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The Arabidopsis Thaliana-Pseudomonas Syringae Interaction

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Introduction

Pseudomonas syringae is a Gram-negative, rod-shaped bacterium with polar flagella (Figure 1; Agrios, 1997). Strains of *P. syringae* collectively infect a wide variety of plants. Different strains of *P. syringae*, however, are known for their diverse and host-specific interactions with plants (Hirano and Upper, 2000). A specific strain may be assigned to one of at least 40 pathovars based on its host range among different plant species (Gardan et al., 1999) and then further assigned to a race based on differential interactions among cultivars of the host plant. Understanding the molecular basis of this high level of host specificity has been a driving force in using *P. syringae* as a model for the study of host-pathogen interactions. In crop fields, infected seeds are often an important source of primary inoculum in *P. syringae* diseases, and epiphytic bacterial growth on leaf surfaces often precedes disease development (Hirano and Upper, 2000). *P. syringae* enters the host tissues (usually leaves) through wounds or natural openings such as stomata, and in a susceptible plant it multiplies to high population levels in intercellular spaces. Infected leaves show water-soaked patches, which eventually become necrotic. Depending on *P. syringae* strains, necrotic lesions may be surrounded by diffuse chlorosis. Some strains of *P. syringae* also cause cankers and galls (Agrios, 1997). In resistant plants, on the other hand, *P. syringae* triggers the hypersensitive response (HR), a rapid, defense-associated death of plant cells in contact with the pathogen (Klement, 1963; Klement et al., 1964; Bent, 1996; Greenberg, 1996; Dangl et al., 1996; Hammond-Kosack and Jones, 1997). In this situation, *P. syringae* fails to multiply to high population levels and causes no disease symptoms.

In the late 1980s, several strains belonging to pathovars *tomato*, *maculicola*, *pisi*, and *atropurpurea* of *Pseudomonas syringae* were discovered to infect the model plant *Arabidopsis thaliana* (reviewed by Crute et al., 1994). The establishment of the *Arabidopsis-P. syringae* pathosystem triggered a period of highly productive research that has contributed to the elucidation of the fascinating mechanisms underlying plant recognition of pathogens, signal transduction pathways controlling plant defense responses, host susceptibility, and pathogen virulence and avirulence determinants. In this chapter we trace the discovery of this pathosystem, overview the most salient aspects of this interaction, and point out the gaps in our knowledge. At the end of this chapter we will also provide a glossary of relevant pathology-related technical terms (Appendix I), a list of people who are studying this interaction so readers can seek help if they have further

questions about the *Arabidopsis-P. syringae* interaction (Appendix II), and several experimental procedures commonly used in the study of the *Arabidopsis-P. syringae* interaction (Appendix III).

1. EARLY DEVELOPMENT OF THE ARABIDOPSIS-PSEUDOMONAS SYRINGAE SYSTEM

1.1. Beginning: Are there any Arabidopsis pathogens?

In the 1980s, *P. syringae* was the first pathogen to be demonstrated to infect *Arabidopsis* and to cause disease symptoms in the laboratory setting. This was achieved by screening many *P. syringae* strains on various *Arabidopsis* accessions (Dong et al., 1991; Whalen et al., 1991; Dangl et al., 1992). The two virulent strains most widely used today, *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *maculicola* ES4326, originated from these early studies. When a suspension of 10^8 bacteria/ml (a high dose of bacteria) is sprayed with a surfactant onto susceptible *Arabidopsis* plants (e.g., ecotype Columbia), the first sign of disease is the appearance of "water-soaked" patches on leaves on day 2. The water-soaked symptom results from massive release of water and, presumably, nutrients from infected *Arabidopsis* cells. The water-soaked patches become necrotic and dark-colored on day 3, and the surrounding leaf tissue shows extensive chlorosis, giving the characteristic appearance of a 'speck' disease (Figure 2, Figure 3E and 3F). In addition, closely related, but avirulent strains (such as *P. syringae* pv. *tomato* JL1965 and *P. syringae* pv. *maculicola* M2) that became sources of some avirulence (*avr*) genes were also identified (Dong et al., 1991; Whalen et al., 1991; Dangl et al., 1992). The main reason for examining *P. syringae* strains as potential pathogens of *Arabidopsis* was because *P. syringae* had already been proven to be an excellent genetically tractable pathogen of soybean, tomato, and bean in the mid-1980s (Keen, 1990). Development of the *Arabidopsis-P. syringae* pathosystem would provide a system in which both the plant and the pathogen are amenable to rigorous genetic analysis. However, it is interesting to note that even after the demonstration of disease symptoms caused by *P. syringae* on *Arabidopsis*, not many people were convinced that this was a good model system for two reasons: there was no report of naturally occurring *P. syringae* disease in *Arabidopsis* in the wild, and in the laboratory inoculation required artificial methods (infiltration or use of surfactant).

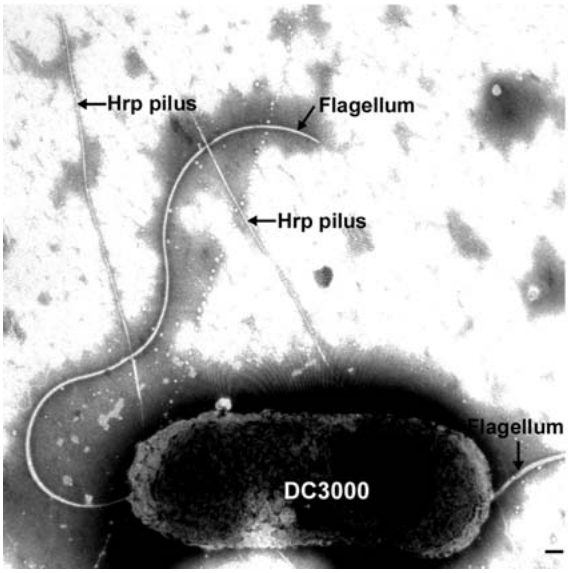


Figure 1. A transmission electron microscope image of *Pseudomonas syringae* pv. *tomato* DC3000. Note that DC3000 produces polar flagella (15 nm in diameter) and a few Hrp pili (8 nm in diameter). The flagella and Hrp pili are indicated with arrows. Flagella enable bacteria to swim toward or away from specific chemical stimuli. Hrp pili are involved in type III secretion of avirulence and virulence proteins.

1.2. Establishing the system: gene-for-gene

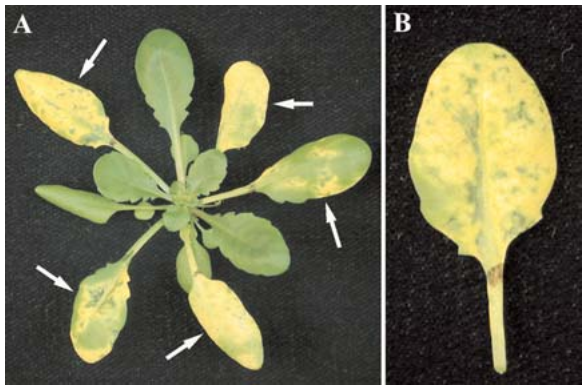


Figure 2. Disease symptoms in *Arabidopsis* leaves caused by DC3000 infection. Leaves (indicated with arrows) were syringe-infiltrated with 5 x 10^5 cfu/mL of *Pst* DC3000 and pictures were taken four days after inoculation. The whole plant is shown in **(A)**. A close-up of a diseased leaf is shown in **(B)**.

interactions

A significant milestone in the development of the *Arabidopsis-P. syringae* system was the demonstration that this pathosystem conforms to the gene-for-gene relationship that underlies many well-known plant-pathogen interactions in nature (such as the flax-rust fungus interaction or the soybean-*P. syringae* interaction) (Keen, 1990). The gene-for-gene hypothesis was advanced by H.H. Flor, based on his work on the flax-rust fungus interaction in the 1940s and 1950s (Flor, 1971). This hypothesis states that when a pathogen (in this case a *P. syringae* strain) has an avirulence (*avr*) gene, and a plant host (in this case the *Arabidopsis* plant) has the corresponding disease resistance (*R*) gene, the plant is resistant to the pathogen (Table 1). It is defined by a single plant *R* gene for a single pathogen *avr* gene, hence the name gene-for-gene resistance. Table 2 defines the terminology involved in gene-for-gene resistance. When the plant is resistant, the pathogen is said to be avirulent and the interaction is said to be incompatible. When the plant is susceptible, the pathogen is said to be virulent and the interaction is said to be compatible. The laboratories of Fred Ausubel and Brian Staskawicz showed that an *avr* gene, *avrRpt2*, from an avirulent *P. syringae* pv. *tomato* strain, JL1065, converted DC3000 and ES4326 into avirulent strains in the *Arabidopsis* ecotype Columbia (Dong et al., 1991; Whalen et al., 1991). Subsequent *Arabidopsis* mutagenesis and screening efforts led to the identification of mutations in the *Arabidopsis RPS2* disease resistance gene (Kunkel et al., 1993; Yu et al., 1993). Thus, a demonstration of the *avrRpt2-RPS2* gene-for-gene interaction was completed. Similar efforts in the laboratory of Jeff Dangl led to the identification of the *avrRpm1* gene in *P. syringae* pv. *maculicola* strain M2 and the *RPM1* resistance gene in *Arabidopsis* ecotype Columbia (Dangl et al., 1992). Interestingly, the *RPM1* resistance gene also recognizes *avrB*, which was isolated initially from *P. syringae* pv. *glycinea* in the soybean-*P. syringae* interaction (Bisgrove et al., 1994). These pioneering efforts spurred subsequent research to identify additional *P. syringae avr* genes and the corresponding *Arabidopsis* resistance gene loci, the eventual cloning of the first *Arabidopsis* resistance gene, *RPS2* (Bent et al., 1994; Mindrinos et al., 1994), and identification of non-*R* gene plant components involved in gene-for-gene resistance (see section 3.1.5).

Early concerns about the lack of natural infection and artificial inoculation methods have still not been addressed, but the *Arabidopsis-P. syringae* system has flourished as a widely recognized model plant-pathogen system. This fact presents us a lesson on what is important in developing a new model system. A model system cannot address every single aspect of a natural system. For example, the *Arabidopsis-P. syringae* system is probably not an appropriate system to model a bacterial invasion process seen in *P. syringae* infection of beans in the field because *P. syringae* infection of *Arabidopsis* requires an artificial infection method. However, it has been a great system in which to study broadly observed phenomena, such as gene-for-gene interactions. Thus, we have to define appropriate questions to ask in a model system; it is not reasonable to dismiss a model system simply because it cannot address every single aspect of natural systems.

Success in the *Arabidopsis-P. syringae* system encouraged development of other *Arabidopsis* pathogen systems (see **chapters by Dangl, Somerville, Innes, Buell, and Crute**). At the same time, comparisons with other plant-pathogen systems, especially the *Arabidopsis-Peronospora parasitica* system, have helped advancement of the *Arabidopsis-P. syringae* system.

2. EARLY INTERACTIONS IN THE ARABIDOPSIS INTERCELLULAR SPACE (APOPLAST)

Table 1. Gene-for-gene resistance

Pathogen	Plant host	
	R/R or *R/r*	*r/r*
avr	Resistant	Susceptible
No *avr*	Susceptible	Susceptible

Table 2. Terminology in gene-for-gene resistance

Interaction	Pathogen	Plant host
Compatible	Virulent	Susceptible
Incompatible	Avirulent	Resistant

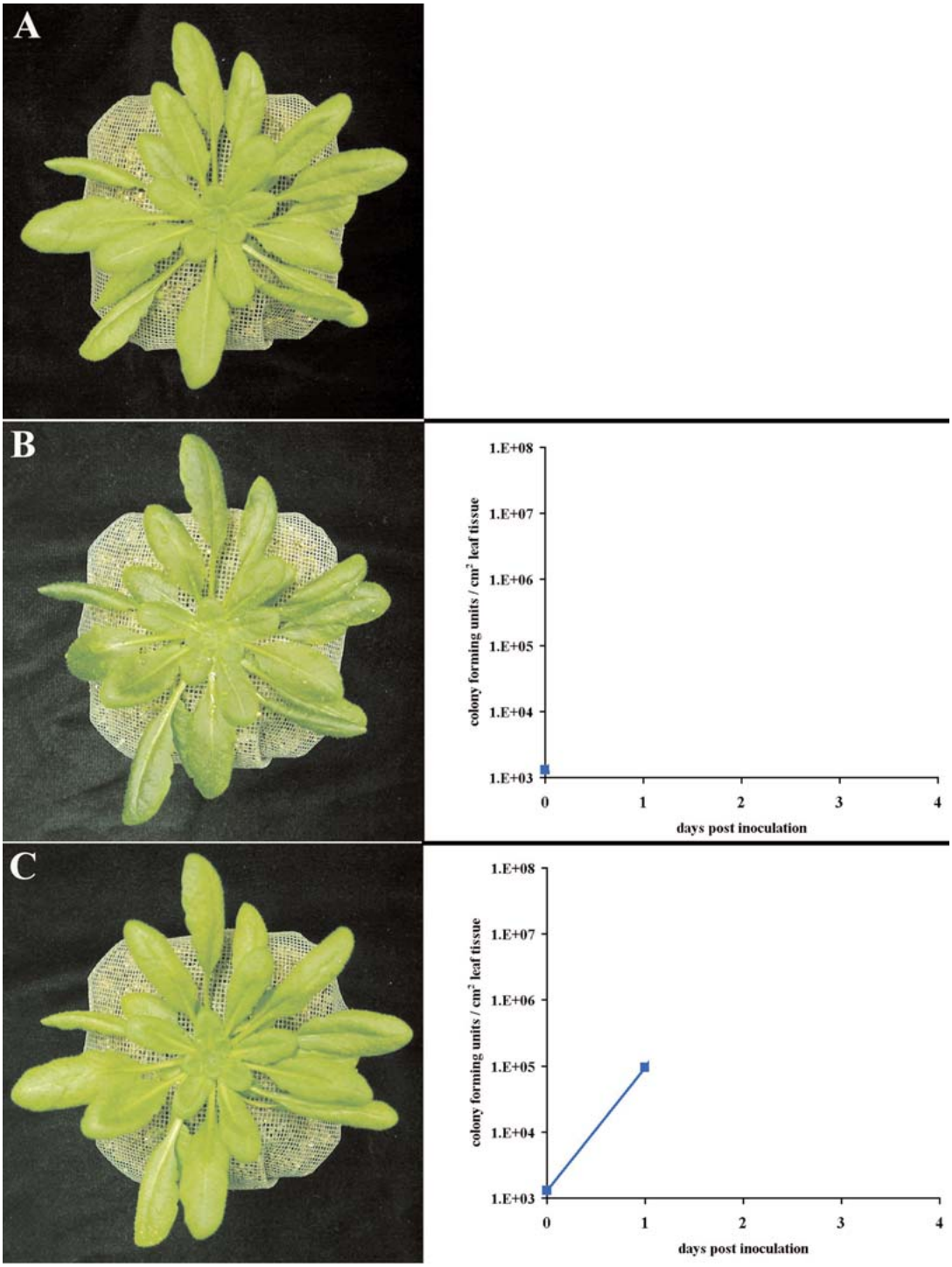
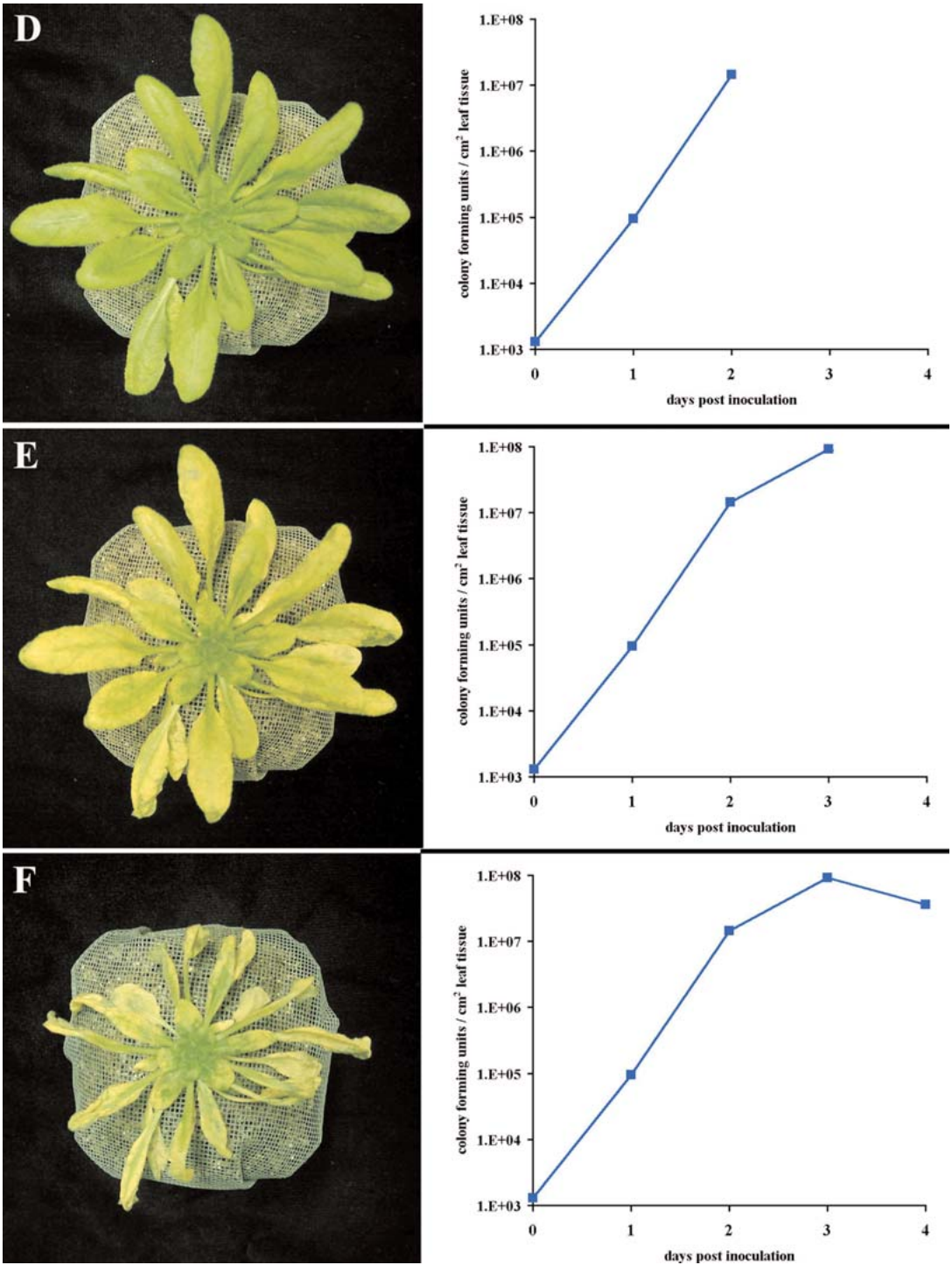


Figure 3. Disease symptom development in a susceptible *Arabidopsis* plant 1, 2, 3 and 4 days after inoculation. Leaves were vacuum infiltrated with 1 x 10^6 bacteria/ml of DC3000. A picture was taken before inoculation **(A)** immediately after vacuum infiltration **(B)** and every day for 4 days (**C** to **F**). To the right of each picture is a plot of the level of bacteria present within the leaves



at that particular time. Note, water-soaking symptoms, appeared at 48 to 60 hours. Significant chlorosis and necrosis occurred at 72 to 96 hours after inoculation. Note that bacteria multiplied to a near maximum level before chlorosis or massive cell death appeared.

As mentioned above, *P. syringae* often first flourishes on the surface of plants as an epiphyte in the wild before it enters the intercellular space to initiate pathogenesis (Hirano and Upper, 2000). The ability of *P. syringae* to grow epiphytically is ecologically important for pathogen survival and spread in the field and is a topic of intensive study. In the laboratory, however, where the *Arabidopsis-P. syringae* system is studied, we often bypass the epiphytic growth phase and place *P. syringae* directly in the intercellular space by syringe injection, or we artificially facilitate the entry of *P. syringae* into the intercellular space by spraying leaves with high doses of bacterial suspension in the presence of a surfactant (e.g., Silwet L-77). In addition, the commonly used strain DC3000 is a very poor epiphyte in the field (Kyle Willis, University of Wisconsin, Madison, personal communication) and therefore, the *Arabidopsis-P. syringae* interaction is not a good model for the study of epiphytic interaction. In this chapter, which is focused on the *Arabidopsis-P. syringae* pathogenic interaction, we therefore begin our discussion with the events occurring immediately after *P. syringae* arrives in the intercellular space, where bacteria are in direct contact with *Arabidopsis* cells that are about 10,000 times larger, are enveloped with a >100 nm thick cell wall, and are full of exploitable photosynthates behind the wall (Figure 4). The mesh size of the cell wall is in the order of 20 to 30 nm, and this is too small for a bacterium of a few μm to simply penetrate. Both *P. syringae* and *Arabidopsis* must react to this situation quickly, obviously for different reasons. Whatever happens in the first few hours of the encounter will determine whether *P. syringae* will be successful in becoming a virulent pathogen of *Arabidopsis* or the *Arabidopsis* plant will effectively stop further infection of *P. syringae.*

The precise details of the early events after *P. syringae* enters the leaf apoplast are still not clear, but a few key steps have been revealed or can be speculated about. From the plant side, it is believed that *Arabidopsis* (and presumably all other plants) have developed mechanisms to detect the invasion of any microbe and respond with the first line (a basal level) of general defense and that this defense is effective enough to stop some microbes (e.g., saprophytes, which lack the ability to flourish in the living tissues). The first line of defense is not well characterized but presumably involves expression of some defense genes (Jakobek et al., 1993). It is relatively benign – it does not sacrifice the plant cell under attack. However, it is still costly to the cell, and that is why it is regulated. How *Arabidopsis* cells detect the presence of a microbe at this stage is not clear, but it likely involves sensing of constitutively expressed extracellular molecules or structures of the microbe. One example may be the bacterial flagellin protein, the structural protein of the bacterial flagellum. Felix et al. (1999) showed that a peptide whose sequence is well conserved among flagellins of eubacteria, including *Pseudomonas*, can elicit general defense responses in plants (Felix et al., 1999). An *Arabidopsis* gene involved in the perception of this peptide, *FLS2*, has been cloned and it encodes a leucine-rich-repeat (LRR) receptor-like protein kinase (Gomez-Gomez and Boller, 2000). Thus, the putative *FLS2* receptor can potentially respond to a wide variety of bacterial pathogens, including *P. syringae*, and activate a general defense response. It is interesting that flagellins of *Agrobacterium* and *Rhizobium*, which do not elicit strong defense in plants, do not have this peptide sequence conserved (Felix et al., 1999).

For *P. syringae*, the plant intercellular space is a potential niche from which to exploit the bulk of photosynthates and other nutrients hidden behind the host cell wall. The normal apoplast is believed to be limited in water and possibly nutrients (although theoretical considerations argue for sufficient nutrients in the apoplast, see Hancock and Huisman, 1981) and is a depository for some plant defense compounds. In order to flourish and attain an extremely high population density (typically $5x10^7$ bacteria/cm^2) in the apoplast, *P. syringae* must produce appropriate virulence factors to cause *Arabidopsis* cells to 'leak' nutrients and water into the intercellular space and at the same time to suppress or evade *Arabidopsis* defense aimed at inhibiting bacterial proliferation. Because no massive host cell death occurs before *P. syringae* has achieved a near maximum population in infected leaves

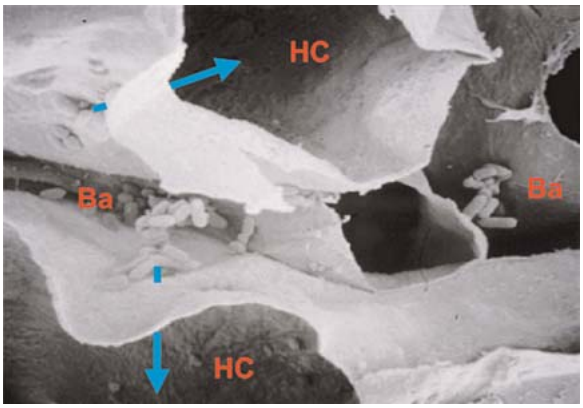


Figure 4. A scanning electron microscopic image of a cross section of an *Arabidopsis* (ecotype Columbia; susceptible) leaf infected with DC3000. HC: host cells. Ba: Bacteria. Arrows indicate the direction of type III secretion from bacteria in the apoplast into the host cell interior. Note that the host cell wall remains intact, physically separating bacteria and host cells until the very late stages of the interaction, when host cells collapse.

(Figure 3), it is believed that nutrient and water release from host cells during the early to mid (probably most critical) stages of *P. syringae* infection is not caused by nonspecific host cell rupture. The exact arsenal of *P. syringae* virulence factors has not been determined, but two virulence systems have been shown to play a key role: the type III protein secretion system that delivers a battery of bacterial avirulence and virulence proteins (type III effectors, hereinafter) to the apoplast and also into the *Arabidopsis* cells (Alfano and Collmer, 1997; Lindgren, 1997; He, 1998; Preston, 2000) and a diffusible toxin coronatine that partially mimics plant hormone methyl jasmonate (MeJA) (Bender et al., 1999; Preston, 2000). Both of these virulence systems are induced in plant tissues, presumably because they are not needed before bacteria encounter plant cells. A detailed description of these two virulence systems will be presented in section 4, but it is important to mention here that direct injection of bacterial virulence proteins into host cells via the type III secretion system is a widespread phenomenon in bacterial pathogens of plants and animals, and is considered to be an evolutionarily critical invention of bacterial pathogens (He, 1998; Hueck, 1998; Galan and Collmer, 1999).

Once *P. syringae* has injected type III effector proteins, which include Avr proteins (see below for more discussion of the relationship between Avr proteins and type III effector proteins), into *Arabidopsis* cells via the type III protein secretion system, there are two outcomes, depending on the genotypes of the infecting *P. syringae* and *Arabidopsis* plants. These two outcomes are most elegantly (albeit in an oversimplified manner) explained by the gene-for-gene hypothesis, i.e., if an infected *Arabidopsis* plant has an *R* gene that recognizes a *P. syringae* type III effector (i.e., an Avr protein in this situation), a rapid defense mechanism of the plant will be triggered. Alternatively, if the infected *Arabidopsis* plant has no corresponding *R* gene and/or the *P. syringae* strain has no *avr* gene, defense responses will be activated slowly, the infection will continue, and the plant will succumb to *P. syringae* and become diseased. In a given *Arabidopsis-P. syringae* system, it is possible that more than one specific combination of *avr* and *R* genes are operating at the same time, and such different combinations are often co-dominant (Table 3). For example, it is possible to have a strain of *P. syringae* carrying *avr* genes interacts with an *Arabidopsis* plant carrying two corresponding *R* genes. The two combinations, *avr1*/*R1* and *avr2*/*R2*, may be co-dominant in the system.

All known *P. syringae avr* genes (with the exception of *avrD*; Keen et al., 1990) that trigger gene-for-gene resistance encode type III effector proteins that are apparently delivered by bacteria to the plant cell via the type III secretion system (Mudgett and Staskawicz, 1998; Collmer et al., 2000; Kjemtrup et al., 2000; White et al., 2000). Why would *P. syringae* inject Avr proteins into *Arabidopsis* cells to trigger host resistance, thus inhibiting bacterial growth? The likely answer is that *avr* genes actually function as virulence genes when host plants do not carry the corresponding *R* genes. In fact, virulence functions of *avrRpm1* and *avrRpt2* on plants lacking the *RPM1* and *RPS2* resistance genes, respectively, have been demonstrated (Ritter and Dangl, 1995; Chen et al., 2000; Guttman and Greenberg, 2001). Thus, a more appropriate view of *avr* genes is probably that these are virulence genes first evolved to promote bacterial parasitism and that plants then counter-evolved surveillance systems to recognize virulence gene-based molecules (effectively turning virulence genes into avirulence genes). When *P. syringae* injects these proteins into *Arabidopsis* cells with the original purpose of parasitizing the *Arabidopsis* cells, it does not “know” that the recipient *Arabidopsis* cells may already be armed with one or more corresponding *R* genes, which would turn these virulence-intended proteins into defense elicitors-an elegant example of the adaptive co-evolution of pathogen virulence and plant resistance traits.

3. P. SYRINGAE ATTACKS AND ARABIDOPSIS COUNTER-ATTACKS

Tremendous progress has been made in understanding how *Arabidopsis* recognizes *P. syringae* Avr proteins and

Table 3. Co-dominance in gene-for-gene resistance

Pathogen	Plant host			
	R1R2	R1r2	r1R2	r1r2
avr1 avr2	Resistant	Resistant	Resistant	Susceptible
- avr2	Resistant	Susceptible	Resistant	Susceptible
avr1 -	Resistant	Resistant	Susceptible	Susceptible
No *avr*	Susceptible	Susceptible	Susceptible	Susceptible

mounts effective defense against *P. syringae*. We now know that the pathogen recognition and defense signal transduction mechanisms underlying the *Arabidopsis-P. syringae* interaction share many common features with those observed in other *Arabidopsis*-pathogen interactions. Readers are encouraged to consult several excellent reviews on this topic (Glazebrook, et al., 1997; Dong, 1998; Innes, 1998; Bent, 2001; Thomma, et al., 2001; Glazebrook, 2001; Dangl and Jones, 2001; Staskawicz et al, 2001). In addition, several chapters of this book describe other *Arabidopsis*-pathogen systems or discuss pathogen recognition and disease signal transduction. We therefore will highlight only examples that particularly illustrate either the important contribution of using *P. syringae* as a model or the uniqueness of the *Arabidopsis-P. syringae* system.

3.1. Pathogen avirulence and plant resistance in incompatible Arabidopsis-P. syringae interactions

3.1.1. Gene-for-gene resistance in the Arabidopsis-P. syringae system

Gene-for-gene incompatibility is prevalent among various plant-pathogen systems and one of the best characterized genetic relationships between plant hosts and pathogens. The prevalence of gene-for-gene resistance and similarities in associated responses among different plant-pathogen systems strongly suggest common underlying molecular mechanisms (Bent, 1996; Hammond-Kosack and Jones, 1997) and, therefore, gene-for-gene resistance was chosen to be the first target of study for the use of this model system. To study gene-for-gene resistance, first, a single *avr* gene was isolated by introducing a cosmid library made from an avirulent strain into a virulent strain. A cosmid clone containing an *avr* gene transformed the virulent strain to an avirulent strain. Use of such a strain carrying only a single *avr* gene created a situation in which only a single gene-for-gene interaction was operating in the plant-pathogen system. This step was crucial because different combinations of *avr* and *R* genes are usually co-dominant. For identification of corresponding *R* genes after genetic isolation of *avr* genes, r¯ plants were identified either by mutational analysis or by screens of various ecotypes. Genetically isolated *avr-R* gene combinations include *avrRpt2-RPS2* (Kunkel et al., 1993; Yu et al., 1993), *avrRpm1* (or nearly identical *avrPpiA1*)-*RPM1* (Dangl et al. 1992), *avrB-RPM1* (originally called *RPS3*, but later shown to be identical to *RPM1*) (Bisgrove et al., 1994), *avrRps4-RPS4* (Hinsch and Staskawicz, 1996), *avrPphB* (formerly known as *avrPph3*)-*RPS5* (Simonich and Innes, 1995), and *avrPphB-PBS1* (Warren et al., 1999).

Breakdowns in the narrowly defined version of the gene-for-gene concept are seen here. The *R* gene *RPM1* corresponds to two *avr* genes, *avrRpm1* and *avrB*. The *avr* gene *avrPphB* corresponds to two *R* genes, *RPS5* and *PBS1*. AvrRpm1 and AvrB are not homologous, neither are RPS5 and PBS1. We should remember that the gene-for-gene hypothesis was forwarded by Flor, based on the study of flax-flax rust fungus interactions in the 1940s and 1950s (Flor, 1971), when knowledge about how genes function at the molecular level was almost non-existent. With the knowledge we currently have about molecular interaction mechanisms, we can easily imagine a number of possible molecular interaction models to explain these situations, and we should interpret the gene-for-gene concept more broadly. In a broader interpretation of the gene-for-gene resistance, the key concepts should be that a plant has pathogen recognition mechanism(s) composed of a repertoire of genetically definable recognition specificities and that pathogen recognition by these mechanism(s) leads to a successful deployment of defense responses in the plant.

3.1.2. R genes in the Arabidopsis-P. syringae system

All of the above-mentioned *Arabidopsis R* genes, *RPS2*, *RPM1*, *RPS4*, *RPS5*, and *PBS1*, have been isolated by a map-based cloning approach (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995; Gassmann et al., 1999; Warren et al., 1998; Swiderski and Innes, 2001). All but *PBS1* belong to the nucleotide binding site-leucine rich repeat (NBS-LRR) class of *R* genes. The protein encoded by an *R* gene of the NBS-LRR class has one NBS structure that is located N-terminal to large imperfect LRRs, which are located close to the C-terminus (Bent, 1996; van der Biezen and Jones, 1998b). NBS-LRR is the dominating class among the *R* genes so far isolated from dicots and monocots (Ellis et al., 2000). Although each class member usually has a high specificity to a particular pathogen of a particular genotype (i.e., with the corresponding *avr* gene), *R* gene members of this class collectively can confer resistance against all major types of plant pests, namely bacteria, oomycetes, fungi, viruses, nematodes, and aphids. This fact provides strong molecular support to the notion of common underlying mechanisms responsible for many gene-for-gene resistance phenomena.

RPS2, *RPM1*, and *RPS5* proteins have coiled-coil (cc) structures (such as a leucine zipper) at their N-termini, and

are classified in the cc subclass of NBS-LRR (cc-NBS-LRR). *RPS4* has a TIR (for Toll and Interleukin-1 Receptor) homology at its N-terminus, which is conserved among the cytoplasmic domains of *Drosophila* Toll protein, mammalian interleukin-1 receptors, and other NBS-LRR R proteins (Whitham et al., 1994). Thus, *RPS4* belongs to the TIR subclass of NBS-LRR (TIR-NBS-LRR). *Arabidopsis* ecotype Col has ~140 NBS-LRR genes in its genome, including ~ 50 cc-NBS-LRRs and ~ 90 TIR-NBS-LRRs. Although it is generally assumed that the role of most, if not all, functional NBS-LRR genes is as *R* genes, it is not known how many of these NBS-LRR genes are functional *R* genes. It is conceivable that some of the NBS-LRR genes might function as a reservoir of sequence materials to create new recognition specificities through recombination.

NBS-LRR proteins are believed to be intracellular, based on computer predictions from their primary sequences. *RPS2* is not secreted or membrane-integrated in a heterologous *in vitro* system (Leister et al., 1996). *RPM1* is peripherally associated with the plasma membrane (Boyes et al., 1998). The plasma membrane localization of *RPM1* seems appropriate because the corresponding Avr proteins, AvrRpm1 and AvrB, are also localized at the plasma membrane (Nimchuk et al., 2000). It is possible that NBS-LRR proteins are localized at different subcellular compartments in the cell for optimal detection of signal molecules generated by the corresponding *avr* genes. For example, it will be interesting to see whether there are any NBS-LRR proteins localized in the nucleus to detect nuclear-transported pathogen signal molecules.

PBS1 encodes a predicted cytoplasmic protein serine/threonine kinase. The tomato *R* gene *PTO* (Martin et al., 1993) is so far the only other example of an *R* gene of the cytoplasmic protein kinase class. Both *PBS1* and the *RPS5* NBS-LRR genes are required for resistance against a *P. syringae* strain carrying *avrPphB* (Warren et al., 1999). This combination of protein kinase and NBS-LRR genes is reminiscent of the tomato *R* genes *PTO* (a protein kinase gene) and *PRF* (NBS-LRR), both of which are required for resistance against a *P. syringae* strain carrying *avrPto* (Salmeron et al., 1996). Although *PBS1* and *PTO* belong to a large subfamily of plant protein serine/threonine kinase genes, within the subfamily they are not very closely related. It is likely that the substrate specificities of these kinases are significantly different (Warren et al., 1998).

3.1.3. avr genes in the Arabidopsis-P. syringae system

Although direct demonstrations are still lacking, all the above-mentioned *avr* gene products, AvrRpt2, AvrRpm1, AvrB, AvrRps4, and AvrPphB, are believed to be delivered from bacteria into the plant cell via the type III secretion system for the following reasons: (i) These *avr* genes require type III secretion genes (called *hrp*/*hrc* genes in *P. syringae* and other pathogenic bacteria) to express their avirulence functions when *P. syringae* strains carrying *avr* genes are inoculated into plants carrying the corresponding *R* genes (Pirhonen et al., 1996; Gopalan et al., 1996). (ii) Direct expression of these *avr* genes in the plant cell leads to the HR, which is dependent on the corresponding *R* genes (Alfano et al., 1997; Gopalan et al., 1996; Leister et al., 1996; Scofield et al., 1996; Tang et al., 1996; McNeillis et al., 1998; Stevens et al., 1998; Nimchuk et al., 2000; Chen et al., 2000; Figure 5) – when expressed in the plant cell, these Avr proteins are predicted to stay in the cytoplasm. (iii) All but AvrRps4 seem to be modified in the plant cell in a host cell-specific manner (Mudgett and Staskawicz, 1999; Nimchuk et al., 2000). Again, it should be emphasized that *P. syringae* stays outside of plant cells (i.e., in the intercellular space) until plant cells start to disintegrate at a very late stage of the interaction.

Host cell-specific modifications of Avr proteins are

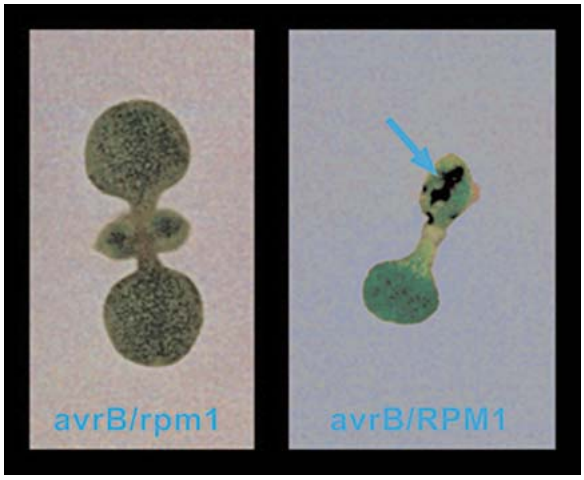


Figure 5. The *RPM1* resistance gene-dependent HR induced by the expression of the *P. syringae avrB* gene directly in *Arabidopsis.* Left panel: An *Arabidopsis rps3-1* (an *rpm1*mutant; Columbia background) seedling expressing *avrB* under the 35S promoter. No HR is present. Right panel: An F1 seedling from a cross between the *rps3-1*/*avrB* plant and a wild-type Columbia plant (*RPM1*$^{+}$). Arrow indicates dark HR necroses on the cotyledon leaf. This seedling died before true leaves emerged because of systemic development of the HR.

intriguing in the light of evolution of virulence/avirulence factors – evolving mechanisms that are dependent on host cell functions. AvrRpt2 protein is cleaved at a specific site when it is incubated with plant cell extracts or when it is directly expressed in the plant cell, whereas it is not cleaved when produced in *P. syringae* or *E. coli* (Mudgett and Staskawicz, 1999). The cleaving activity was not detected in *Arabidopsis* intercellular fluids, so the cleavage event is predicted to occur inside the plant cell. When a *P. syringae* strain carrying *avrRpt2* is inoculated into the leaf, the inoculated tissue accumulates the cleaved form of AvrRpt2. Therefore, AvrRpt2 seems to be transported into the plant cell and specifically cleaved inside the cell.

AvrRpm1, AvrB, and the processed form of AvrPphB (AvrPphB is rapidly cleaved when expressed in bacteria or plants) have canonical eukaryotic acylation sequences at their N-termini (Nimchuk et al., 2000). Mutations in the potential N-terminal myristoylation sites in AvrRpm1 and AvrB dramatically decreased their Avr activities when they were delivered by *P. syringae* (it is not known whether the mutations affected translocation of the proteins) or when they were expressed in the plant cell. When the proteins were expressed in the plant cell, they were myristoylated and localized to the plasma membrane in a myristoylation site-dependent manner. Because overexpression of the Avr proteins in the plant cell can overcome the requirement for the myristoylation site, myristoylation seems to be a mechanism to increase the concentration of the protein at the site of action, which is probably the intracellular side of the plasma membrane. AvrPphB protein expressed in the plant cell is cleaved and plasma membrane-localized in a myristoylation site-dependent manner. This observation demonstrates that proteins with canonical sites can be myristoylated post-translationally and supports the notion that proteins with canonical sites can be myristoylated after they are delivered from bacteria via the type III system.

3.1.4. Models for molecular mechanisms of gene-for-gene relationships

We have yet to determine how Avr-based signals are recognized by the *R*-mediated mechanism. Below we discuss two popular hypotheses.

Two observations led to the ligand-receptor model (or elicitor-receptor model) (Gabriel and Rolfe, 1990): (i) both *avr* and *R* genes are usually dominant, and (ii) in most cases, genetic single gene-single gene correspondence can be seen. In this model: (i) an *avr* gene generates a specific molecular signal (elicitor) directly (with the Avr protein as the signal) or indirectly (e.g., Avr protein is an enzyme that makes the signal molecule; Keen et al., 1990); (ii) the corresponding *R* gene encodes the receptor for the molecular signal; and (iii) this ligand-receptor interaction initiates signal transduction to induce downstream responses (Figure 6). Because the general concept of specific interactions between ligands and the cognate receptors has been known in biology for a long time, this model is an intuitively obvious one. Although the R protein in Figure 6 is depicted as a membrane receptor, the model based on genetic relationships does not specify the nature of the receptor, and the R protein could be an intracellular receptor. If an Avr protein itself is the specific molecular signal, this model predicts that the Avr protein and the corresponding R protein physically interact. The *P. syringae* AvrPto protein and the corresponding tomato Pto protein interact in the yeast two-hybrid assay, and the specificity for this interaction tightly correlates with the specificity in their *avr* and *R* functionalities (Scofield et al., 1996; Tang et al., 1996; Frederick et al., 1998). *PTO* belongs to a rare *R* gene class of protein kinase genes. The rice blast fungus Avr-Pita protein and the corresponding rice Pi-ta R protein interact in the yeast two-hybrid assay and *in vitro* (Jia et al., 2000). Pi-ta belongs to the NBS-LRR class, although its LRRs do not have a typical consensus sequence (Bryan et al., 2000). These observations of physical interactions between Avr and R proteins support the ligand-receptor model.

Although the ligand-receptor model is an obvious one, it does not provide simple explanations to some observations. (i) Plants do not have an efficient mechanism to create new pathogen recognition specificities and select good ones, compared with the vertebrate adaptive immune system, in which somatic recombination creates a vast repertoire of recognition specificities and clonal selection provides a way to select good specificities. Given this limitation, how can plants

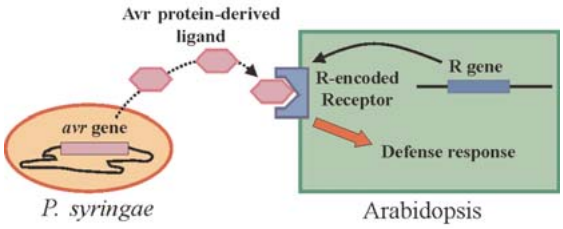


Figure 6. The ligand-receptor model of *R* gene and *avr* gene interaction. A specific signal molecule is directly or indirectly generated by the *avr* gene in *P. syringae*. The signal molecule is recognized by the receptor encoded by the corresponding *R* gene in *Arabidopsis*. This moleuclar recognition leads to rapid induction of defense response.

have enough recognition specificities, which are limited by the number of *R* genes in the genome, to effectively fend off most potential pathogens, when pathogens, which are in most cases microbes, can evolve much faster than plants? For example, *Arabidopsis* has only ~140 NBS-LRR genes. (ii) The same or very similar *R* genes can confer resistance against very different types of pathogens. The tomato *Mi* gene can confer resistance against both nematodes and aphids (Rossi et al., 1998). The potato *Rx* and *Gpa2* genes confer resistance against potato virus X and nematode, respectively, and are highly homologous (Bendahmane et al., 1999; van der Vossen et al., 2000). Similarly, the *Arabidopsis RPP8* and *HRT* genes, which are highly homologous, confer resistance against the *Peronospora parasitica* and turnip crinkle virus, respectively (McDowell et al., 1998; Kachroo et al., 2000). How could molecular signals derived from very different types of pathogens be recognized by the same or very similar *R* genes? (iii) In many cases, cloned *R* genes cannot function in different families of plants. The NBS-LRR based mechanism, for example, apparently evolved before major diversification of angiosperms. It is difficult to imagine that the downstream mechanism has become incompatible with the NBS-LRR upstream factors. (iv) Avr proteins in general appear to be virulence factors when the plant does not have the appropriate *R* genes. Is there any reason that the factors to be recognized by plants as signals of pathogen attack should have virulence functions in nature?

To explain these phenomena, the "guard model" has been put forward recently (van der Biezen and Jones, 1998a). According to this model: virulence factors originating from pathogens have targets in the host to express their virulence functions; the function of an R protein is to guard such a target of a virulence factor; when the target is attacked by the virulence factor, the R protein somehow senses it and initiates signal transduction to induce defense responses (Figure 7). The guard model has been gaining popularity despite the lack of directly supporting evidence, because it can provide simple explanations to the above questions. (i) Assuming that the number of targets for virulence factors is limited, plants may not need to have a large number of *R* genes, nor do they have to generate new specificities quickly. A population genetic study of *RPM1* alleles among ecotypes supported the trench warfare hypothesis in the evolution of *avr* and *R* genes, rather than the arms race hypothesis (Stahl et al., 1999). The trench warfare hypothesis images a battle between a host and its pathogen in which one wins sometimes and loses other times at the front line, but the overall situation does not change drastically. The arms race hypothesis, on the other hand, images a battle in which one acquires a new weapon and almost eliminates the other, then the other fights back with another new weapon. The *RPM1* gene was not defeated by the pathogen for a long time, and its occurrence among the population fluctuates during this time. This observation is consistent with the trench warfare hypothesis and could be explained by assuming a relatively limited number of potential virulence targets (so that it is not easy for a pathogen to evolve a totally new virulence factor) and a balance between benefit and cost of resistance. (ii) If the same or very similar molecules are targeted by virulence factors derived from different types of pathogens (this situation is more likely to occur if the number of potential virulence targets is limited), it is conceivable that the same or very similar R proteins can guard the same or very similar virulence target molecules. (iii) A combination of a virulence factor target and the guarding R protein can co-evolve and drift, so that it is conceivable that after some evolutionary time, partners in orthologous combinations in different taxa of plants become unexchangeable. (iv) That virulence factors are the molecules to be recognized as signals of pathogen attack is a built-in assumption of the model.

From the viewpoint of molecular recognition mechanisms, the guard model appears to be a small extension of the ligand-receptor model. The combination of the virulence factor target and the R protein can be considered as a receptor complex. However, the guard model adds more restrictions in this figure from the viewpoint of biological functions – the ligand must be intrinsically a virulence factor, and the receptor complex must contain the virulence factor target in addition to the R

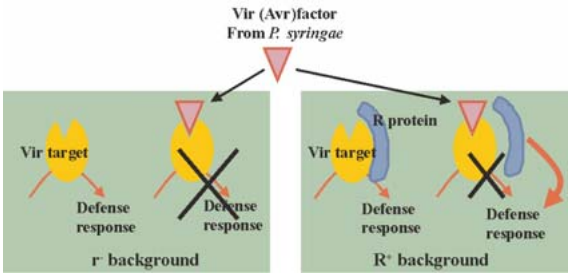


Figure 7. The guard model of *R* gene and *avr* gene interaction. When a plant does not have an appropriate *R* gene (r^- background; left), a virulence factor derived from *P. syringae* interacts with the plant virulence target molecule. The virulence target molecule has a role in defense response induction in the plant cell, and this function is inhibited by the interacting virulence factor. When a plant has the appropriate *R* gene (R^+ background; right), the virulence target is guarded by the R protein. When the target is attacked by the virulence factor, the R protein senses the attack and rapidly induces defense response.

protein. These restrictions are the reason that the guard model can provide the above simple explanations.

The result from co-immunoprecipitation of AvrRpt2 and RPS2 after expressing the proteins in *Arabidopsis* protoplasts was consistent with the notion of receptor complex (Leister and Katagiri, 2000). They were co-immunoprecipitated together with some other plant proteins from the plant extracts; they were not co-immunoprecipitated by themselves in vitro. However, this study does not tell whether the other complex components are necessary for gene-for-gene recognition, or whether one of the components is the AvrRpt2 virulence factor target.

3.1.5. Other genes involved in gene-for-gene resistance

A few *Arabidopsis* genes in which mutations affect a group of *R* gene-mediated resistance are known. A mutation in *NDR1* strongly affects resistance mediated by some cc-NBS-LRR *R* genes, whereas a mutation in *EDS1* strongly affects resistance mediated by TIR-NBS-LRR *R* genes (Aarts et al., 1998). The most simple-minded model is that cc-NBS-LRR and TIR-NBS-LRR use different signal transduction pathways, and *NDR1* and *EDS1* are signal transducers in each pathway. For gene-for-gene resistance against *P. syringae*, resistance mediated by cc-NBS-LRRs (namely *RPS2*, *RPM1*, and *RPS5 R* genes) is affected by the *ndr1*mutation but not much by the *eds1*mutation, whereas resistance mediated by the *RPS4* TIR-NBS-LRR is affected by *eds1* and not by *ndr1*. This does not conflict with the model if we assume both pathways can independently induce a set of defense responses that are important for resistance against *P. syringae*. Alternatively, these pathways may induce two different sets of defense responses, and both sets are effective against *P. syringae*. However, there are many other simple models to explain the behavior of *ndr1* and *eds1* mutations. In addition, there is no reason to believe that *NDR1* and *EDS1* have comparable positions in the sequence of events. For example, these proteins might be needed to produce functional R proteins in a subclass-specific manner (e.g., modification, localization); one of them might be affecting pathogens directly but show differential effects due to differences in the sensitivity of R-mediated recognition (the other functions in a different way); in the guard model, they might be the targets of multiple virulence factors, guarded by multiple R proteins. It should be emphasized that the effects of *ndr1* and *eds1* mutations on *R* gene-mediated resistance are not always clear-cut. For example, *RPS2*-mediated resistance is strongly suppressed by the *ndr1* mutation, but *RPM1*-mediated resistance is only partially suppressed (Century et al., 1995). *NDR1* might be a quantitative factor for NBS-LRR *R* gene functions (Tao et al., 2000).

A mutation in *PBS2* affects resistance mediated by *RPS2*, *RPM1*, and RPS5, but does not affect resistance mediated by *RPS4*. Within this set of *R* genes, it appears that *pbs2* suppresses cc-NBS-LRR mediated resistance, although a larger set of *R* genes needs to be tested to obtain a general conclusion about the *R* gene subclass specificity. There are also NBS-LRR genes whose functions are independent of any of *NDR1* , *EDS1* , *PAD4*, and *PBS2* (Bittner-Eddy and Beynon, 2001). Some of such complications could stem from a signal transduction network (involving not only divergent pathways but also convergent pathways) and quantitative dynamics of the network.

3.2. General resistance in the Arabidopsis-P. syringae interaction

Here we use the term “general resistance” as resistance that contributes to reduction in growth of virulent and avirulent pathogens to a similar extent. As you will see in this section, even the growth of a virulent pathogen is limited by general resistance of the plant. Note that the growth of an avirulent pathogen is limited by the gene-for-gene resistance in addition to general resistance. Even when the gene-for-gene resistance component is intact, a defect in general resistance component may allow an avirulent pathogen to grow well enough to cause disease symptoms. But in this case, without the gene-for-gene resistance component, the pathogen would grow even better.

3.2.1. General resistance against virulent P. syringae: a new view of compatible Arabidopsis-P. syringae interactions

Establishment of a genetic model plant-pathogen system opened up new areas of experiments, some of which ended up challenging dogmas. One new area of experimentation is intensive plant mutant screens under well-controlled conditions: because *Arabidopsis* plants are

small, mutant screens can be performed in growth chambers (even when you don't have access to a large volume of growth chamber space) instead of greenhouses. Well-controlled conditions are crucial in reproducibility of subtle phenotype differences. This is especially important because plant-pathogen interactions are often significantly affected by environmental factors and the developmental stage of the plants. One subtle phenotype difference that was pursued was the difference in the degree of disease symptoms during compatible interactions (Glazebrook et al., 1996). Mutational analysis of *Arabidopsis* in response to virulent *P. syringae* strains led to identification of *Arabidopsis* mutants (e.g., *npr1*, *eds*, *pad4*; see below) that show enhanced disease susceptibility to normally virulent *P. syringae* strains (Glazebrook et al., 1997b). These *Arabidopsis* mutants are impaired in the activation of defense responses during compatible interactions. A long-held dogma in plant pathology textbooks led people to believe that incompatible and compatible interactions are qualitatively different. The idea was that plants are susceptible to certain pathogens because they simply cannot mount a resistance response to the pathogens. However, studies on these mutants unambiguously demonstrated that even when they are susceptible to a pathogen, plants defend themselves to slow down the pathogen-but the general defense is not effective enough to completely stop the pathogen.

This change of the view of compatible interactions also raises a question about whether the distinction between compatible and incompatible interactions is necessarily correlated with differences in the underlying molecular mechanisms. One can easily imagine the following situation: each plant has a pool of *R* genes with different affinities that detect various *P. syringae* Avr proteins. The affinity will be highest if an Avr protein is perceived by a cognate *R* gene in a resistant plant and is responsible for induction of an effective defense. Although a susceptible plant lacks the cognate *R* gene to recognize an "Avr-like" protein of a virulent *P. syringae* strain, some *R* genes in the susceptible plant may still weakly "interact" with one or more of these "Avr-like" proteins produced by the virulent *P. syringae* strain, so the resistance pathway is activated, but too slowly and/or too weakly to completely stop the virulent *P. syringae* infection. Nevertheless, loss-of-function mutations in these "weak" *R* genes or resistance signaling component genes would increase the susceptibility of the plant to virulent *P. syringae*. In short, compatible/incompatible interactions may not be determined by distinctive molecular mechanisms, but by quantitative or kinetic variations in the same molecular mechanism. We should be aware that traditional classification of biological phenomena may not be correlated with distinctive molecular mechanisms.

An interesting observation is that some *Arabidopsis* mutations initially identified based on defects in gene-for-gene resistance against avirulent pathogens also affect general resistance against virulent pathogens or *vice versa*. For example, virulent *Pst* DC3000 grows better in the *eds1* mutants, which were initially identified in screens for gene-for-gene interaction mutants (Aarts, et al., 1998). Mutations in PAD4 were identified initially by reduced general resistance, but *pad4* affects some R gene-mediated resistance and general resistance to *P. syringae* (Glazebrook, et al., 1997b). When expressed in the plant lacking *RPS2*, the virulence function of AvrRpt2 affects both general resistance to virulent DC3000 and specifically *RPM1*-mediated gene-for-gene resistance (Chen, et al., 2000). These observations suggest that R-mediated resistance and general resistance against virulent pathogens are closely related. Responses to virulent strains might use both *EDS1*- and *PAD4*- dependent pathways, if EDS1 and PAD4 are indeed signal transducers. Furthermore, recognition of virulent strains might even use NBS-LRR type proteins but the response could be slower and/or weaker. In the guard model, the pathogens' virulence factors may attack some molecular components for general resistance and resistance mediated by R proteins that guard the particular components could also be affected.

3.2.2. Role of SA in general resistance and gene-for-gene resistance to P. syringae

Genes that contribute to general resistance against *P. syringae* and *P. parasitica* appear to be mainly involved in the SA-dependent signaling pathway for defense response regulation. Mutations in all such genes (e.g., *EDS* and *PAD4*; Aarts et al., 1998; Glazebrook et al., 1997b), except for *NPR1* and *DTH9*, reduce SA accumulation during pathogen attack (Glazebrook, 2001). NPR1 seems to be a signal transducer downstream of SA (Delaney et al., 1995; Cao et al., 1994; Kinkema et al., 2000). Studies that involve plants that carry the *NahG* transgene (encoding salicylate hydroxylase which degrades SA) and general resistance mutants clearly indicate that the SA-dependent pathway is crucial for general resistance against many pathogens including *P. syringae*. Furthermore, many pathogen-responsive genes are regulated in a SA-dependent manner. SA is accumulated at a higher level during incompatible interactions than during compatible interactions at early stages of interaction. The SA-dependent pathway might be the link between general resistance and gene-for-gene resistance. However, the role of SA in gene-for-gene resistance is not that clear.

NahG plants can develop an HR upon infection of avirulent bacteria. They are not strongly affected in the gene-for-gene resistance mediated by at least some R genes, whereas the general resistance component is strongly impaired (Delaney et al., 1994). It should be noted that not only is SA a major signal transducer after recognition of pathogen attack, but it also can potentiate pathogen recognition sensitivity at a low level (Shirasu et al., 1997). One role of SA in gene-for-gene resistance may be to potentiate the recognition mechanism. In this way, only gene-for-gene interactions with relatively low sensitivities might be strongly affected by a lower SA level.

3.2.3. Role of JA and ethylene in general resistance to P. syringae

In addition to the SA-dependent pathway, involvement of the JA/ethylene-dependent pathway in general defense has been observed. In many cases, the SA-dependent pathway and the JA-dependent pathway act antagonistically (Felton et al., 1999a, 1999b; Pieterse and van Loon, 1999; Thomma et al., 2001). Activation of the JA-pathway could suppress the SA-pathway and reduce general resistance against pathogens, such as *P. syringae*, against which plants mainly use the SA-pathway. In fact, some virulent *P. syringae* strains appear to use this antagonistic interaction to suppress *Arabidopsis* defense (see section 4.2.1). However, the JA/ethylene pathway, not the SA pathway, is important for induced systemic resistance (ISR), which causes resistance against virulent *P. syringae* strains (see section 3.5). Whereas in many cases JA and ethylene appear to function in concert, ethylene response seems to be important for general resistance or tolerance against virulent *P. syringae* (Ton et al., 1999a; 1999b; Bent et al., 1992). The interactions among SA-, JA-, and ethylene-dependent pathways do not appear to be simple. Complications could arise from different roles of these pathways in different stages of plant-pathogen interactions. Therefore, it will be important to obtain various measurements of a given interaction with high spatial and temporal resolution.

3.3. What defense responses are responsible for Arabidopsis resistance to P. syringae?

We have very limited knowledge about which particular defense responses are important for resistance against *P. syringae* or any other pathogen. Phytoalexins are known to be involved in pathogen resistance in some pathosystems (Hammerschmidt and Dann, 1999). The *Arabidopsis PAD3* gene encodes a P450 monooxygenase, which is likely to be one of the enzymes required for synthesis of the *Arabidopsis* phytoalexin camalexin (Zhou et al., 1999). A mutation in this gene abolishes camalexin accumulation after infection by *P. syringae* but does not affect other tested defense responses (Glazebrook and Ausubel, 1994). General and gene-for-gene resistance against *P. syringae* is not significantly affected in the *pad3* mutant (Glazebrook and Ausubel, 1994), whereas general resistance against the fungus *Alternaria brassicicola* is reduced in the *pad3* mutant (Thomma et al., 1999). Although camalexin exhibits toxicity to *P. syringae in vitro* (Rogers et al., 1996), it is likely that camalexin does not have a major contribution to resistance against *P. syringae* in *Arabidopsis.* We should, however, keep in mind that resistance to a given pathogen is likely to be composed of complex combinations of different defense responses. Some responses may act additively, some may act synergistically, and some may be functionally redundant. We may not detect an effect of loss of a redundant function. Reactive oxygen species, PR proteins, and host cell wall modification are additional candidate defense compounds/mechanisms, but none of them have so far been linked directly to *P. syringae* resistance *in vivo*.

3.4. Systemic resistance responses

An initial localized infection of an avirulent *P. syringae* strain causes plant cell death (an HR in this case), which could further trigger a whole-plant level of relatively weak resistance against secondary infection by a broad range of pathogens, including normally virulent strains of *P. syringae* (Ryals et al., 1996). This phenomenon is called systemic acquired resistance, or SAR (see **chapter by Dangl** for details). SA accumulation in the systemic tissues is essential for SAR, but SA is unlikely to be the long-distance signal that initiates SAR in the uninfected parts of the plant (Vernooij et al., 1994). Because the SA-dependent mechanism is also crucial for local gene-for-gene and general defense, many *Arabidopsis* mutants isolated based on their deficiency in local defense against pathogens have defects in the SA signaling pathway and are also deficient in SAR (Glazebrook et al., 1997a). The level of SA accumulated in the infected local tissues is

much higher than that in the systemic tissue. It seems that plants use the same SA pathway at different degrees for local defense and for defense in systemic tissues in SAR. This would be a very practical solution for plants because (i) plants would not have had to evolve two distinct signaling pathways for two purposes and (ii) full-blown activation of the SA pathway appears deleterious not only to pathogens, but also to the plant itself, as suggested by growth defects among plant mutants that have the SA pathway constitutively on.

3.5. Rhizobacteria-mediated induced systemic resistance (ISR)

Before we leave the subject of *P. syringae* resistance, we would like to mention an interesting systemic resistance mechanism that is effective against *P. syringae*, but is mechanistically distinct from SAR described above. This resistance mechanism is called induced systemic resistance (ISR) and is triggered by non-pathogenic, root-colonizing rhizobacteria, such as *Pseudomonas fluorescens* strain WCS417r or *P. putida* strain WCS358r (Pieterse et al., 1996). In contrast to pathogen-induced SAR, ISR is not associated with SA accumulation or activation of *PR* genes. Jasmonic acid and ethylene appear to play an important role in this SA-independent pathway (Pieterse et al., 1998; Knoester, 1999). The *Arabidopsis* jasmonate response mutant *jar1* and the ethylene response mutant *etr1*, which show a normal response to inducers of SAR, are unable to express ISR after root treatment with *P. fluorescens* WCS417r. Although ISR and SAR seem to follow distinct signaling pathways, they are both blocked in the *npr1* mutant (Pieterse et al., 1998). Thus, the NPR1 protein is not only required for the SA-dependent expression of *PR* genes that are activated during SAR, but also for the jasmonate- and ethylene-dependent activation of so-far-unidentified defense responses resulting from ISR. Because the expression of none of the SAR-associated or jasmonate/ethylene-induced genes is affected during ISR, ISR is believed to be associated with an increase in *Arabidopsis* sensitivity to the defense hormones JA and ethylene (van Wees et al., 1999, 2000).

4. Pathogen virulence and host susceptibility in the compatible Arabidopsis-P. syringae interactions

In the preceding section, we discussed how *Arabidopsis* recognizes and defends against *P. syringae* infection. What happens when *Arabidopsis* fails to rapidly recognize a *P. syringae* strain, due to lack of appropriate *R* genes? How do *Arabidopsis* plants become susceptible to virulent *P. syringae* strains? What is the molecular basis of *P. syringae* pathogenicity? To be a successful extracellular pathogen, virulent *P. syringae* (e.g., strain DC3000 in *Arabidopsis* ecotype Columbia) must evolve an array of pathogenic mechanisms to suppress or evade *Arabidopsis* defense responses that are apparently effective in preventing virulent infection by the vast majority of potential parasites. It must also develop mechanisms to release nutrients and water to the apoplast, where bacteria live. How *P. syringae* succeeds in doing this is not known. However, molecular genetic analysis of *P. syringae* pathogenicity has revealed two virulence systems that play an important role in *P. syringae* infection of plants: the type III protein secretion system and a toxin called coronatine.

4.1. Pathogen virulence

4.1.1. The type III protein secretion system: A key pathogenicity factor in P. syringae

The type III protein secretion system was discovered first in the human pathogens *Yersinia* spp., but has now been found to be widespread among Gram-negative bacterial pathogens of plants and animals (He, 1998; Hueck, 1998; Galan and Collmer, 1999; Cornelis and Van Gijsegem, 2000). The most intriguing feature of this protein secretion system is the ability of this system to actively inject bacterial virulence proteins directly into host cells. The importance of the type III secretion system in *P. syringae* pathogenicity is underscored by the observation that *hrp/hrc* mutations that block type III secretion completely eliminate *P. syringae* infectivity in susceptible *Arabidopsis* plants (Roine et al., 1997). The ability of *P. syringae* to inject virulence proteins directly into the host cell is believed to be highly significant in pathogen evolution, because this injection mechanism enables the pathogen to gain access to a vast number of intracellular host targets that would not be available for bacterial virulence proteins delivered to the surface of host cells. As mentioned in section 3.1.3, in *P. syringae* and other plant bacterial pathogens, the majority of the known type III effectors are Avr proteins, which are identified based on their ability to trigger resistance responses following recognition by the *R* gene products in the incompatible interactions. How *P.*

syringae delivers Avr and other type III effector proteins from its cytoplasm to the host cell cytoplasm is not known. However, a *P. syringae* surface pilus (called the Hrp pilus) assembled by the type III secretion system has been shown to play a key role in this process, possibly by providing a bridge for protein transfer (Roine et al., 1997; Wei et al., 2000; Hu et al., 2001; Jin et al., 2001).

With the DC3000 genome sequencing project near completion (http://www.tigr.org/tdb/mdb/mdbinprogress.html), several groups are addressing a pressing question: how many type III effectors are produced by this bacterium? Two strategies of systematic search for type III effectors are underway. First, all known type III effector genes in *P. syringae* contain a characteristic '*hrp*-box' motif in their promoters (Shen and Keen, 1993; Innes et al., 1993; Xiao et al., 1994). Therefore, a BLAST search using the consensus '*hrp*-box' motif should identify putative type III effector genes. Second, a type III secretion reporter system has been developed. A truncated AvrRpt2 protein lacking the N-terminal type III secretion signal but maintaining HR-eliciting activity once inside *RPS2*$^{+}$ *Arabidopsis* cells (Mudgett et al., 2000; Guttman and Greenberg, 2001) can be used to generate random fusions in the DC3000 genome. An in-frame fusion of the N-terminal secretion signal of a type III effector with the truncated AvrRpt2 reporter will target the fusion protein into *Arabidopsis* cells to trigger an AvrRpt2/RPS2-dependent HR. With a combination of these two strategies, we shall soon know the exact number of type III effectors in DC3000.

How do type III effectors function inside the host cell to promote plant susceptibility? This has been a major mystery in the field of plant-pathogen interactions. To date, no plant 'susceptibility' pathway has been clearly identified in any plant-bacteria interaction. In animal-pathogen interactions, increasing evidence suggests that the MAP kinase defense pathway (Orth et al., 1999), delivery of reactive oxygen-generating enzymes (Vazquez-Torres et al., 2000, 2001; Feng et al., 2001; Vazquez-Torres and Fang, 2001), ubiquitin-like molecules (Orth et al., 2000), and actin cytoskeleton (Galan and Zhou, 2000) in the host are the targets of type III virulence proteins, demonstrating that type III effectors are 'smart bombs' sent by pathogens. Unfortunately, the primary sequences of the identified *P. syringae* type III effector genes provide little clue to their functions in modulating plant signaling and metabolic processes. In *Xanthomonas* spp., however, there are some clues to the functions of several Avr proteins in compatible hosts. For example, AvrRxv/AvrBsT from *X. campestris* pv. *vesicatoria* shares sequence homology with YopJ/P of *Yersinia* and AvrA of *Salmonella* (Orth et al., 2000). YopJ is a MAP kinase pathway inhibitor that suppresses macrophage defenses and appears to have protease activity (Orth et al., 1999, 2000). Whether AvrRxv and AvrBsT target a MAP kinase pathway for their virulence functions in susceptible host plants is not known, but the putative protease activity of AvrBsT appears to be required for elicitation of plant resistance response (Orth et al., 2000). AvrBs2 shows similarity with agrocinopine synthase (opine production in tumors) of *Agrobacterium tumefaciens*, suggesting a possible (but not proven) role in the nutrition of the pathogen (Swords et al., 1996). AvrBs3 family members, which are widespread in pathogenic *Xanthomonas* spp., appear to be transcription factors, although the host genes directly transcribed by the AvrBs3 family proteins remain to be identified (Bonas et al., 1989; Yang and Gabriel, 1995; Zhu et al., 1998; Zhu et al., 1999; Yang et al., 2000).

It is almost certain that a major function of *P. syringae* type III effector proteins is to suppress plant defense responses in the host. Supporting evidence is accumulating. For example, *hrp* mutant bacteria appear to induce the expression of several general defense-associated genes, such as phenylalanine ammonia lyase and chitinase genes (Jakobek et al., 1993), and papillae formation (Bestwick et al., 1995; Brown et al., 1995), whereas the wild-type strains suppress expression of these defense genes and formation of papillae. There is also bacterial genetic evidence that some *avr* gene products, including AvrRpt2, interfere with the function of other *avr* gene products in the elicitation of host resistance (Ritter and Dangl, 1996; Reuber and Ausubel, 1996; Jackson et al., 1999; Chen et al., 2000; Tsiamis et al., 2000). An increasingly popular idea, as discussed in the guard model (Figure 7), is that type III effectors, including Avr proteins, may target key components of general defense in susceptible plants, presumably to suppress host defense. However, the exact molecular mechanisms by which type III effectors modulate the plant resistance responses remain to be elucidated.

Experiments involving heterologous expression of *avr* genes inside plant cells suggest that Avr proteins can be deleterious even in the absence of a known cognate *R* gene if expressed too strongly (Gopalan et al., 1996; McNeillis et al., 1998; Nimchuk et al., 2000). Whether these effects result from interaction with susceptibility targets in the host is unknown. However, the sensitivity of *Arabidopsis* to AvrB over-expressed in the susceptible plant cell depends on a single gene, which suggests that a specific plant target is involved in this phenomenon (Nimchuk et al., 2000). As extracellular pathogens, *P. syringae* and other bacterial pathogens must also cause host cells to release water and nutrients into the apoplast. Consequently, some type III effectors may be involved in water and nutrient release (Figure 8). Despite these accumulating clues, in no case has a specific plant 'susceptibility' target been identified for any type III effector in plant pathogenic bacteria. Identification of host

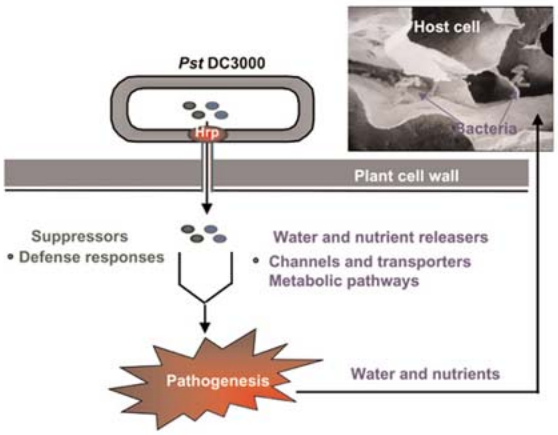


Figure 8. A hypothetical model of the potential targets of type III effector proteins in the host cell. *P. syringae* is an extracellular pathogen, living and multiplying in the leaf apoplast. Some effector proteins must therefore be involved in releasing water, carbohydrates and other nutrients from the host cell. Other effector proteins are likely involved in suppressing or evading host defense responses. In the top right corner is a scanning electron microscopic image of a cross section of an *Arabidopsis* leaf infected with DC3000 (see Figure 4).

susceptibility targets/pathways is therefore a major challenge in the field.

4.1.2. The coronatine toxin-A molecular mimic of methyl jasmonate

In addition to the type III secretion system, strains DC3000 and ES4326 also produce a toxin (called coronatine) that plays a significant role in modulating host susceptibility (Bender et al., 1999). However, unlike mutations affecting the type III secretion system, mutations affecting coronatine production do not completely eliminate pathogen virulence in susceptible *Arabidopsis* plants, rather they have only a quantitative effect on pathogen virulence, most notably coronatine-deficient bacterial mutants cause substantially weaker disease symptoms (chlorosis and necrosis) and a subtle reduction of bacterial multiplication in host leaves (Bender et al., 1987; Mittal and Davis, 1995).

The structure of coronatine (Figure 9) has two distinct components: the polyketide coronafacic acid (CFA) and coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine. The primary symptom elicited by coronatine in plants is tissue chlorosis (Gnanamaickam et al., 1982). However, exogenously applied coronatine induces purpling of *Arabidopsis* leaves (Bent et al., 1992), presumably resulting from anthocyanin production. Coronatine also inhibits root elongation and stimulates ethylene production in plants (Ferguson and Mitchell, 1985; Kenyon and Turner, 1992; Feys et al., 1994).

Coronatine bears remarkable structural and functional homologies to methyl jasmonate (MeJA). Furthermore, coronatine and MeJA induce similar biological responses in *Arabidopsis* seedlings (Feys et al., 1994) and other plants (Weiler et al., 1994; Greulich et al., 1995; Koda et al., 1996), leading to the suggestion that coronatine functions as a molecular mimic of MeJA. MeJA accumulates in response to wounding or insect chewing and is involved in a systemic defense response to invading insects via production of defense compounds, including proteinase inhibitors (Ryan and Pearce, 1998). Why would bacteria produce a toxin whose function mimics that of MeJA? As mentioned above, coronatine-deficient DC3000 mutants have reduced virulence in susceptible *Arabidopsis*, so one must hypothesize that coronatine somehow conditions host plants to be more susceptible for bacterial infection via activation of the JA signaling pathway. Emerging evidence suggests that the JA-mediated insect defense and SA-controlled pathogen resistance are sometimes antagonistic to each other so that activation of one pathway leads to inhibition of the other (Felton et al., 1999a, 1999b; Pieterse and van Loon, 1999; Thomma et al., 2001). This has been interpreted as plants prioritizing energy-consuming defense responses towards specific insults (e.g., insects vs. some microbial pathogens). If this is true, coronatine could act as a suppressor of the plant's SA-dependent microbial defense system by triggering JA-mediated insect defense response. Consistent with this idea, a study shows that a coronatine mutant of DC3000 induces the expression of two pathogen defense-associated genes (phenylalanine ammonia lyase and

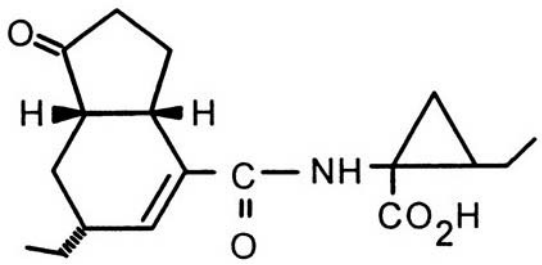


Figure 9. The structure of coronatine.

glutathione S-transferase) more highly than wild-type DC3000 (Mittal and Davis, 1995). However, we have yet to resolve the precise mechanism by which coronatine tricks *Arabidopsis* and other plants to turn on the JA pathway and to presumably inhibit effective plant defense against *P. syringae*. Remember that ISR against *P. syringae* is actually dependent on the JA pathway components (see section 3.5). How can the same pathway be involved in processes leading to opposing effects on *P. syringae* resistance/susceptibility?

4.2. Host susceptibility

4.2.1. Arabidopsis coi1 mutant-Blocking the action of the coronatine toxin

Based on the ability of coronatine to inhibit *Arabidopsis* root growth, Feys et al. (1994) isolated a coronatine-insensitive (*coi1*) mutant of *Arabidopsis*. Interestingly, the same mutant is also insensitive to MeJA, further suggesting a similar mode of action of coronatine and MeJA. Consistent with the role of coronatine in the virulence of toxin-producing *P. syringae* strains, the *coi1* mutant plants are highly resistant to *P. syringae* pv. *atropurpurea* (Feys et al., 1994), and pv. *tomato* DC3000 (Kloek et al., 2001; R. Thilmony and S. Y. He, unpublished), with markedly reduced disease symptoms and bacterial multiplication. However, the *coi1* mutant has increased susceptibility to insects and certain necrotrophic fungal pathogens and is male sterile (Feys et al., 1994; Thomma et al., 1998). At present, it is not known how the same COI1 protein participates in seemingly different pathways leading to pathogen defense/susceptibility and pollen development. The COI1 protein belongs to a family of conserved proteins (e.g., human Skp2 involved in cell division, yeast Grr1 involved in cell division and nutrient uptake, and *Arabidopsis* TIR1 involved in auxin response) that contain an F box and leucine-rich repeats (Xie et al., 1998). Proteins of this family are known to be involved in selectively recruiting substrate regulatory proteins (e.g., transcription repressors) into a complex for ubiquitination and subsequent removal by proteolysis (Latres et al., 1999; Spencer et al., 1999; Spiegelman et al., 2001). Thus, the COI1 protein could control pathogen defense responses and pollen development by targeting distinct repressors for degradation in different cell types and possibly in response to different signals.

Recently, Barbara Kunkel's lab isolated additional alleles of the *coi1* mutant from a direct screening for *Arabidopsis* mutants with enhanced resistance (Kloek et al., 2001). They made the important finding that DC3000 resistance (restriction of DC3000 multiplication) in the *coi1* plants is largely abolished when the *coi1* plants are crossed to SA-deficient *nahG* plants. These results suggest that the inhibition of DC3000 multiplication in the *coi1* mutant is dependent on accumulation of SA. Perhaps the *coi1* plants can mount a more aggressive SA-mediated pathogen defense in the absence of the otherwise antagonistic JA defense pathway. Interestingly the *coi1nahG* plants still show less severe disease symptoms than wild-type plants after DC3000 infection. This observation argues that the COI1 protein is required for DC3000-induced symptom development, in addition to its involvement in cross-talk with the SA-mediated defense pathway.

Compared with the bacterial *cor-* mutant phenotype, the *Arabidopsis coi1* mutant phenotype is much more drastic, suggesting that lack of perception of coronatine is not the only defect in the *coi1* mutant. Specifically, whereas DC3000 multiplies about 100-fold less in *coi1* plants (compared with in wild-type Col plants) and causes no disease symptoms when 10^6 cfu/ml of DC3000 are infiltrated into leaves, DC3000 *cor-* mutants multiply similarly as the wild-type bacteria in Col plants (at most, a 10-fold reduction) and still cause some disease symptoms, albeit at a lower frequency (Kloek et al., 2001; R. Thilmony and S. Y. He, unpublished results). COI1 could be required for the action of additional DC3000 virulence factors. One possibility is that some type III effectors also target a COI1-dependent pathway to modulate host susceptibility, which, if proven, could assign a major host target for *P. syringae* type III effectors. Alternatively, coronatine may activate only a subset of COI1-dependent, SA-antagonistic pathways, whereas the *coi1* mutation may eliminate all COI1-mediated, SA-antagonistic pathways.

As discussed in section 3.2, a variety of other *Arabidopsis* mutants also influence susceptibility to virulent *P. syringae* infection via their effects on general defense in the plant. Some mutants have increased susceptibility owing to a block in the accumulation or perception of SA (e.g., *npr1*, *eds*, *ndr1*, *pad4*, *eds5*, *sid2*); others have enhanced resistance owing to constitutive expression of SA and defense genes (e.g., *cpr*, *dnd*, *acd*, lesion mimic mutants, MAP kinase mutants; see chapter by Dangl). A key distinction between the *Arabidopsis coi1* mutant and these other *Arabidopsis* mutants is that we know that the COI1 pathway is the target of a known *P. syringae* virulence factor (coronatine). As we continue to unravel the host components targeted by the DC3000 virulence factors, it would not be surprising to learn that some of the known *Arabidopsis* defense pathway components are additional targets of DC3000 virulence factors during compatible interactions. This prediction is

in line with the adaptive co-evolution theory that both plant and pathogen evolve to overcome each other's lethal weapons, and with the guard model of *R* gene/*avr* gene interaction.

5. ARABIDOPSIS AND P. SYRINGAE GENOMICS

In addition to the genetic tractability of both host and pathogen genomes, the *Arabidopsis-P. syringae* pathosystem now offers another advantage: rapidly accumulating genomics resources. Completion of *Arabidopsis* genome sequencing in 2000 (The *Arabidopsis* Genome Initiative, 2000) and expected completion of the *P. syringae* pv. *tomato* DC3000 genome sequencing in 2002 (http://www.tigr.org/tdb/mdb/mdbinprogress.html) are making the *Arabidopsis-P. syringae* system an attractive genomics-amenable system. Powerful structural, computational, and functional genomics approaches can be used to examine this interaction in ways that cannot yet be done with many other pathosystems. For example, genome-wide monitoring of changes in gene, protein, and metabolite expression will give us a global picture of the intricate cross-talk among interconnecting signaling and metabolic pathways during the *Arabidopsis-P. syringae* interaction. Knowledge of host and pathogen genomes will also facilitate systematic mutational analysis of host and pathogen determinants involved in incompatible and compatible interactions.

5.1. Host genomics

Analysis of the *Arabidopsis* genome has identified hundreds of genes that show significant sequence similarities with known disease resistance genes, disease signal transduction components, and downstream defense response genes, confirming the belief that *Arabidopsis* (and likely other plants) devotes a substantial portion of its genome to combating pathogens. Some of these genes likely encode additional recognition components of distinct specificities, checkpoints of sub-branches of disease signaling pathways, and redundant defense substances. They represent a rich resource for discovery of new genes controlling *Arabidopsis-P. syringae* interactions.

Global gene expression profiling is already an established technology for *Arabidopsis* (Zhu and Wang, 2000). It has been used in the study of host gene regulation in interaction with *P. syringae*. One of the discoveries made by Maleck et al. (2000) is that many of the genes regulated by DC3000/*avrRpt2* in the systemic leaves clustered with genes regulated by *Peronospora parasitica* and also by the SA analog BTH (within the first 4 h), suggesting that the DC3000/*avrRpt2*-induced SAR is transcriptionally related to *P. parasitica*-induced SAR and to an early phase of BTH-induced SAR (Maleck et al., 2000). The inclusion of the DC3000/*avrRpt2* treatment in this study thus helped to sort out the biologically relevant transcripts that are strongly associated with SAR. The identified novel SAR-associated genes are candidates for further study using functional genomics approaches.

5.2. P. syringae genomics

At the writing of this edition, the DC3000 genome (approximately 6.2 mb) has not been annotated completely. However, even the incompletely annotated DC3000 genome is giving us an opportunity to perform many specific analyses. For example, a Blast search using the '*hrp* box' motif, which is present in the promoters of all known *P. syringae avr* genes, has revealed about 70 putative open reading frames (J. Zwiesler-Vollick, A. Plovanich-Jones, and S. Y. He, unpublished). Some of these genes could encode *bona fide* type III effectors and are therefore candidates for further verification by experimentation. The DC3000 genome also contains genes involved in cell wall degradation, extracellular polysaccharides, and production of phytohormones (e.g., IAA), which have been shown to be important in the virulence of other bacterial pathogens (Alfano and Collmer, 1996). These potential virulence factors (in addition to the type III secretion system and coronatine toxin) could therefore contribute to DC3000 aggressiveness in *Arabidopsis* tissues.

In summary, we are entering an exciting phase of research on the *Arabidopsis-P. syringae* interaction using a variety of functional genomics tools. We anticipate that many more structural, computational, and functional genomics studies of both *Arabidopsis* and *P. syringae* will be completed in the near future, which will provide a comprehensive picture of interconnecting host and pathogen pathways that define incompatible and compatible interactions. We also anticipate the construction of a comprehensive plant-*P. syringae* functional genomics interaction database soon, so expect a much expanded version of this section in the next edition!

6. CONCLUDING REMARKS

Since the discovery of *P. syringae* as a pathogen of *Arabidopsis* in the late 1980s, tremendous progress has been made in our understanding of pathogen virulence and avirulence determinants, the mechanism of host recognition of pathogen avirulence factors, resistance signal transduction pathways, and some aspects of host susceptibility. This progress is made possible because of the vision of a few pioneering scientists in the late 1980s. The genetic tractability of both *Arabidopsis* and *P. syringae* has played a critical role in the success of developing the *Arabidopsis-P. syringae* pathosystem into a remarkable model for research on plant-pathogen interactions.

Despite the exciting progress, a number of fundamental questions remain to be answered. For incompatible *Arabidopsis-P. syringae* interactions, we still have no idea of the actual mechanisms/compounds that stop *P. syringae* infection in the apoplast of a resistant plant. Although the gene-for-gene interactions have been molecularly defined and provide a clear explanation for host resistance to avirulent strains of a compatible *P. syringae* pathovar (i.e., a pathovar in which some strains can cause disease in *Arabidopsis*), we do not know the molecular basis of the more prevalent nonhost resistance of *Arabidopsis* that is extremely effective against the vast majority of pathogens that are capable of causing disease in some other plants, but never *Arabidopsis.* For example, why don't any of the *P. syringae* pv. *phaseolicola* strains, which infect bean, infect *Arabidopsis*? Is this because *Arabidopsis* contains all the resistance genes that could recognize all *P. syringae* pv. *phaseolicola* strains? Or is this because the virulence factors of *P. syringae* pv. *phaseolicola* that are adapted to the bean 'susceptibility' targets are not optimized for *Arabidopsis?* Recently, an *Arabidopsis* mutant, *nho1* (for nonhost resistance 1), has been isolated that allows *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *tomato* DC3000 *hrp* mutants, and the saprophyte *P. fluorescens* to grow significantly (Lu et al., 2001). In addition, the *nho1* mutation also compromised resistance mediated by *RPS2*, *RPS4*, *RPS5*, and *RPM1*, providing evidence that nonhost resistance is controlled, at least in part, by general resistance that functions in gene-for-gene resistance. Further characterization of this mutant will shed light on the molecular basis of *Arabidopsis* nonhost resistance against *P. syringae*. For compatible *Arabidopsis-P. syringae* interactions, the main challenge is to identify the susceptibility targets of *P. syringae* virulence factors and to learn how *P. syringae* has evolved to circumvent *Arabidopsis* defense and at the same time to cause *Arabidopsis* cells to release water and nutrients into the apoplast.

It is important to point out that we are at the beginning of an era when we can apply powerful scientific approaches toward understanding biological systems at a global level, in addition to understanding narrowly reduced, specific parts of the systems. This capability will drastically change the way biological research can be done, the way we think about biology, and the level of understanding we have in biology. Starting with a model biological system that is powerful in both genetics and genomics makes sense for collective efforts toward global levels of understanding. For the study of plant-pathogen interactions, the *Arabidopsis-P. syringae* system is such an ideal model system. We anticipate a new wave of system-based discoveries, which will reveal the dynamic, interconnecting, and flexible nature of the *Arabidopsis* and *P. syringae* signaling networks during interaction. In the end, we hope that study of this model interaction will contribute to our appreciation of how plants and bacterial pathogens have evolved to survive each others' attacks and counterattacks, which will in turn help us to develop sustained control measures by guided interception of bacterial virulence and/or by selective activation of plant defense.

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APPENDIX I: A LIST OF TECHNICAL TERMS TO BE LINKED TO THE MAIN TEXT

Apoplast: Intercellular space in the plant tissue. For most bacterial pathogens, this is their native habitat.

Avirulence (*avr*) genes: Pathogen genes that encode proteins (usually secreted) that are recognized by plant disease resistance gene products to trigger plant defense responses. Some Avr proteins function as enzymes to produce secondary elicitors. Many avirulence genes play a role in bacterial virulence in the absence of *R*-gene-mediated recognition in susceptible hosts.

Avirulent pathogen: A pathogen in a resistant plant. An avirulent pathogen still causes disease in susceptible host plants.

Compatible interaction: An interaction between a susceptible plant and a virulent pathogen, resulting in disease.

Coronatine: A polyketide phytotoxin (see Figure 9) produced by several pathovars of *Pseudomonas syringae*; it is required for the full virulence of the pathogen. The molecular basis by which coronatine contributes to bacterial virulence is not understood, but in tomato leaves it promotes disease chlorosis. Increasing evidence suggests that coronatine mimics, at least in part, the plant wounding response hormone jasmonic acid.

Disease chlorosis: A common disease symptom in pathogen infection in which the leaf tissue appears yellow due to the loss of chlorophyll. In the case of *Pseudomonas syringae* infection of *Arabidopsis* leaves, the chlorosis symptom occurs relatively late, usually on day 3 after pathogen infection. The molecular basis of tissue chlorosis is not known, but pathogen toxins, coronatine in the case of *Pseudomonas syringae* infection of tomato leaves, may be responsible.

Disease necrosis: A common, slow-developing disease symptom caused by necrotrophic pathogens. In the case of *Pseudomonas syringae* infection of *Arabidopsis* leaves, tissue necrosis appears at very late stage of disease development. The molecular basis of disease necrosis and its relationship to the much more rapid HR necrosis during incompatible interactions are not known.

Epiphytic growth: Many bacterial pathogens, including *Pseudomonas syringae*, can survive and multiply on the plant surface as epiphytes without causing disease.

Ethylene: A gaseous plant growth regulator involved in plant responses as diverse as fruit ripening, leaf senescence, and plant tolerance/resistance of microbial pathogens.

Gene-for-gene theory: H.H. Flor proposed this theory in the 1940s to explain the results from his study of the inheritance of rust resistance in flax (*Linum usitatissimum*) and pathogenicity in the flax rust fungus (*Melampsora lini*). Flor stated that "Host-parasite interaction in flax rust may be explained by assuming a gene-for-gene relationship between rust reaction in the host and pathogenicity in the parasite." The quadratic checks (Table 2) are used to illustrate these reactions in which an incompatible reaction occurs when the host has a resistance gene and the pathogen contains a corresponding avirulence gene. We now know that the gene-for-gene interaction occurs in many other genetically defined pathosystems, although in some cases the interactions may be more complicated, involving multiple host resistance genes and/or multiple pathogen avirulence genes (see Table 3).

Growth curve: An unfitted curve generated from plotting log (culturable bacterial number/cm^2 leaf tissue) against time after pathogen inoculation (usually in days). This is a standard means of evaluating how well a bacterial pathogen multiplies in plant tissues.

Host resistance: A form of plant resistance by which some cultivars of a plant species prevent infection by some strains of a virulent pathogen. In all cases known, the mechanism involves gene-for-gene interaction.

***hrp* genes:** Genes required for bacteria to elicit the hypersensitive reaction in resistant plants and to cause disease in susceptible plants, hence Hypersensitive Reaction and Pathogenicity genes. Most *hrp* genes are involved in the regulation and assembly of a type III protein secretion apparatus. These genes, usually clustered in the genome or on a plasmid, are present in most Gram-negative bacterial plant pathogens. Nine *hrp* genes are also conserved in all animal and human pathogenic bacteria that contain a functional type III secretion system and are therefore called *hrc*, for hrp

gene conserved.

Hypersensitive response (HR): Rapid, localized plant cell death upon contact with avirulent pathogens. HR is considered to be a key component of multifaceted plant defense responses to restrict attempted infection by avirulent pathogens.

Incompatible interaction: An interaction between a resistant plant and an avirulent pathogen, resulting in no disease.

Induced systemic resistance (ISR): A long-lasting, broad-spectrum resistance induced throughout otherwise susceptible plants by prior local inoculation of certain plant growth-promoting rhizobacteria (PGPR). Components of jasmonate and ethylene signaling pathways, but not the SA signaling pathway, are required for ISR.

Jasmonic acid: Jasmonic acid and methyl jasmonate (MeJA) are plant growth regulators derived from the octadecanoid signaling pathway that is elicited by wounding and insect chewing. JA signaling is required for pollen development, insect resistance, and resistance to certain fungal pathogens.

Local resistance: Plant defense mounted locally in the infected leaves/other tissues in response to infection of avirulent and virulent pathogens. The local resistance mechanism involving gene-for-gene recognition usually triggers SAR throughout the infected plants. PR genes are activated locally around the infection sites, suggesting some mechanistic overlap between local resistance and SAR.

Nonhost resistance: A form of plant resistance by which most plant species prevent infection of most species of pathogens. The underlying mechanisms are not understood, but could involve preformed defense barriers and chemicals, gene-for-gene resistance, and/or mismatch of pathogen virulence factors and host susceptibility targets.

Oxidative burst: Upon pathogen infection, plants rapidly accumulate reactive oxygen species, such as H_2O_2 and O^-_2, as part of an early defense response. The magnitude and duration of an oxidative burst are important in determining its function in plant responses. In plant-bacteria interactions, for example, a transient and nonspecific oxidative burst occurs at 30 min after inoculation with either a virulent or avirulent pathogen. However, a second and longer-lasting oxidative burst is activated only by an avirulent pathogen, which is correlated with host resistance. An oxidative burst could potentially kill the invading pathogens directly as well as serve as second messengers for activating other plant defense responses.

Pathogenicity: A term to describe the qualitative capability of a pathogen to cause disease.

Pathovar: This is a unique infrasubspecific taxonomical term used only for plant pathogenic bacteria. The pathovar is used to distinguish among bacteria within the species that exhibit different host ranges. Nutritional, biochemical, physiological, and nucleic acid-based tests (e.g., DNA hybridization, restriction fragment length polymorphism, and repetitive DNA PCR-based genetic fingerprinting) are generally in agreement with the groupings made on the basis of host range.

Pathogenesis Related (PR) proteins: These proteins are induced throughout the infected plant in response to pathogen infection and are associated with SAR. Some PR proteins (such as chitinases and b-1,3-glucanases) exhibit antifungal activity *in vitro*.

Phytoalexins: Small anti-microbial compounds produced by plants in response to infection.

Papilla: A structure often observed at the pathogen infection site between the primary cell wall and the plasma membrane of a host cell. Papilla contains cell wall materials (callose and lignin).

Resistance (R) genes: Plant genes involved in recognition of pathogen avirulence factors. These genes encode putative receptors of avirulence factors and the majority of them are leucine-rich repeat proteins and/or kinases.

Resistant plant: A plant that is able to resist pathogen infection and exhibits no or few disease symptoms. A plant may be resistant to one pathogen, but susceptible to another.

Salicylic acid (SA): Salicylic acid, or 2-hydroxybenzoic acid [$C_6H_4(OH)CO_2H$], is an endogenous messenger for activation of multiple plant resistance responses against microbial pathogens. SA accumulation is a hallmark of SAR.

Saprophyte: A microbe that does not feed on living plant tissues or cause disease in any plants. A saprophyte may be present in or on plants and feed as a secondary scavenger.

Susceptible plant: A plant that is not able to resist infection of a pathogen and exhibits disease symptoms. A plant may be susceptible to one pathogen, but resistant to another.

Systemic acquired resistance (SAR): A long-lasting, broad-spectrum resistance induced throughout otherwise susceptible plants by prior local infection of necrotizing pathogens or pretreatment of certain chemical inducers (such as salicylic acid [SA], benzothiadiazol [BTH], and 2,6-dichloroisonicotinic acid [INA]). SAR is accompanied by accumulation of SA, which is required for activation of a set of 'pathogenesis-related' (PR) genes.

Type III secretion: A specialized bacterial protein secretion process that delivers some bacterial virulence protein to the host apoplast and others directly into the host cell cytoplasm or nuclei. This secretion process is conserved in both plant and mammalian bacterial pathogens.

Virulent pathogen: A pathogen in a susceptible plant. A virulent pathogen becomes avirulent when it is in a resistant plant.

Virulence: A quantitative descriptor of the ability of a pathogen to colonize a host and cause disease.

Water-soaking: A common disease symptom in bacterial

infection. Water-soaking is caused by release of water and, presumably, nutrients into the apoplast from infected plant cells. In the case of *Pseudomonas syringae* infection of *Arabidopsis* leaves, the water-soaking symptom appears first in the infected leaves. The water-soaked regions will become necrotic eventually. The molecular basis of water-soaking is not known.

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APPENDIX III: BACTERIAL PATHOGEN INOCULATION TECHNIQUES

The following is a presentation of several common methods used for bacterial pathogen inoculation of *Arabidopsis*. The first section will briefly describe growth of *Arabidopsis* plants, specifically, a special case in which the plants are grown in pots covered with mesh. The next section will explain bacterial inoculum preparation, followed by a presentation of three methods of inoculating *Pseudomonas syringae* bacterial pathogens onto *Arabidopsis* leaves. The three inoculation methods are:

A. Syringe injection of individual leaves.
B. Dipping or spray inoculation of pots or flats of plants.
C. Vacuum infiltration of plants in screened pots.

These protocols are used in Sheng Yang He's laboratory.

Growing Arabidopsis Plants for Inoculation:

For syringe injection or spray inoculation, the plants can be grown by standard methods (without mesh), but if plants are going to be used for dipping or vacuum infiltration it is recommended that they be grown in pots with mesh. This is important for helping contain the soil during inversion in the inoculum. The soil mix we use is an equal mix of Baccto high porosity professional plant mix, perlite and vermiculite. The moist soil mix is mounded into 3-inch square pots and has a thin layer of fine vermiculite spread over the top of the soil. This soil mix should rise about 0.5 to 1 inch above the edge of the pot. The pot is then covered with mesh (we use plastic window screen), which is held firmly to the surface of the soil with a rubber band. The pots are placed in flats and soaked with a fertilizer solution.

Arabidopsis seed is sown in the screened pots and covered with a plastic dome to maintain high humidity for efficient germination. If necessary, the flats may be placed in the cold (4°C) for 2 days and then moved to the growth chamber. The cold treatment will help to synchronize germination. The growth chamber conditions are 20°C and 70-80% relative humidity with 12 hours of fluorescent light (a light intensity of approximately 100 to 150 μEinsteins/m^2/sec). After about 1 week, the seeds will germinate and emerge on top of the screen. The plastic domes are then opened slightly for a couple of days and then removed completely. At this time any excess plants are removed from the pot (usually 4 to 6 well-distributed plants are grown in each pot). The plants are watered from the bottom up (adding water to the flat) once or twice a week. It is important not to let the soil completely dry out between watering. At the same time, it is important not to overwater plants. The plants have fertilizer added during

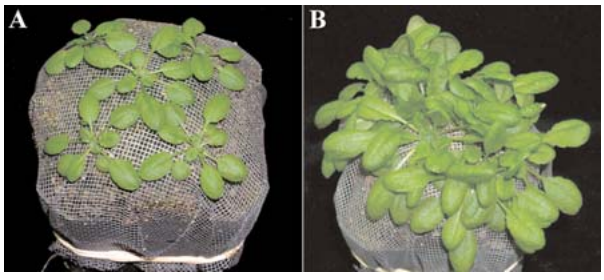

Figure 10. *Arabidopsis* grown in pots with mesh. **(A)** A pot of four-week-old *Arabidopsis* plants. **(B)** A pot of six-week-old *Arabidopsis* plants.

watering about every two weeks, or more often if necessary.

Plants 4 to 6 weeks old are used for inoculation (at this point they usually have numerous large leaves but have not started to flower). Pictures of Col-0 plants grown in screened pots are shown in Figure 10.

Pseudomonas Inoculum Preparation

1. Bacteria are streaked out from a –80°C glycerol stock onto a plate of King's medium B or a low salt Luria Bertani (LB) medium (10 g/L Tryptone, 5 g/L Yeast Extract and 5 g/L NaCl pH=7.0) with appropriate antibiotics and grown for 1 or 2 days at 28°C. Many *P. syringae* strains do not grow well in a high pH medium. Adjust the medium pH to pH 7 or slightly lower.

2. Bacteria from the fresh streak are transferred to a liquid culture with appropriate antibiotics and grown with shaking at 28°C for 8 to 12 hours, when bacterial culture should reach mid to late log phase growth (OD_{600}=0.6 to 1.0). (Alternatively, the bacteria can be plated and grown on solid medium, and then scraped off the plate for use in preparation of the inoculum.)

3. The bacteria from the liquid culture are harvested. If the culture overgrows the OD_{600} estimate of viable bacteria will not be as accurate because of the increasing number of dead bacteria and *Arabidopsis* leaf symptom development will be more variable.

4. The culture is centrifuged at 2500 x g for 10 minutes in a swinging bucket rotor to pellet the bacteria.

5. The culture supernatant is poured off and the bacteria are resuspended in sterile water or 10 mM $MgCl_2$. We have used both, and water seems to work as well as 10 mM $MgCl_2$.

6. (Optional) The cells can be washed 1 or 2 times in water (in volumes equal to that used to grow the bacteria) by repeating steps 4 and 5.

7. The Optical Density (OD) of the bacterial cell suspension is quantified using a spectrophotometer set at 600 nm.

For *Pst* DC3000 an OD_{600}=0.2 is approximately 1 x 10^8 colony-forming units/mL. Injection of dense bacterial suspensions (~10^8 cfu/mL) of avirulent bacteria is used to elicit a confluent hypersensitive response (dry-looking necrosis) in resistant plants in a relatively short time (approximately 8 to 12 hours after injection). This is because most plant cells have direct contact to the bacteria and undergo the HR with this high density of the bacteria inoculum. In this way, the HR can be macroscopically observed. Dense bacterial suspensions of virulent bacteria cause a slower disease necrosis (at 18 to 24 hours) if injected into leaves. Dense bacterial suspensions are also used in dipping or spraying inoculation. In these cases, disease symptoms will develop within 3 or 4 days in susceptible plants, whereas no disease symptoms will appear in resistant plants.

A lower level of inoculum (OD_{600}=0.002 of *Pst* DC3000 is 1 x 10^6 cfu/mL) is used for syringe or vacuum infiltration. Avirulent bacteria, when injected or vacuum infiltrated into a resistant host at 10^6 cfu/mL, usually produces no disease symptoms, whereas the virulent bacterial strain will cause chlorosis and necrosis of the infiltrated tissue of a susceptible host plant within 3 days.

8. The inoculum is made by calculating the proper dilution necessary for the desired bacterial concentration and then diluting that volume of bacteria in sterile water.

Note that a plant's response to bacteria could vary for different growth conditions. Even subtle differences, such as differences in the watering program or airflow around plants can significantly change the response. The dose of bacteria may have to be empirically adjusted in each laboratory. For example, in Fumi Katagiri's laboratory, typically 2 to 10 times lower bacterial doses are used for these purposes.

Syringe Injection

Plants are grown by standard techniques and the inoculum is prepared as described above. Individual leaves can be infiltrated easily using a syringe. The steps are illustrated below:

1. A leaf is selected and marked so that it can be identified later. A blunt-ended permanent marker works well for this.

2. The leaf is carefully inverted, exposing the abaxial (under) side. A 1-mL needleless syringe containing a bacterial suspension is used to pressure-infiltrate the leaf intracellular spaces. Avoid the vascular system of the leaf for injection; damage of the midrib will have obvious detrimental effects on the viability of the leaf tissue (see Figure 11).

3. Only a small amount of inoculum (approximately 10 µL) will infiltrate the leaf. As this occurs, water-soaking of the leaf is apparent.

4. The intercellular spaces of the infiltrated leaves are allowed to dry and then the plants are covered with a plastic dome to maintain humidity for 2 to 3 days. Leaves that have been syringe-inoculated with 5 x 10^5 cfu/mL of *Pst* DC3000 four days after inoculation are shown in Figure 2.

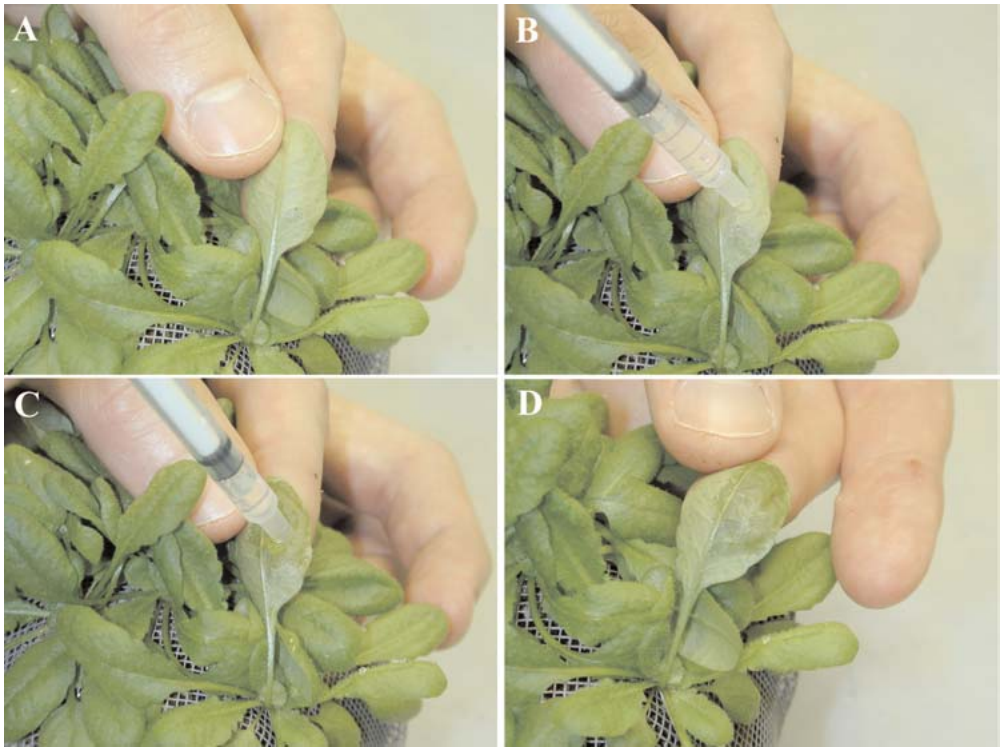

Figure 11. Syringe infiltration of *Arabidopsis* leaves. **(A)** The abaxial (under) side of the *Arabidopsis* leaf to be syringe-infiltrated. **(B)** Placement of the syringe on the right side of the leaf, avoiding the midvein. **(C)** Gentle infiltration of a portion of the leaf's intercellular space. **(D)** The syringe-infiltrated leaf. Note that the infiltrated area appears water-soaked.

Spray or Dipping Inoculation

The normal infection route for *Pseudomonas syringae* and other foliar bacterial pathogens is through wounds or natural openings such as stomata. Dipping or spraying bacterial suspensions on *Arabidopsis* leaves mimics this natural method of entry into the apoplastic space.

Spray Inoculation:

The plants are grown and the bacterial suspension prepared as previously described.

Plants in pots or flats are sprayed with a bacterial suspension containing 2 to 5 X 10^8 cfu/mL in water with 0.02 to 0.05% Silwet L-77 (Union Carbide)1. A normal spray bottle with the nozzle set to spray a fine mist is used. Continue to spray the bacterial suspension onto leaves until there is imminent runoff. By this point, the leaf surfaces should be coated with the bacterial suspension and appear evenly wet (Figure 12).

Dipping Inoculation:

Dipping inoculation is much like spray inoculation, it is simply a different way of coating the leaves with the bacterial suspension. Plants grown in pots with mesh are dipped into a bacterial suspension like that used for spray inoculation. The inverted pot of plants is fully submerged in the bacterial suspension for 2 to 3 seconds and then removed. The leaf surfaces should be evenly coated with the bacterial suspension.

Following inoculation, the plants are immediately placed under a plastic dome to maintain high humidity for 2 to 3 days. The high humidity (80 to 90%) supports disease symptom development. It is important to ensure that the

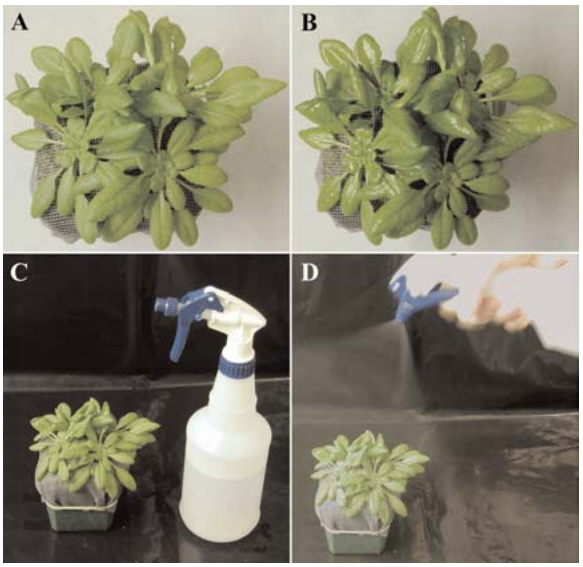

Figure 12. Spray inoculation of *Arabidopsis* plants. **(A)** *Arabidopsis* plants before inoculation. **(B)** The same pot of plants after spray-inoculation. **(C)** The spray bottle. **(D)** Spraying inoculum onto the plants.

humidity is not too high (~100%), otherwise the leaf intracellular spaces will become completely saturated, giving abnormal disease symptom development.

[1] Note, Silwet L-77 is a surfactant believed to improve the access of bacteria to the leaf apoplastic space. The amount of L-77 necessary in the inoculum (but below the level of phytotoxicity) may vary depending on the ecotype/genotype of the plants inoculated and the conditions in which they are grown. For *Pst* DC3000 inoculation of Col-0 plants, we typically use 0.05% Silwet L-77. As with each inoculation technique, the conditions should be carefully optimized before experimental use. If L-77 is not used in the bacterial suspension, the bacterial suspension will bead up into droplets on the hydrophobic surface of the leaves and rapidly run off the leaves. This significantly reduces the reliability of symptom development on any particular plant or leaf, although some leaves will still develop disease symptoms without the use of Silwet L-77.

Vacuum Infiltration

1. The inoculum is prepared as described above. Note the surfactant L-77 Silwet is added to the inoculum at the level of 0.004% (40 µl/L). The Silwet aids in vacuum infiltration; without it not all the leaves will be infiltrated. Note, a relatively large volume of inoculum is needed, usually several liters; it depends on the container used for vacuum infiltration and the number of plants to be infiltrated.

2. The vacuum infiltration apparatus (Figure 13) is assembled and the refrigerated condensation trap is turned on.

3. The inoculum is poured into a container (a 1-L glass beaker is shown), which supports the inverted pot (so that the whole pot is not submerged) while allowing the plants to be entirely immersed in the inoculum.

4. The beaker with the plants in the inoculum is placed in the vacuum chamber and the vacuum pump is turned on.

5. When the vacuum pressure reaches a level of approximately 20 inches of mercury, it is maintained for 1 minute while the pump continues to pull a vacuum.

The vacuum pressure and the time necessary for complete infiltration of the leaves without inflicting damage to the plants may vary for other vacuum systems, but the optimal settings can be determined by trial and error. After 1 minute, the vacuum pressure gauge reads 22 to 25 inches mercury and bubbles will appear on the surface of the leaves as well as on the top of the inoculum.

6. After the incubation, the vacuum pressure is rapidly released by removing the valve stopcock. When the vacuum pressure returns to zero, the plants can be removed from the chamber. During this rapid return to atmospheric pressure the leaves will become infiltrated with the bacterial suspension. Pictures of steps 3 through 6 are shown in Figure 14.

7. A successful inoculation results in almost all the leaves being fully infiltrated with the inoculum. The effectiveness of the vacuum treatment can be easily assessed by examining the plant leaves. Infiltrated leaves look darker green (water-soaked) due to the presence of the bacterial suspension within the leaf intercellular spaces (see Figure 14 F).

8. If more plants are to be treated, the soil-contaminated bacterial suspension is discarded and replaced with fresh inoculum and steps 4 through 7 are repeated.

9. After inoculation, the plants are allowed to dry completely (for 1 to 3 hours), until the leaves do not look water-soaked any more. The inoculated plants are then covered with a plastic dome for 2 to 3 days to maintain high humidity.

For Col-0 plants inoculated with *Pst* DC3000 at a dose of OD_{600}=0.002 *Pst* DC3000 (10^6 cfu/mL), the water-soaked symptom will develop within 2 to 3 days followed by chlorosis and necrosis of the inoculated tissue occurring 3 to 4 days post-inoculation (Figure 15).

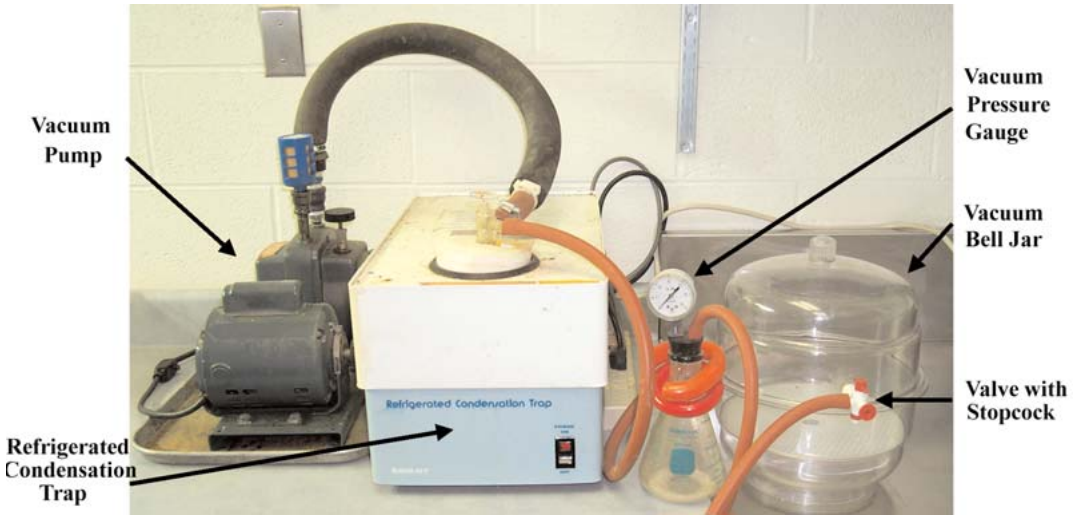

Figure 13. The vacuum-infiltration apparatus. The vacuum pump, refrigerated condensation trap, vacuum pressure gauge, bell jar, and valve with stopcock are indicated by arrows.

APPENDIX IV: BACTERIAL PATHOGEN ENUMERATION PROCEDURE

The classic phytopathological technique for quantifying bacterial virulence is an assay measuring bacterial multiplication within the host tissue. Virulent pathogens (e.g., *Pst* DC3000) inoculated at low concentrations (e.g., $<10^4$ colony-forming units/cm^2 leaf tissue, which approximately corresponds to an inoculation of $1x10^6$ cfu/ml) can colonize the host tissue and in the course of several days multiply more than 10,000-fold within the host tissue (to a level of 1 x 10^8 colony-forming units/cm^2 leaf tissue). In contrast, nonpathogenic mutant strains (e.g., *Pst* DC3000 *hrpH*$^-$ mutant) or avirulent pathogens (e.g., *Pst* DC3000 carrying the *avrRpm1* gene) in the same time course will either not multiply significantly or grow only 10- to 100-fold within the host tissue (Figure 16). The massive multiplication of the virulent bacteria correlates well with symptom development, such that the bacterial strain attains the maximal population immediately in advance of significant symptom development, which in the case of *Pst* DC3000 infection is characterized by necrotic lesions surrounded by diffuse chlorosis. The nonpathogenic strains or avirulent strains do not multiply to high populations and also do not produce disease symptoms.

A standard enumeration procedure involves pathogen inoculation (see Appendix II) followed by assaying bacterial populations present within host tissues at regular intervals (usually daily, including the day of inoculation, to establish the bacterial level immediately following inoculation). Typically the preferred inoculation techniques are either syringe injection or vacuum infiltration. From our experience, these two methods of inoculation produce more reproducible starting bacterial populations within the host leaves. Inoculum densities are usually relatively low, from 1 x 10^4 to 1 x 10^6 cfu/mL, to allow the maximum room for bacterial multiplication to occur within the host tissue. Plotting log (culturable bacterial number/cm^2 leaf tissue) against time (usually in days) after pathogen inoculation produces an unfitted curve, commonly known as a growth curve. This is a standard means of evaluating how well a bacterial pathogen multiplies in plant tissues. An example of a growth curve is shown in Figure 16.

Procedure:

1. Leaves are harvested and surface sterilized[1] as follows:

Whole leaves are removed from the host plant and placed in a 70% ethanol solution for 1 minute. The leaves are gently mixed in the solution occasionally. The leaves are then removed, blotted briefly on paper towels and then rinsed in sterile distilled water for 1 minute. The leaves are then removed and blotted dry on paper towels. Leaf disks are excised from leaves with a 0.5 cm^2 or smaller cork

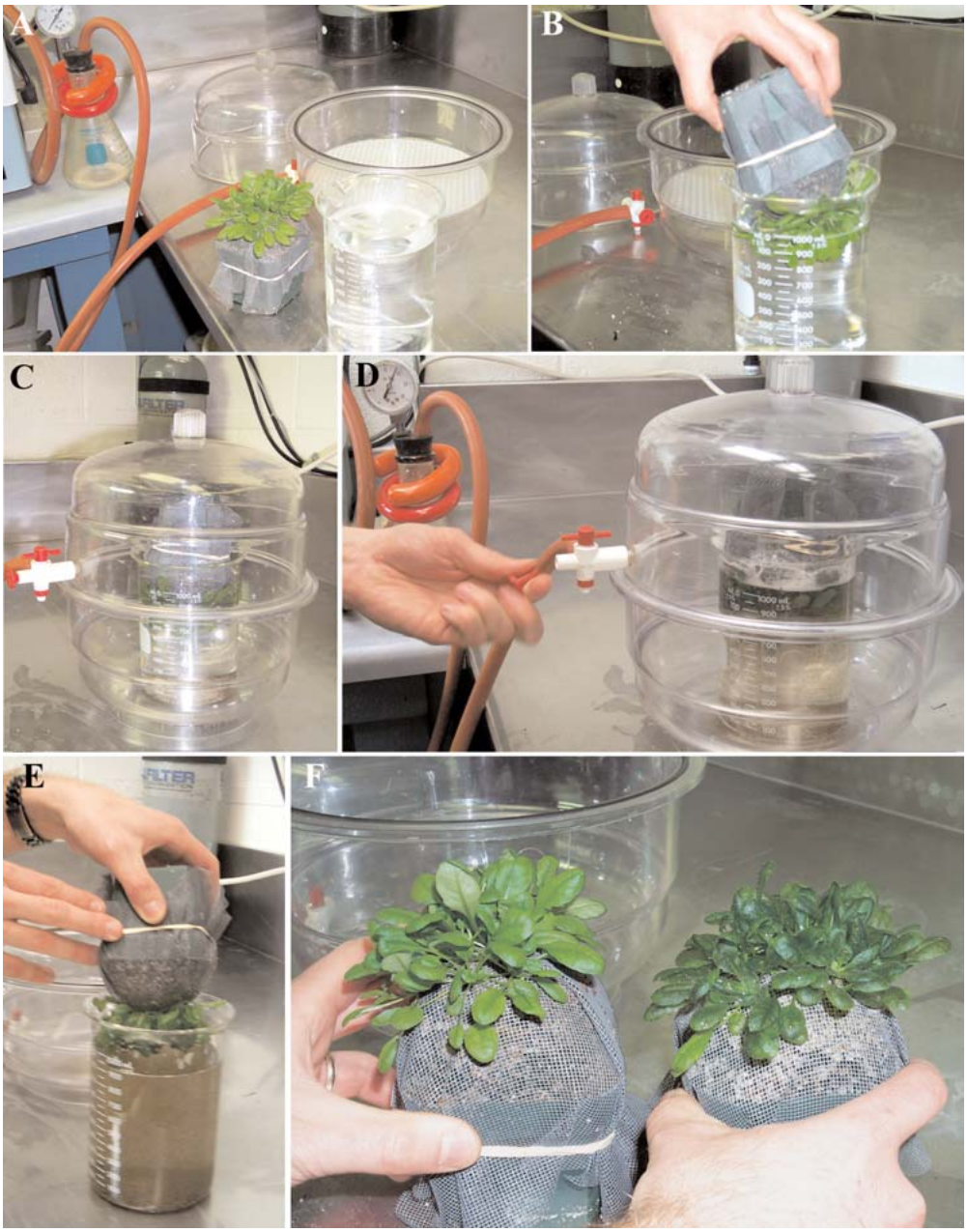

Figure 14. Vacuum infiltration procedure steps 3 through 6. **(A)** The plants and bacterial suspension before infiltration. **(B)** Inverting the pot of *Arabidopsis* plants in the bacterial suspension. **(C)** Vacuum infiltration of the plants while in the sealed bell jar. **(D)** Release of the vacuum pressure by removal of the valve stopcock. Note that the surface of the bacterial suspension and the leaf surface are covered with bubbles before the vacuum pressure is released. **(E)** Removal of the pot of plants from the bacterial suspension. **(F)** Comparison of uninoculated (left) and vacuum-infiltrated plants (right). The vacuum-infiltrated leaves have inoculum within their intercellular space and appear water-soaked.

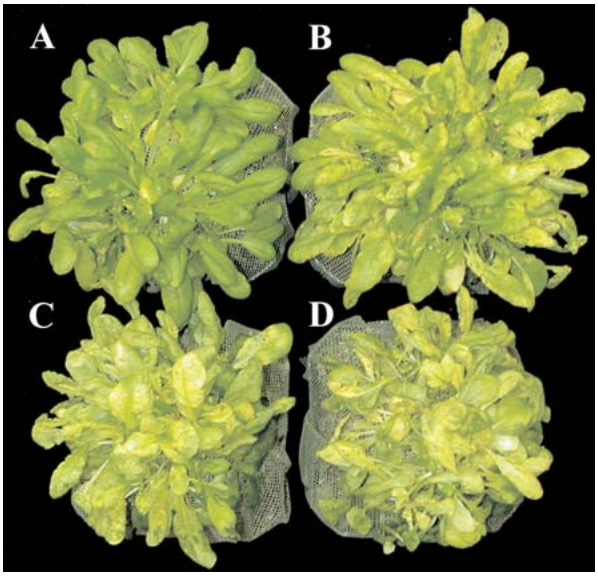

Figure 15. Disease symptoms following vacuum infiltration. Plants 4 days after inoculation with different densities of *Pst* DC3000 are shown. Plants vacuum infiltrated with 1 x 10^4 cfu/mL **(A)** 1 x 10^5 cfu/mL **(B)** 1 x 10^6 cfu/mL **(C)** and 1 x 10^7 cfu/mL **(D).**

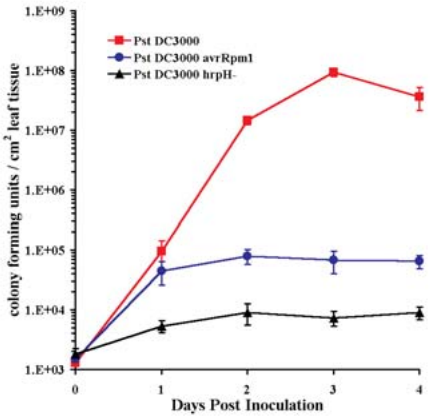

Figure 16. Multiplication of *P. syringae* pv. *tomato* DC3000 strains in *Arabidopsis* leaves. Leaves were inoculated with 1 x 10^5 cfu/mL of bacteria and *in planta* bacterial populations were determined daily. Multiplication of *P. syringae* pv. *tomato* DC3000 (virulent), DC3000/*avrRpm1* (avirulent), and the DC3000 *hrpH*$^-$ mutant (nonpathogenic), in *Arabidopsis* Columbia leaves is plotted on a log scale. The error bars indicate the standard deviation within the 3 replicate samples for each treatment.

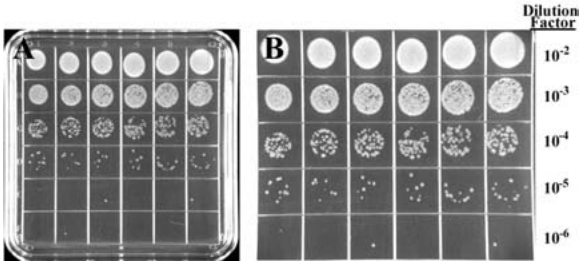

Figure 17. Determination of the bacterial population in inoculated leaf tissue. **(A)** A square plate containing agar medium with the appropriate antibiotics was spotted six times with 10 µL of six 10-fold dilutions of a homogenate of *Pst* DC3000-inoculated *Arabidopsis* leaves. The plate was incubated at 28°C for 2 days. **(B)** A close-up of a portion of the plate from **(A)** is shown. The dilution factor of each sample is indicated. Countable colonies are visible in spots from sample dilutions of 10^{-4} and/or 10^{-5}.

borer depending on the size of the sample leaves.

Typically, leaf disks from the leaves of 2 or more independent replicate plants are pooled for a single tissue sample. Three or more samples are needed for each time point to generate statistically analyzable data.

2. The leaf disks for a single sample are placed in a 1.5-mL microfuge tube with 100 µL sterile distilled water. Steps 1 and 2 are repeated for each sample.

3. The tissue samples are ground with a microfuge tube plastic pestle, either by hand or, if many samples are involved by using a small hand-held electric drill. The samples are thoroughly macerated until pieces of intact leaf tissue are no longer visible.

4. The pestle is rinsed with 900 µL of water, with the rinse being collected in the original sample tube such that the sample is now in a volume of approximately 1mL.

5. Steps 3 and 4 are repeated for all the samples.

6. Following grinding of the tissue, the samples are thoroughly vortexed to evenly distribute the bacteria within the water/tissue sample. A 100-µl sample is removed and diluted in 900 µl sterile distilled water. A serial 1:10 dilution series is created for each sample by repeating this process. The number of serial dilutions necessary to get countable colonies must be determined for each sample empirically, but dilutions to 10^{-7} are usually sufficient for any bacterial strain.

7. The samples are plated on the appropriate medium (e.g., King's medium B) supplemented with the necessary antibiotics to select for the inoculated bacterial strain. Plating can be done in the traditional way (100 µL of a single sample is spread on a single plate) or several 10 µL aliquots of the 1:10 serial dilutions can be spotted on to a single plate and allowed to dry onto the surface.

8. The plates are placed at 28°C for approximately 2 days and then the colony-forming units for each dilution of each sample are counted. A plating of a typical sample dilution series is shown in Figure 17.

For the 10-µL spotting technique, a single spot should be used for estimating the bacterial population only if it has >10 and < ~70 colonies (or whatever is reliably countable) present in the spotted sample dilution. The population present within the tissue is calculated based on the dilution factor divided by the amount of tissue present in each sample.

[1]Note, leaf surface sterilization is optional, but recommended. It removes bacteria present on the surface of the leaf (those present from the inoculation as well as any initially present epiphytic populations). Thus, the populations assayed are those bacteria present within the apoplastic space (and thus protected from surface sterilization). These bacteria within the apoplastic space and not those on the leaf are responsible for disease development. Obviously, leaf surface sterilization cannot be used on the leaf samples from spray or dip inoculated plants from day 0, since immediately following inoculation the inoculated bacteria are present on the surface and susceptible to the surface sterilization procedure.