any one of many different pathways may results in the *noc1* phenotype, but if the function of NOC1 is to destabilize the chloroplasts in some manner, it is probably targeted to the chloroplast. Of the 193 genes, 24 encode proteins predicted to be targeted to the chloroplast. These included 11 expressed proteins, and 2 hypothetical proteins. The other chloroplast targeted genes are listed in Table 4-2. Fine mapping is currently being conducted.

Table 4-2. Genes of chromosome 4 located near *NOC1* that encode proteins predicted to be targeted to the chloroplast.

Locus	Description	cTP	R.C.
AT4G22590	trehalose-6-phosphate phosphatase, putative, similar to trehalose-6-phosphate phosphatase (AtTPPA) GI:2944178; contains Pfam profile PF02358: Trehalose-phosphatase	0.781	5
AT4G22690	cytochrome P450 family protein, flavonoid 3',5'-hydroxylase Hf1, Petunia x hybrida, PIR2:S38985	0.649	4
AT4G22745	methyl-CpG-binding domain-containing protein, contains Pfam profile PF01429: Methyl-CpG binding domain	0.627	4
AT4G22760	pentatricopeptide (PPR) repeat-containing protein, contains Pfam profile PF01535: PPR repeat	0.903	2
AT4G22840	bile acid:sodium symporter family protein, low similarity to SP|Q12908 Ileal sodium/bile acid cotransporter {Homo sapiens}; contains Pfam profile PF01758: Sodium Bile acid symporter family	0.400	5
AT4G22910	WD-40 repeat family protein, contains 6 WD-40 repeats (PF00400); similar to fizzy-related protein (GI:5813825) Drosophila melanogaster, PID:g2326419;	0.969	2
AT4G23100	glutamate-cysteine ligase / gamma-glutamylcysteine synthetase (GSH1), identical to glutamate-cysteine ligase SP:P46309 from (Arabidopsis thaliana); contains Pfam profile: PF04107 glutamate-cysteine ligase family 2(GCS2)	0.514	4
AT4G23450	zinc finger (C3HC4-type RING finger) family protein, contains Pfam profile: PF00097 zinc finger, C3HC4 type	0.929	2
AT4G23650	calcium-dependent protein kinase, putative / CDPK, putative, similar to calcium-dependent protein kinase (Marchantia polymorpha) gi|5162877|dbj|BAA81748; contains protein kinase domain, Pfam:PF00069; contains EF hand domain (calcium-binding EF-hand), Pfam:PF00036, INTERPRO:IPR002048	0.949	1
AT4G23800	high mobility group (HMG1/2) family protein, similar to HMG2B (Homo sapiens) GI:32335; contains Pfam profile PF00505: HMG (high mobility group) box	0.809	4
AT4G23940	FtsH protease, putative, contains similarity to zinc dependent protease GI:7650138 from (Arabidopsis thaliana)	0.984	1

Protein sequences were analyzed by TARGETP (http://www.cbs.dtu.dk/services/TargetP/)
The 13 genes not listed in this table are annotated either as hypothetical proteins or expressed proteins.

Discussion

The molecular basis of plant susceptibility to pathogen infection remains elusive in spite of the intense effort to understand bacterial pathogenesis in plants. We isolated an *Arabidopsis* mutant (*noc1*) that has altered symptom development upon *Pst* DC3000 infection. *noc1* plants undergo water-soaking and necrosis similarly to wild-type plants, but do not exhibit chlorosis. To our knowledge, this is the first report of a mutant in which chlorosis and necrosis have been uncoupled. Despite the altered symptom development, *Pst* DC3000 multiplication in wild-type plants and in the *noc1* mutant are comparable. These results suggest that we have identified a host mutation that specifically affects the development of disease chlorosis.

We demonstrated that the decreased chlorosis seen in this mutant is associated with a greater amount of chlorophyll compared with the wild-type plants. We do not currently know whether the increased abundance of chlorophyll is caused by a defect in the chlorophyllase-mediated degradation pathway or in other cellular pathways. However, we have provided evidence that transcription of chlorophyllase is still up-regulated, albeit to a slightly lower level, in *noc1* plants after infection with *Pst* DC3000. Therefore, the chlorophyll degradation pathway is at least partially intact in the *noc1* mutant. Whether the mutation affects a step downstream of chlorophyllase in this degradation pathway remains to be determined.

The microarray analysis reveals that, in fact, very few genes are differentially regulated in this mutant. Although mutants that are deficient in the perception of certain phytohormones (ethylene and JA) also have decreased symptom development after bacterial infection, not all phenotypes of these mutants are shared with *noc1*. Both ethylene and JA are perceived by *noc1* plants. This is consistent with the microarray results which showed that ethylene and JA responsive genes were not differentially regulated during infection. Thus it is unlikely that these pathways are altered in *noc1* plants.

From preliminary physical mapping, we know that the mutated gene is located on the long arm of chromosome 4. The delimited region contains 193 genes and, although this number is too large to apply a candidate gene approach, there are several interesting possibilities. One candidate is a tyrosine aminotransferase (At4g23600), which was later reclassified as a cysteine lyase. Cysteine lyases may be involved in sulfur metabolism, glucosinolate biosynthesis, or indole acetic acid metabolism. This gene is an interesting candidate because it is induced by the phytotoxin coronatine, which is known to cause chlorosis in tomato plants (30). There is also a gene that is annotated as a senescence-associated family protein (At4g23410). The physiological role of this protein has not been studied. Although *noc1* does not have delayed or altered senescence (data not shown), it is possible that a senescence-related gene is altered in the mutant.

Theoretically, *noc1* may be allelic to one of the senescence mutants found in the previous screen by Oh *et al.* (11). However, *noc1* plants are sensitive to ethylene, suggesting that *noc1* cannot be allelic to *ore2, ore3, or ein2,* which are all ethylene insensitive. In addition, *noc1* cannot be allelic to *ein2, ore1,* or *ore9* because they are located on different chromosomes (*ein2* (12) and *ore1* (11) are on chromosome 5; *ore9* is on chromosome 2 (11)). The physical map locations for *ore10* and *11* have not yet been published and it is possible that *noc1* is allelic to one of these two mutants. *noc1* is not allelic to *AtCLH1* or *AtCLH2* because those genes are located on chromosomes 1 and 5, respectively (3). Interestingly, *AtCLH1* anti-sense lines are not distinguishable from wild-type plants at a whole-plant phenotype level (5). However, the authors did not report on the phenotype of the anti-sense lines during pathogen infection or during senescence.

It is feasible that the *noc1* mutation causes the gain-of-function of a gene that inhibits chlorosis. Two examples that lead to delayed senescence and a stay-green phenotype are over-expression of *GF14λ*, a 14-3-3 protein (31), and over-expression of cytokinin (32, 33). Whether cytokinin perception or signaling is altered in *noc1* mutants remains to be determined.

Fine mapping and cloning of *noc1* is currently underway. Determining the identity of the gene responsible for the *noc1* phenotype will be an important step toward increasing our understanding of the processes involved in disease symptom development during *Pst* DC3000 infection of *Arabidopsis*.

Acknowledgements-

I would like to thank R. Nantais, V. Ndlovu, and A. E. Plovanich-Jones for conducting the original mutant screen and isolating the *noc1* mutant; N. Pumplin, Y. B. Kwack, K. McLellen, and M. Trapp for help with scoring the F2 population; and N. Raikhel for use of the initial mapping primer set from Invitrogen, Carlsbad, CA.

References

1. JANAVE, M. T. (1997) Enzymic degradation of chlorophyll in Cavendish bananas: *In vitro* evidence for two independent degradative pathways. *Plant Physiol Biochem*, 35, 837-846.

2. TAKAMIYA, K. I., TSUCHIYA, T. & OHTA, H. (2000) Degradation pathway(s) of chlorophyll: what has gene cloning revealed? *Trends Plant Sci*, 5, 426-431.

3. TSUCHIYA, T., OHTA, H., OKAWA, K., IWAMATSU, A., SHIMADA, H., MASUDA, T. & TAKAMIYA, K. (1999) Cloning of chlorophyllase, the key enzyme in chlorophyll degradation: finding of a lipase motif and the induction by methyl jasmonate. *Proc. Natl. Acad. Sci. U S A*, 96, 15362-15367.

4. TREBITSH, T., GOLDSCHMIDT, E. E. & RIOV, J. (1993) Ethylene induces de novo synthesis of chlorophyllase, a chlorophyll degrading enzyme, in Citrus fruit peel. *Proc. Natl. Acad. Sci. U S A*, 90, 9441-9445.

5. BENEDETTI, C. E. & ARRUDA, P. (2002) Altering the expression of the chlorophyllase gene *ATHCOR1* in transgenic *Arabidopsis* caused changes in the chlorophyll-to-chlorophyllide ratio. *Plant Physiol*, 128, 1255-1263.

6. THOMAS, H. & HOWARTH, C. J. (2000) Five ways to stay green. *J Exp Bot*, 51 Spec No, 329-337.

7. THOMAS, H. & SMART, C. M. (1993) Crops that stay green. *Ann Appl Biol*, 123, 193-219.

8. VICENTINI, F., HORTENSTEINER, S., SCHELLENBERG, M., THOMAS, H. & MATILE, P. (1995) Chlorophyll breakdown in senescent leaves - identification of the biochemical lesion in a stay-green genotype of F*estuca-pratensis* Huds. *New Phytologist*, 129, 247-252.

9. ROCA, M., JAMES, C., PRUZINSKA, A., HORTENSTEINER, S., THOMAS, H. & OUGHAM, H. (2004) Analysis of the chlorophyll catabolism pathway in leaves of an introgression senescence mutant of *Lolium temulentum*. *Phytochem*, 65, 1231-1238.

10. GUIAMET, J. J., SCHWARTZ, E., PICHERSKY, E. & NOODEN, L. D. (1991) Characterization of cytoplasmic and nuclear mutations affecting chlorophyll and chlorophyll-binding proteins during senescence In soybean. *Plant Physiol*, 96, 227-231.

11. OH, S. A., PARK, J. H., LEE, G. I., PAEK, K. H., PARK, S. K. & NAM, H. G. (1997) Identification of three genetic loci controlling leaf senescence in *Arabidopsis thaliana*. *Plant J*, 12, 527-535.

12. ALONSO, J. M., HIRAYAMA, T., ROMAN, G., NOURIZADEH, S. & ECKER, J. R. (1999) EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science*, 284, 2148-2152.

13. WOO, H. R., CHUNG, K. M., PARK, J. H., OH, S. A., AHN, T., HONG, S. H., JANG, S. K. & NAM, H. G. (2001) ORE9, an F-box protein that regulates leaf senescence in *Arabidopsis*. *Plant Cell*, 13, 1779-1790.

14. OH, M. H., MOON, Y. H. & LEE, C. H. (2003) Increased stability of LHCII by aggregate formation during dark-induced leaf senescence in the *Arabidopsis* mutant, *ore10*. *Plant Cell Physiol*, 44, 1368-1377.

15. KATAGIRI, F., THILMONY, R. & HE, S. Y. (2002) The *Arabidopsis thaliana-Pseudomonas syringae* interaction. *The Arabidopsis Book*, 1.

16. SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989) *Molecular cloning: a laboratory manual* (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press).

17. DEBROY, S., THILMONY, R., KWACK, Y. B., NOMURA, K. & HE, S. Y. (2004) A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. *Proc. Natl. Acad. Sci. U S A*, 101, 9927-9932.

18. HAUCK, P., THILMONY, R. & HE, S. Y. (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. *Proc. Natl. Acad. Sci. U S A*, 100, 8577-8582.

19. SCHAFFER, R., LANDGRAF, J., ACCERBI, M., SIMON, V., LARSON, M. & WISMAN, E. (2001) Microarray analysis of diurnal and circadian-regulated genes in *Arabidopsis*. *Plant Cell*, 13, 113-123.

20. ARNON, D. I. (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol*, 24, 1-15.

21. FEYS, B. J. F., BENEDETTI, C. E., PENFOLD, C. N. & TURNER, J. G. (1994) *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell*, 6, 751-759.

22. GUZMAN, P. & ECKER, J. R. (1990) Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. *Plant Cell*, 2, 513-523.

23. LUKOWITZ, W., GILLMOR, C. S. & SCHEIBLE, W. R. (2000) Positional cloning in *Arabidopsis*. Why it feels good to have a genome initiative working for you. *Plant Physiol*, 123, 795-805.

24. JANDER, G., NORRIS, S. R., ROUNSLEY, S. D., BUSH, D. F., LEVIN, I. M. & LAST, R. L. (2002) *Arabidopsis* map-based cloning in the post-genome era. *Plant Physiol*, 129, 440-450.

25. BENEDETTI, C. E., COSTA, C. L., TURCINELLI, S. R. & ARRUDA, P. (1998) Differential expression of a novel gene in response to coronatine, methyl jasmonate, and wounding in the Coi1 mutant of *Arabidopsis*. *Plant Physiol*, 116, 1037-1042.

26. NAKABAYASHI, K., ITO, M., KIYOSUE, T., SHINOZAKI, K. & WATANABE, A. (1999) Identification of *clp* genes expressed in senescing *Arabidopsis* leaves. *Plant Cell Physiol*, 40, 504-514.

27. GNANAMANICKAM, S. S., STARRATT, A. N. & WARD, E. W. B. (1982) Coronatine production *invitro* and *invivo* and its relation to symptom development in bacterial-blight of soybean. *Can J Bot Rev*, 60, 645-650.

28. XIE, D. X., FEYS, B. F., JAMES, S., NIETO-ROSTRO, M. & TURNER, J. G. (1998) *COI1*: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science*, 280, 1091-1094.

29. BENT, A. F., INNES, R. W., ECKER, J. R. & STASKAWICZ, B. J. (1992) Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. *Mol Plant Microbe Interact*, 5, 372-378.

30. JONES, P. R., MANABE, T., AWAZUHARA, M. & SAITO, K. (2003) A new member of plant CS-lyases. A cystine lyase from *Arabidopsis thaliana*. *J Biol Chem*, 278, 10291-10296.

31. YAN, J., HE, C., WANG, J., MAO, Z., HOLADAY, S. A., ALLEN, R. D. & ZHANG, H. (2004) Overexpression of the *Arabidopsis* 14-3-3 protein GF14 lambda in cotton leads to a "stay-green" phenotype and improves stress tolerance under moderate drought conditions. *Plant Cell Physiol*, 45, 1007-1014.

32. GAN, S. & AMASINO, R. M. (1995) Inhibition of leaf senescence by autoregulated production of cytokinin. *Science*, 270, 1986-1988.

33. SMART, C. M., SCOFIELD, S. R., BEVAN, M. W. & DYER, T. A. (1991) Delayed leaf senescence in tobacco plants transformed with *tmr*, a gene for cytokinin production in *Agrobacterium*. *Plant Cell*, 3, 647-656.

Chapter 5: Summary and future directions

The overall goal of the described research was to increase our understanding of the compatible plant-pathogen interaction. Although we know that bacterial type III effectors are important for pathogenesis, their specific functions in the plant cell are just beginning to be elucidated. Studying susceptibility is technically challenging. Bacterial mutagenesis is not sufficient to explore the role of effectors because effectors are functional redundant to each other or each effector only has a small effect on virulence. The tools used to measure symptom development or disease progression are often not sensitive enough to discern subtle differences in bacterial growth or symptom development. To gain further understanding of pathogenesis, alternative approaches need to be explored. I applied two different approaches to study the compatible *A. thaliana-P. syringae* interaction: first, *A. thaliana* plants that over-express the *avrPto* effector were characterized; second, an *A. thaliana* mutant (*noc1*) that has reduced disease-associated symptom development was isolated and studied.

AvrPto

AvrPto, one effector from *Pseudomonas syringae* pv. *tomato* JL1065, has been well characterized as an avirulence protein, but its virulence function, until now, has only minimally been addressed. In fact, only a few reports have investigated the function of AvrPto in susceptible hosts. These studies demonstrate the addition of *avrPto* to certain *Pst* strains increases their virulence (1, 2). Although *avrPto* mutants do not have a virulence phenotype, this result may be due to functional redundancy and/or the lack of sensitivity in the methods currently used to assay virulence.

I have chosen to study the virulence role of the *Pst* DC3000 effector, AvrPto, by creating *Arabidopsis* plants that over-express this effector driven by an inducible promoter (Chapters 2 and 3). After induction of the transgene, plants become paler than wild-type plants within 24 hours. This paleness progresses into chlorosis by 3 days after induction.

In Chapter 2, I showed that over-expression of *avrPto* causes gene expression changes in the host similar to those induced by *Pst* DC3000. Remarkably, 78% and 85% of the genes induced by, or repressed by *Pst* DC3000, respectively, are regulated in a similar fashion in *avrPto* plants. If the transcriptional profile in *avrPto* plants mimics the response to *Pst* DC3000 infection so closely, then maybe the need for other, presumably functionally redundant, type III effectors has been bypassed and rendered unnecessary in *avrPto* plants. Indeed, a *hrp* mutant which cannot secrete any type III effectors into the host cell and therefore, is not pathogenic, was able to multiply in *avrPto* plants up to 500-fold more than in wild-type plants. In addition, *avrPto* plants also allowed *Pseudomonas fluorescens* 55 (*Pf* 55), which is a non-pathogenic bacterium, and an avirulent strain, *Pst* DC3000 (*avrRpt2*), to grow over 600- and 900- fold, respectively, more than in wild-type plants (Chapter 3).

The biased down-regulation of *Arabidopsis* genes that encode putatively secreted proteins suggests that AvrPto may be disrupting extracellular protein secretion in the host. One process that is believed to require functional protein secretion is papilla formation. Papillae form between the plasma membrane and the cell wall. They are composed of hydroxy proline-rich glycoproteins, phenolic compounds, and callose, and are thought to be structural barriers against invading microbes. Papilla deposition is elicited by *hrp* mutants, but not by the wild-type pathogen, *Pst* DC3000. In order to determine if AvrPto alters secretion in the host, I assayed for papilla formation in response to various bacterial strains. I found that *avrPto* plants suppress papilla deposition in response to two different *hrp* mutants (*hrpA* and *hrcC*), *Pf* 55, and *Pst* DC3000 (*avrRpt2*) (Chapters 2 and 3). One concern using callose deposition as a marker for secretion is that callose is synthesized at the plasma membrane. Our lab is currently trying to find other, more reliable, markers for secretion.

Results from yeast-two-hybrid experiments (Appendix A) to find host interactors of AvrPto also seem to support the theory that AvrPto may be inhibiting protein secretion. AvrPto interacts with members of the *Arabidopsis* RabE family. Rabs are small GTPases that are involved in protein transport from the Golgi to the plasma membrane. Possibly, AvrPto is preventing protein secretion by binding to RabE and either interfering with proper RabE function or localization. Currently, our lab is studying the effects of wild-type, dominant-negative, and constitutively active RabE expression in transgenic *Arabidopsis* plants on resistance.

Another interesting candidate identified from the AvrPto yeast-two-hybrid screen was a kinase. This protein is interesting because Pto, the corresponding R gene in tomato, is also a kinase. In addition, a kinase has been shown to interact with Rab8, the rat homologue of RabE. Confirmation of the interaction of this kinase with AvrPto, as well as characterization of T-DNA insertion lines is presently underway.

An alternative method for assaying host protein secretion is to measure the abundance of proteins in the apoplast. The intercellular wash fluid (IWF) was collected from wild-type plants after infection with high concentrations of *Pst* DC3000 and *Pst* DC3000 (*avrRpt2*). At least four protein bands were present in the fluid from the *Pst* DC3000 (*avrRpt2*)-inoculated tissue but absent in fluid from the *Pst* DC3000-inoculated tissue. There are at least three possibilities that would account for this result: 1) *Pst* DC3000 (*avrRpt2*) could be causing leakage of these proteins, perhaps as part of the cell death process; 2) *Pst* DC3000 (*avrRpt2*) is inducing the specific secretion of these proteins; 3) or *Pst* DC3000 (*avrRpt2*) causes higher expression levels of these proteins and then the same amount of leakage or secretion occurs in both *Pst* DC3000- and *Pst* DC3000 (*avrRpt2*)- inoculated tissue. Interestingly, these proteins are absent in the apoplastic fluid from *avrPto* plants inoculated with *Pst* DC3000 (*avrRpt2*). This result indicates that AvrPto is either interfering with the leakage, secretion or expression of these proteins. Future experiments will test the above hypotheses by comparing the abundance of these proteins in whole leaf extracts under the different treatments. If the protein concentration is the same in all the treatments, then I propose to distinguish between leakage and secretion by using the above mentioned dominant negative RabE transgenic plants or inhibitors of secretion. If I do not detect the same proteins in the IWF of these plants or after treatment with the inhibitors, then I will conclude that these proteins are secreted into the apoplastic space.

It was previously shown that *Arabidopsis* secretes several PR proteins into the apoplast during SAR induced by 2, 6-dichloroisonicotinic acid (INA) (3). I conducted western blots on the IWFs and found that PR proteins were detectable in all the different treatments, probably due to the infiltration process. I believe that *avrPto* plants were able to secrete PR proteins. This result suggests that PR protein secretion is not affected by AvrPto.

To test whether *avrPto* over-expression leads to excessive leakage of nutrients, I inoculated the IWF from these plants and wild-type plants with the *hrcC* mutant. The *hrcC* mutant grew to similar levels in the IWF from either wild-type or *avrPto* plants. From this result, we can conclude that at least water-extractable nutrients are not limiting in the apoplastic space. However, it is possible that these nutrients may not be available to the bacteria under physiological conditions in wild-type plants, but are available in *avrPto* plants.

It is important to note that my results were obtained from over-expressing *avrPto* in plants. Therefore, at this time, I cannot exclude the possibility that I observed a gain-of-function effect. Shan *et al.* (4) showed that mutation of the myristylation site disrupts AvrPto function in resistant tomato plants and that the plasma membrane localization is essential for this avirulence function. An N-terminal 6x His-tagged version of *avrPto* was expressed in *Arabidopsis*. In contrast to wild-type *avrPto* plants, the 6x His-*avrPto* plants do not undergo chlorosis or allow growth of *hrp* mutants (K. Nomura and W. Underwood, unpublished results). The 6x His tagged AvrPto is no longer localized to the host plasma membrane (K. Nomura, unpublished results) suggesting that not only the production, but host membrane localization is also necessary for AvrPto to carry out its virulence function in *Arabidopsis*.

The *noc1* mutant

No-chlorosis (*noc1*) was identified from a screen to identify *Arabidopsis* mutants that had decreased symptom development in response to *Pst* DC3000. Water soaking, necrosis and bacterial growth are normal in this mutant, but it lacks chlorosis development in response to infection with *Pst* DC3000. To our knowledge, this is the first instance in which disease-associated bacterial growth, necrosis, and chlorosis have been uncoupled.

To test the hypothesis that there was more chlorophyll in *noc1* leaves than in wild-type leaves, I assayed chlorophyll abundance. I showed that the stay-green phenotype of *noc1* was due to higher amounts of chlorophylls *a* and *b* in the *noc1* leaves compared to those in wild-type plants after *Pst* DC3000 infection.

There are at least two possibilities that could explain the *noc1* phenotype: the rate of chlorophyll synthesis exceeds the rate of chlorophyll breakdown, or there is a defect in chlorophyll degradation. I assayed the transcript abundance of *AtClh1,* which is the first gene in a chlorophyll degradation pathway, to address this question. I found that this gene is up-regulated in the *noc1* mutant after *Pst* DC3000 infection, albeit to a slightly lower level than in wild-type plants. This result suggests that signaling leading up to the chlorophyllase degradation pathway is not drastically disrupted in the *noc1* mutant. Transcript abundance of downstream genes of this step could not be assayed because they have not yet been identified.

Microarray results also confirm that the *AtClh1* gene is expressed similarly in *noc1* and wild-type plants. In fact, there were very few genes that were differential regulated in the *noc1* plants compared to wild-type plants. Salicylic acid (SA), jasmonic acid (JA), and ethylene are signaling molecules in plants that are essential for the *Arabidopsis* response to pathogens. There are several mutants that have altered perception of these compounds that exhibit some phenotypes in common with the *noc1* mutant. However, there were no SA, JA or ethylene responsive genes that were differentially expressed in *noc1*plants, even though the microarray was enriched with SA, JA and ethylene response genes.

Seedling growth in the presence of these hormones confirms the microarray results. The *ein2* mutant is also interesting with respect to the *noc1*mutant because *ein2* plants have reduced symptom development in response to *Pst* DC3000 infection without decreased growth. Because *noc1* plants also have altered symptom development without altered levels of growth, we tested *noc1* plants for insensitivity to ethylene. Etiolated wild-type and *noc1* seedlings had tight apical hooks, thickened hypocotyls, and reduced hypocotyl elongation, whereas the *ein2* mutant does not exhibit this triple response upon exposure to ethylene. This result suggests that the *noc1* mutant is not affected in ethylene perception.

Coronatine, a toxin produced by *Pst* DC3000, has been shown to elicit chlorosis on tomatoes and has structural similarity to MeJA. Therefore, altered perception of MeJA could explain the lack of chlorosis in the *noc1* mutant. However, when *noc1, coi1* (which are resistant to *Pst* DC3000), and wild-type seeds were germinated on MeJA plates, the roots of *noc1* and wild-type seedlings were stunted, while those of the *coi1* seedlings were not. This result demonstrates that *noc1* plants were able to perceive MeJA.

The *noc1* mutation was mapped to a 619-kb region on the long arm of chromosome 4, which contains 193 genes. Only a few of these genes were present on the microarray slide mentioned above and none of them were differentially regulated in the *noc1* mutant compared to wild-type plants. Of the 193 genes, 24 encode proteins predicted to be targeted to the chloroplast. These proteins are potential candidates because *noc1* plants have more chlorophyll than wild-type plants and chlorophyll is located in the chloroplast, but I cannot exclude the possibility that NOC1 is not localized to the chloroplast. Future work will focus on the fine mapping of the *noc1* mutation and cloning of the gene.

We are a long way from knowing how to prevent disease without chemicals or *R* genes, but we are steadily getting the information we need. Once we understand the role of bacterial effectors and how bacterial disease progresses, we will then be able to design strategies that will allow increased protection of crop plants.

References

1. CHANG, J. H., RATHJEN, J. P., BERNAL, A. J., STASKAWICZ, B. J. & MICHELMORE, R. W. (2000) avrPto enhances growth and necrosis caused by *Pseudomonas syringae* pv.*tomato* in tomato lines lacking either Pto or Prf. *Mol Plant Microbe Interact*, 13, 568-571.

2. SHAN, L., HE, P., ZHOU, J. M. & TANG, X. (2000) A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto. *Mol Plant Microbe Interact*, 13, 592-598.

3. UKNES, S., MAUCH-MANI, B., MOYER, M., POTTER, S., WILLIAMS, S., DINCHER, S., CHANDLER, D., SLUSARENKO, A., WARD, E. & RYALS, J. (1992) Acquired resistance in *Arabidopsis*. *Plant Cell*, 4, 645-656.

4. SHAN, L., THARA, V. K., MARTIN, G. B., ZHOU, J. M. & TANG, X. (2000) The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane. *Plant Cell*, 12, 2323-2338.

Appendix A: Putative AvrPto interactors

Supplementary Material for Chapter 3

Proteins that are able to interact with AvrPto were screened by using the LexA-based yeast-two-hybrid system (Clonetech Laboratories, Inc. Palo Alto, CA). Bogdanove and Martin (1) conducted a similar screen using a tomato cDNA library. While Bogdanove and Martin (1) used *avrPto* from *Pst* JL1065, my screen was conducted using *avrPto* from *Pst* DC3000. The two versions of *avrPto* differ from each other in just four bases that cause four amino acid changes. There may be new information gained from my screen.

avrPto was amplified by PCR and cloned into pNLexA using the sense primer 5' GCGAATTCCGAACCATGGGAAATATATGTGTC 3' and the antisense primer, 5' GCCTCGAGATTGCCAGTTACGGTA 3'. The construct was transformed into the EGY48 strain carrying the *lacZ* reporter plasmid. The construct was tested for autoactivation and protein expression (data not shown). Two independent *Arabidopsis* cDNA libraries (kindly provided by J. Jones) made using infected and uninfected *Landsberg erecta* plants were screened.

Originally, 203 blue colonies were identified. Of these, 147, retained their blue color and grew on plates lacking leucine. These clones were then PCR-amplified using pB42AD primers and the products were digested with *Hae*III. The clones were grouped into classes based on the digestion pattern. Representatives from 27 classes were sent for sequencing. Only 21 sequences were readable and Blast analysis of these sequences revealed a total 15 different genes (listed in Table A-1). As yet, autoactivation and confirmation of AvrPto interaction of these clones has not been conducted. In addition, it is possible that more than one plasmid could be retained in some yeast colonies.

AvrPto is localized to the plasma membrane of the host. Therefore, the predicted targeting of these proteins is pertinent to this study and is also listed in Table A-1.

Both the Bogdanove and Martin (1) and my screen found a Rab8 homologue in tomato and *Arabidopsis*, respectively. This small GTP binding protein is thought to be involved in host cell trafficking from the Golgi apparatus to the plasma membrane. Representatives of different Rab families (A-G) were then investigated further by transforming yeast containing AvrPto as the bait. Figure A-1 shows that AvrPto interacts with only the five RAB8 homologues (RabE in plants) and none of the other Rab families in *Arabidopsis*.

Table A-1. Putative interactors of AvrPto.

# of clones found	At Locus	Description	Predicted locations*	Score
1	AT1G02870	expressed protein	M	0.646
1	AT1G11290	pentatricopeptide (PPR) repeat-containing protein	C	0.661
1	AT1G29930	light-harvesting chlorophyll a/b binding protein	C	0.778
1	AT2G35260	expressed protein	C	0.954
1	AT2G46220	expressed protein	C	0.637
1	AT3G26600	expressed protein	-	
1	AT3G50700	zinc finger protein	C	0.845
1	AT5G16840	RRM-containing protein	-	
2	AT1G71920	histidinol-phosphate aminotransferase	C	0.957
2	AT4G28030	GCN5-related N-acetyltransferase (GNAT) family	C	0.843
3	AT3G23050	auxin-responsive protein IAA7 (Indoleacetic acid-induced protein 7)	-	
3	AT4G04320	malonyl-CoA decarboxylase -related	M	0.669
4	AT5G59840	Ras family GTP-binding protein	-	
6	AT3G57870	ubiquitin-conjugating enzyme, putative, strong similarity to SP|P50550 Ubiquitin-like protein SUMO-1 conjugating enzyme (Ubiquitin-conjugating enzyme UbcE2A)	M	0.700
8	AT4G11890	protein kinase family	-	

* TARGETP-predicted locations (http://www.cbs.dtu.dk/services/TargetP/)
M = Mitochondrion; C = Chloroplast; - = other, location unknown

Rabs: trafficking and secretion

A typical plant cell contains 5000 to 10,000 different polypeptide sequences and billions of individual protein molecules. For cells to function properly, it must direct these proteins to specific places within the cell (2). Protein trafficking is crucial for all cellular processes (3). It is central to the interaction of cells with their environment because it is the route by which components of the extracellular matrix and secreted enzymes are released into the surrounding milieu (2). Although crucial for a number of many other house-keeping functions in plants, such as the formation of the phragmoplast during cell division, cell polarization during development, general protein metabolism, and plant wall biogenesis, the secretory pathway is also involved in specialized plant processes such as responses to abiotic and biotic stresses (4).

Proteins traverse the secretory system in vesicles that bud off one compartment and dock, then fuse with the next. Within the secretory pathway, proteins travel from the endoplasmic reticulum (ER) to the Golgi, and through the Golgi into the trans-Golgi network (TGN). At the TGN, secreted proteins are sorted from vacuolar proteins and packaged into secretory vesicles (5). The proteins then travel to the plasma membrane for secretion or to the tonoplast for delivery to the vacuole. There is also the endocytic pathway in which proteins are internalized into early endosomes, and transported by means of late endosomes to the lysosome, or vacuole. Anterograde transport carries membrane and cargo proteins through the exocytotic and endocytotic pathways. Retrograde transport retrieves "resident" proteins and membrane components, returning them to their original compartments (6). The large number of different transport intermediates in cells and the numerous potential targets for those carriers seem to demand a means of ensuring that the carriers fuse at the appropriate destination and time (7). If docking and fusion were unregulated, all of the organelles in the cytoplasm might become stuck together as part of a giant sandwich (8). Two classes of proteins have emerged as specific and essential players in many vesicle transport processes. One class, the SNAREs {soluble N-ethylmaleimide-sensitive fusion factor (NSF) attachment protein receptors} is composed of integral membrane proteins which serve as receptors for soluble factors that are necessary for docking and fusion. The other class is comprised of a branch of the RAS superfamily of small GTPases, called Rab proteins (RAS-related in brain) (9). Rab proteins are thought to determine the fusion competence of membranes (10) and have been primarily implicated in vesicle docking as regulators of SNARE pairing (9).

Rab functions are conserved across all eukaryotes (10) and they control cellular events ranging from secretion and endocytosis to signal transduction and development (11). Each Rab protein has a characteristic distribution on cell membranes and, with only a few exceptions, participates in a specific trafficking step of vesicular transport (9). The specificity of Rab localization is provided by structural determinants unique to each family member that appear to be recognized by distinct sets of proteins on organelle surfaces (8).

A newly synthesized Rab is recognized in the cytoplasm by a REP (Rab escort protein) which presents the Rab to the geranylgeranyl transferase (12). The reversible membrane localization of Rabs depends on this post-translational modification of a cysteine motif at the very carboxyl terminus with one or two highly hydrophobic geranylgeranyl groups (12). Mutant mono-prenylated Sec4 proteins in yeast are unable to localize to the correct subcellular compartment and as a result are non-functional (11). REP then functions as a chaperone which keeps the Rab soluble and delivers it to the appropriate membrane (12).

Rab proteins exist in two states: an active form, which is bound to GTP, and an inactive form, which is bound to GDP. REP and GDI (GDP dissociation inhibitor) show a marked preference for GDP-bound Rabs (11). GDI has structural similarity to REP and like REP, GDI can present geranylgeranylated, GDP-bound Rabs to specific membranes (12). Despite the sequence conservation, however, GDI cannot replace REP in the prenylation reaction. REP is therefore a specialized GDI that plays a dual role in Rab prenylation and membrane association (9).

Membrane delivery is catalyzed by a GDF (GDI displacement factor) (11, 12), which recruits Rabs to specific donor membranes (3). GDF is secretory-compartment-specific and at least has specificity for a subgroup of Rabs (6). It may play a role in the regulation of trafficking by controlling GDI dissociation and the shift to GTP association (6). Membrane delivery precedes the exchange of GDP with GTP (11). The exchange of GDP for GTP results in a conformational change that releases REP and exposes an isoprenoid lipid anchor attached to the C-terminal cystein residue. This hydrophobic tail allows Rabs to bind to membranes. Rab proteins exchange GDP for GTP with the help of GEF (guanine nucleotide exchange factor) (2). Since GEFs are specific for particular Rabs, they make an important additional contribution to the fidelity of Rab targeting (11). Alternatively, Rabs can be maintained in the inactive state by GDI (2). GDI prevents indiscriminant membrane binding and contributes to the process of recruitment to the membrane (9).

Although they demonstrate a low level of intrinsic ATPase activity, Rab proteins must interact with a GAP (GTPase activating protein) to hydrolyze GTP effectively (2). GAPs may also be specific for some Rab family members (9), but, although newly identified GAPs show some substrate preference, they do not exhibit absolute specificity *in vitro*, suggesting that they might also be promiscuous *in vivo* (3).

Upon GTP hydrolysis, the Rab may be released from the membrane due to a conformational change that facilitates the efficient dissociation of Rab-GDP from the membrane (2). This is mediated by GDI, which is capable of retrieving the geranylgeranylated, GDP-bound Rab from intracellular membranes (12). Current models propose that GDI extracts Rabs from target membranes after the vesicle fusion event (6) and recycles them back to their membranes of origin (8). GDI, which is more abundant than REP, thus serves as a recycling factor that allows several rounds of membrane association and retrieval of the Rabs (12). The (GTP/GDP) cycle imposes temporal and spatial regulation to membrane transport, with the Rabs acting like timers whose clocks are set depending on the intrinsic and catalyzed rates of nucleotide exchange and hydrolysis (13). Their on/off regulatory functions are restricted to the membrane compartments where they are localized (13).

As previously mentioned, SNAREs also play a role in protein trafficking. SNAREs, initially defined as a category of proteins that bind α-SNAP (soluble NSF attachment protein), has come to denote a more general class of vesicle trafficking proteins. Thus SNARE proteins may not bind α-SNAP, but they do possess the hallmark characteristics of being relatively small and compartment-specific (14). In general, SNAREs are C-terminally anchored integral membrane proteins with most of their mass present in the cytoplasm (7) in order to engage in protein trafficking interaction (14). All SNAREs bear an approximate 60-65 amino acid residue 'SNARE motif' in the membrane proximal region. Conserved in this motif are hydrophobic heptad repeats indicating a propensity to form α-helical coiled-coil structures (7). Specific complex formation between SNARES on opposing membranes is required for membrane fusion reactions (14). The assembled trans-SNARE complex consists of a bundle of four helices, one of which is supplied by the v-SNARE (vesicle SNAREs which are anchored on the cargo vesicle) and the other three by the t-SNARE (target SNAREs which reside on the target membrane) (5, 15). The t-SNAREs always include a syntaxin, which contributes one helix, whereas the remaining two helices are either from a single SNAP 25-type protein or from two separate t-SNARE light chains (16). Trimeric SNARE complexes involving a SNAP25 homologue have been described in plasma membrane fusion events, whereas tetrameric SNARE complexes prevail in endomembrane fusion processes (15). Membrane docking is mediated by the formation of this four helical bundle of SNARE proteins (16). The matching of v- and t-SNARES is believed to provide specificity to membrane fusion reactions (8).

SNAREs cannot be the sole membrane targeting determinants because some SNAREs act at multiple steps *in vivo* and can be found in more than one SNARE complex. Also, non-cognate sets of SNAREs can form complexes *in vitro* and non-cognate complexes exhibit comparable stabilities. Lastly, although plasma membrane t-SNAREs are dispersed over a wide area, secretion only occurs in well defined subdomains (7). Any given organelle will contain SNARE complexes that must remain inactive unless they are at their specific place of function (13). Thus targeting specificity cannot be determined solely by the specific localization of t-SNAREs and the relative affinities of v/t-SNARE interactions (7). Genetic experiments implicate Rabs in the processes by which transport vesicles recognize their cognate fusion targets (8). Rather than being mere regulators of SNARE protein complexes, Rab GTPases and their effectors are primary determinants of compartmental specificity in the organelles of eukaryotic cells (13).

Early in the targeting process Rabs mediate the tethering of an incoming vesicle to the correct target organelle (7, 13). It is not clear whether tethering is completely independent of all SNARE functionality. If tethering is ignorant of the downstream SNAREs, then it would be the critical targeting event in membrane traffic (7). Alternatively, Rabs may coordinate tethering with downstream catalysis of cognate SNARE complex assembly (7). This tethering proposal differs from current models which hold that Rabs act though the tethering factor (for example by simply conferring tether localization or by acting as physical components of the tether). The Rab protein may coordinate tethering with SNARE assembly by interacting either directly or indirectly with t-SNARE complex on the target membrane (7). Rabs, in their GTP – bound conformations, may recruit transport step-specific docking factors from the cytosol that facilitate v-/ t-SNARE pairing. Thus Rabs act either directly or indirectly to facilitate SNARE complex formation but are not core elements of such complexes (8). If the initial Rab recruitment onto a nascent transport vesicle is coupled (or quickly followed by) its conversion to Rab GTP, only functional transport vesicles will recruit docking factors. Therefore, docking will only take place between transport vesicles and their targets, rather than between entire organelles (8). Rabs may also act by stabilizing the v-/t-SNARE interaction (17). Regardless, vesicle docking is the accepted role of Rabs in vesicular transport (6).

The t-SNARE membrane and v-SNARE membrane fusion event is traditionally assigned to SNAREs. Once docked, the coiled-coil motifs of the v- and t-SNAREs interact in a head to head manner, bringing the vesicle and target membranes into close contact (2). This interaction is thought to be a key step in the reaction leading to vesicle fusion. Experimental support that SNAREs can directly mediate membrane fusion comes from studies of liposomes containing purified SNAREs (18). This work shows that liposomes containing v-SNAREs are able to fuse with t-SNARE bearing liposomes. Another study with isolated vacuolar SNARE proteins and liposomes also showed that fusion automatically follows after trans-SNARE pairing unless a special mechanism exists to prevent it, as in regulated exocytosis (19). In other words, pairing of these SNARE proteins by itself results in spontaneous and efficient bilayer fusion. Studies in permeabilized cells also show that trans-SNARE complex assembly is coincident with membrane fusion and the ability of SNAREs to fuse membranes is roughly proportional to their binding affinities, as reflected by their thermal stabilities (7).

Alternatively, in an *in vitro* system that reconstitutes vacuole fusion, the vast majority of v/t-SNARE complexes could be enzymatically disassembled by NSF, yet membrane fusion could still take place (7). It is important to note that whereas NSF disrupts cis-SNARE complexes, under the same conditions, the trans-SNARE complexes engaged in fusion are resistant to NSF (19). Whether a small amount of fusion competent trans-SNARE complexes persisted to catalyze fusion could not be ruled out by this approach (7). Experiments with sea urchin eggs also suggest that the fusion of secretory vesicles can proceed in the absence of SNARE complexes (7). Thus, the role of SNAREs in fusion is still controversial.

After fusion, the complex is disassembled by NSF and α-SNAP. α -SNAP binds the v- / t-SNARE complex and recruits NSF to it (2). NSF uses the energy of ATP hydrolysis to unravel the coiled-coil interaction between the helical domains of the SNARE proteins (20) freeing the v-SNAREs and t-SNAREs for subsequent fusion events (2). The requirement for NSF-mediated reactivation of SNAREs may also allow the cell to control when and where membranes fuse (20). Although NSF and SNAP are required to sustain continuous fusion, they are not required for bilayer fusion *per se* (19). Lastly, the v-SNARE can recycle back to the donor membrane (7).

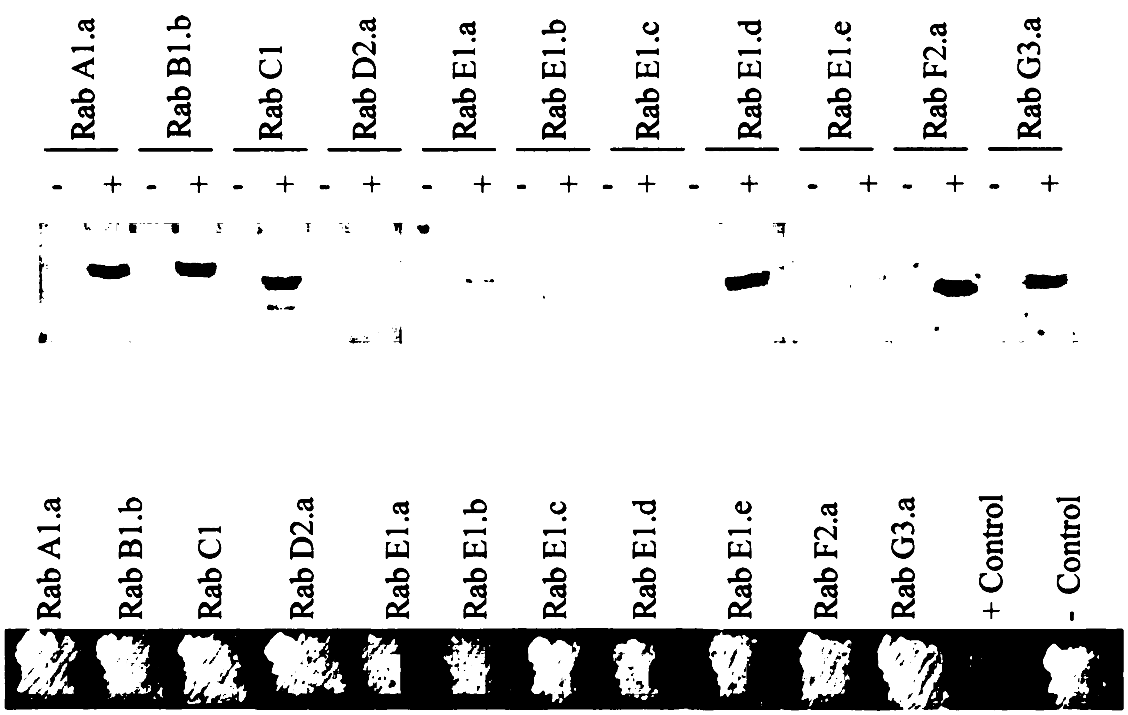


Figure A-1. AvrPto interacts with the Rab E family. AvrPto was fused to the DNA binding domain in pNLexA and the Rabs were fused to the transcriptional activation domain in pB42AD. (A) Immunoblot of each Rab probed with antibody raised against the hemagglutinin epitope. Yeast strains were grown in media containing glucose (-) or galactose (+). Galactose is necessary for protein expression. (B) Physical interaction between AvrPto and Rab proteins in the LexA two hybrid system. Yeast strains were grown at 30°C for 5 days on galactose X-gal plates. Blue color indicates interaction whereas white color indicates no interaction. AvrPto interacts with members of the RabE family.

This next section will focus specifically on the last step of the secretion pathway, from the trans-Golgi network to the plasma membrane. The specific class of Rabs involved at this step includes Rab8 in mammals, Sec4 in yeast, and RabE in plants. While there is abundant information available for this class of Rabs in mammals in yeast, there is very little information about them in plants.

Although there is some evidence that some Rabs function in vesicle budding, Rab8 has not been shown to be involved in this step of vesicle transport. It has been shown, on the other hand, to play a role in the motility of vesicles as suggested from both *in vivo* and *in vitro* studies. Specifically, ectopic expression of wild-type or activated Rab8 in cells resulted in reorganization of the cytoskeleton and changes in cell morphology (6, 21). Rab8 was shown to be able to promote the reorganization of actin and microtubules (9). In addition, genetic interactions have been uncovered in yeast between sec4, and the myosin heavy chain Myo2p, indicating a possible mechanism whereby vesicles are propelled by motor proteins along polarized actin cables towards the site of exocytosis (13).

Hattula *et al.* (22) found a novel human protein, called Rabin8, which binds only to Rab8 when it is bound to GDP. They show that both Rabin8 and Rabin3 (from rat) are GEFs that facilitate the release of GDP from Rab8, and GTP association. They also found that a non-Rab8-binding region of Rabin8's carboxy terminus is essential for targeting Rab8 vesicles to the cell surface. Rabin8 co-localized with cortical actin. However, they were not able to demonstrate a direct *in vitro* association between purified actin and Rabin8. Rabin8 localizes to the plasma membrane, but when co-expressed with the dominant negative form of Rab8 (Rab8 T22N), Rab8 T22N relocalized from the perinuclear region to numerous vesicles and Rabin8 was translocated from the plasma membrane to the Rab8-T22N containing vesicles. Hattula *et al.* (22) conclude that Rab8's activation takes place on intracellular vesicles (the donor compartments). They acknowledge that Rabin8-mediated activation of Rab8 might also occur on the plasma membrane. Lastly, they demonstrated that Rabin8's carboxy terminus is essential for Rabin8 to move to Rab8-containing vesicles and for polarized delivery of these vesicles to the cell surface (22). In yeast, it is has been shown that Sec2, Rabin8, and Rabin3 are GEF's for Sec4 (6), and genetic studies suggest that Gyp1 acts as a GAP for Sec4 (13).

The Exocyst complex is an effector for Sec4 (3). It plays a role in fusion of trans-Golgi vesicles with the plasma membrane (6) at a point upstream from the SNAREs (7). The Exocyst marks the sites of exocytosis on the plasma membrane and mediates vesicle targeting (13). The Exocyst is a large (1000- to 2000-kD (6)) 19.5S particle complex that contains Sec3, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and exo70p (8). One of the Exocyst components, Sec3, stably associates with the secretion-active site on the plasma membrane, even in the absence of membrane trafficking (7, 8), which suggests that Sec3 may serve as a spatial landmark for this site (3, 6, 7). Another Exocyst subunit, Sec15, can bind to the GTP-bound form of Sec4p, which localizes to secretory vesicles (6, 7). Interestingly, incorporation of Sec3p into the Exocyst requires Sec4p Rab function, suggesting that the Rab may regulate assembly of the Exocyst (7).

Rab 8-interacting protein (Rab8ip) interacts with the GTP-bound form of Rab8 (17). It is serine/threonine protein kinase that is a component of the stress-activated protein kinase pathway in humans. Although Rab8ip was primarily found in the cytosol, substantial amounts of the kinase were associated with sedimentable membranes in a salt-sensitive linkage (17). Immunofluorescence microscopy showed that it was concentrated in the plasma membrane and the Golgi region (similarly to Rab8) (17). Protein phosphorylation-dephosphorylation events are known to be required for many stages in intracellular protein traffic. Protein kinases have been implicated in the generation of secretory vesicles in polarized secretion. One possible mechanism is that the kinase serves to regulate Rab8 by selectively phosphorylating active Rab8 molecules and, consequently, modulating their function in vesicular transport. However, Ren *et al.* (17) found that, *in vitro*, Rab8 is not a substrate for Rab8ip. An alternative model is that rab8 regulates the kinase activity of Rab8ip, which serves as its effector. It is even possible that this phosphorylation may render v-, t-SNAREs and/or v-/t-SNARE complexes competent for vesicle targeting and/or fusion (17). It is well established that phosphorylation can prevent the assembly of t-SNAREs and dephosphorylation may relieve such inhibitory effects on membrane fusion (19). If Rab8 affects the kinase activity of this effector, then Rabs may modulate secretion in response to stress stimuli or Rab regulated protein phosphorylation may be important for vesicle targeting and fusion (6).

Rabs may also play a role in plant defense against pathogens. The expression of *AtSNAP33*, a homolog of the t-SNARE SNAP 25 in *Arabidopsis*, is induced after inoculation with pathogens in inoculated leaves as well as in systemic leaves (5). The local induction is partially SA-independent whereas the systemic induction requires SA. SA increases the level of expression of *AtSNAP33* in the absence of a pathogen. Interestingly, *AtSNAP33* is induced in *npr1* (n̲on-expressor of P̲R̲ genes) plants after pathogen infection. Wick *et al.* (5) hypothesize that after pathogen attack, increased vesicle fusion may be required to permit increased secretion of PR proteins. In addition, increased vesicle fusion may be required for repair of damage to the plasma membrane provoked by reactive oxygen species. Reactive oxygen species are produced after pathogen attack and mechanical stimulation. It has been shown in sea urchin eggs that SNAP-25 is required for membrane resealing after injury (5). Ethylene, another plant hormone that plays a role in plant defense, rapidly and transiently increased expression and GTP binding activity of Rab8 (23).

PEN1 is a syntaxin that is localized to the plasma membrane and is required for resistance of *Arabidopsis* to *Blumeria graminis hordei* (24). It was later shown that PEN1is required for timely papillae association (25). Bogdanove and Martin (1) screened a tomato cDNA library for proteins that interact with the bacterial effector protein, AvrPto. They suggest that one of the interactors, RabE, might regulate the polarized secretion of antimicrobial compounds and/or components involved in mounting cellular responses to attack by bacterial pathogens.

References

1. Bogdanove, A. J. & Martin, G. B. (2000) AvrPto-dependent Pto-interacting proteins and AvrPto-interacting proteins in tomato. *Proc Natl Acad Sci U S A*, 97, 8836-8840.

2. Buchanan, B. B., Gruissem, W. & Jones, R. L. (2000) *Biochemistry & molecular biology of plants* (Rockville, Md., American Society of Plant Physiologists).

3. Segev, N. (2001) Ypt and Rab GTPases: insight into functions through novel interactions. *Curr Opin Cell Biol*, 13, 500-511.

4. Pimpl, P. & Denecke, J. (2002) Protein-protein interactions in the secretory pathway, a growing demand for experimental approaches in vivo. *Plant Mol Biol*, 50, 887-902.

5. Wick, P., Gansel, X., Oulevey, C., Page, V., Studer, I., Durst, M. & Sticher, L. (2003) The expression of the t-SNARE AtSNAP33 is induced by pathogens and mechanical stimulation. *Plant Physiol*, 132, 343-351.

6. Segev, N. (2001) Ypt/rab gtpases: regulators of protein trafficking. *Sci STKE*, 2001, RE11.

7. Waters, M. G. & Hughson, F. M. (2000) Membrane tethering and fusion in the secretory and endocytic pathways. *Traffic*, 1, 588-597.

8. Schimmoller, F., Simon, I. & Pfeffer, S. R. (1998) Rab GTPases, directors of vesicle docking. *J Biol Chem*, 273, 22161-22164.

9. Novick, P. & Zerial, M. (1997) The diversity of Rab proteins in vesicle transport. *Curr Opin Cell Biol*, 9, 496-504.

10. Jurgens, G. & Geldner, N. (2002) Protein secretion in plants: from the trans-Golgi network to the outer space. *Traffic*, 3, 605-613.

11. Seabra, M. C. & Wasmeier, C. (2004) Controlling the location and activation of Rab GTPases. *Curr Opin Cell Biol*, 16, 451-457.

12. STENMARK, H. & OLKKONEN, V. M. (2001) The Rab GTPase family. *Genome Biol*, 2, REVIEWS3007. Epub 2001 Apr 3027.

13. ZERIAL, M. & MCBRIDE, H. (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol*, 2, 107-117.

14. HAY, J. C. & SCHELLER, R. H. (1997) SNAREs and NSF in targeted membrane fusion. *Curr Opin Cell Biol*, 9, 505-512.

15. FUKUDA, R., MCNEW, J. A., WEBER, T., PARLATI, F., ENGEL, T., NICKEL, W., ROTHMAN, J. E. & SOLLNER, T. H. (2000) Functional architecture of an intracellular membrane t-SNARE. *Nature*, 407, 198-202.

16. HEESE, M., GANSEL, X., STICHER, L., WICK, P., GREBE, M., GRANIER, F. & JURGENS, G. (2001) Functional characterization of the KNOLLE-interacting t-SNARE AtSNAP33 and its role in plant cytokinesis. *J Cell Biol*, 155, 239-249.

17. REN, M., ZENG, J., DE LEMOS-CHIARANDINI, C., ROSENFELD, M., ADESNIK, M. & SABATINI, D. D. (1996) In its active form, the GTP-binding protein rab8 interacts with a stress-activated protein kinase. *Proc Natl Acad Sci U S A*, 93, 5151-5155.

18. WEBER, T., ZEMELMAN, B. V., MCNEW, J. A., WESTERMANN, B., GMACHL, M., PARLATI, F., SOLLNER, T. H. & ROTHMAN, J. E. (1998) SNAREpins: minimal machinery for membrane fusion. *Cell*, 92, 759-772.

19. MCNEW, J. A., PARLATI, F., FUKUDA, R., JOHNSTON, R. J., PAZ, K., PAUMET, F., SOLLNER, T. H. & ROTHMAN, J. E. (2000) Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature*, 407, 153-159.

20. ALBERTS, B. (2002) *Molecular biology of the cell* (New York, Garland Science).

21. PERANEN, J., AUVINEN, P., VIRTA, H., WEPF, R. & SIMONS, K. (1996) Rab8 promotes polarized membrane transport through reorganization of actin and microtubules in fibroblasts. *J Cell Biol*, 135, 153-167.

22. HATTULA, K., FURUHJELM, J., ARFFMAN, A. & PERANEN, J. (2002) A Rab8-specific GDP/GTP exchange factor is involved in actin remodeling and polarized membrane transport. *Mol Biol Cell*, 13, 3268-3280.

23. MOSHKOV, I. E., MUR, L. A., NOVIKOVA, G. V., SMITH, A. R. & HALL, M. A. (2003) Ethylene regulates monomeric GTP-binding protein gene expression and activity in Arabidopsis. *Plant Physiol*, 131, 1705-1717.

24. COLLINS, N. C., THORDAL-CHRISTENSEN, H., LIPKA, V., BAU, S., KOMBRINK, E., QIU, J. L., HUCKELHOVEN, R., STEIN, M., FREIALDENHOVEN, A., SOMERVILLE, S. C. & SCHULZE-LEFERT, P. (2003) SNARE-protein-mediated disease resistance at the plant cell wall. *Nature*, 425, 973-977.

25. ASSAAD, F. F., QIU, J. L., YOUNGS, H., EHRHARDT, D., ZIMMERLI, L., KALDE, M., WANNER, G., PECK, S. C., EDWARDS, H., RAMONELL, K., SOMERVILLE, C. R. & THORDAL-CHRISTENSEN, H. (2004) The PEN1 Syntaxin Defines a Novel Cellular Compartment upon Fungal Attack and Is Required for the Timely Assembly of Papillae. *Mol Biol Cell*, 15, 5118-5129.