Bacterial Growth Restriction During Host Resistance to *Pseudomonas syringae* Is Associated with Leaf Water Loss and Localized Cessation of Vascular Activity in *Arabidopsis thaliana*

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The physiological mechanisms by which plants limit the growth of bacterial pathogens during gene-for-gene resistance are poorly understood. We characterized early events in the *Arabidopsis thaliana*–*Pseudomonas syringae* pathosystem to identify physiological changes for which the kinetics are consistent with bacterial growth restriction. Using a safranin-O dye solution to detect vascular activity, we demonstrated that *A. thaliana* Col-0 resistance to *P. syringae* pv. *tomato* DC3000 cells expressing *avrRpm1* involved virtually complete cessation of vascular water movement into the infection site within only 3 h postinoculation (hpi), under the conditions tested. This vascular restriction preceded or was simultaneous with precipitous decreases in photosynthesis, stomatal conductance, and leaf transpiration, with the latter two remaining at detectable levels. Microscopic plant cell death was detected as early as 2 hpi. Interestingly, suppression of bacterial growth during AvrRpm1-mediated resistance was eliminated by physically blocking leaf water loss through the stomata without altering plant cell death and was nearly eliminated by incubating plants at high relative humidity. The majority of the population growth benefit from blocking leaf water loss occurred early after inoculation, i.e., between 4 and 8 hpi. Collectively, these results support a model in which *A. thaliana* suppresses *P. syringae* growth during gene-for-gene resistance, at least in part, by coupling restricted vascular flow to the infection site with water loss through partially open stomata; that is, the plants effectively starve the invading bacteria for water.

Bacterial foliar pathogens have been a common subject of studies aimed at identifying the molecular and physiological basis of pathogen interactions with plants. One of the best-studied pathosystems is that of *Arabidopsis thaliana* and *Pseudomonas syringae* pv. *tomato*; this pathogen infects both tomato and *A. thaliana*. Like many other bacterial pathogens, *P. syringae* has developed the ability to suppress basal defenses and promote disease by injecting protein effectors directly into plant cells via the type III secretion system (T3SS) (DeRoy et al. 2004; Galan and Wolf-Watz 2006; Hauck et al. 2003).

Effectors promote disease and suppress resistance, in part, by interfering with defense-related molecular signaling pathways (Jamir et al. 2004; Kang et al. 2004; Mattoo et al. 2007). Plant resistance (*R*) genes facilitate recognition of specific intracellular effectors, triggering a disease resistance response. This gene-for-gene mediated resistance is often accompanied by a hypersensitive response, which is a rapid plant programmed cell death (PCD) that is localized to the site of infection (Dangl and Jones 2001; Huang et al. 1988). Disease resistance may thus involve localized tissue necrosis as well as the production of antimicrobial compounds, the induction of defense-related genes, and the activation of systemic resistance that is effective against a broad range of pathogens (Heath 2000; Mittler et al. 1997; Morel and Dangl 1997; Mur et al. 2008).

Bacterial growth is limited during gene-for-gene mediated resistance. For example, *P. syringae* strains often attain populations as high as 10^8 to 10^9 cells per square centimeter in *A. thaliana* leaves of susceptible hosts, but populations are 10- to 200-fold lower in resistant hosts (Jambunathan et al. 2001; Whalen et al. 1991). Identifying the physiological mechanisms that are directly responsible for limiting bacterial growth has been problematic because the effects of plant PCD are difficult to separate from the myriad other changes that occur during gene-for-gene mediated resistance. Interestingly, PCD is not necessarily required for bacterial inhibition, as demonstrated by the ability of *A. thaliana* ecotype Columbia (Col-0) to inhibit the growth of *P. syringae* pv. *tomato* DC3000 expressing *avrRps4* (Hinsch and Staskawicz 1996) or *hopPsyA* (Gassmann 2005) in the absence of a visible hypersensitive response (HR). Similarly, *A. thaliana dnd1* mutants express effector-mediated resistance in response to some bacterial pathogens without inducing the HR (Clough et al. 2000; Yu et al. 1998). Consistent with this uncoupling of the HR and restricted pathogen growth, *A. thaliana ndr1* mutants display exaggerated PCD in the presence of certain effector-expressing *P. syringae* strains without showing a proportional suppression of the bacterial populations (Century et al. 1995).

Leaf water status may play a key role in determining the outcome of plant-pathogen interactions. Maintaining water-soaked leaves in high humidity can suppress macroscopic HR necrosis and increase pathogen growth (Cook and Stall 1977; Young 1974) as well as promote the growth of saprophytic bacteria introduced into leaves (Stall and Cook 1979). Furthermore, high humidity can suppress PCD in some *Arabidopsis* lesion-mimic defense gene mutants, including *cpr22*, *cnp1*, and *lsd6* (Jambunathan et al. 2001; Weymann et al. 1995; Yoshioka et al. 2001), and can also suppress *Cf-4/Avr4*- and

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Cf-9/Avr9-dependent hypersensitivity and defense gene expression in transgenic tomato seedlings (Wang et al. 2005). These studies implicate leaf-water status as a factor influencing plant defense.

Leaf-water availability may have a direct impact on bacterial growth during gene-for-gene mediated disease resistance. Previously, we demonstrated that pathogen growth restriction was correlated with low water availability in the early stages of a gene-for-gene resistance response (Wright and Beattie 2004). We used *P. syringae* strains containing transcriptional fusions responsive to low water potentials to monitor bacterial exposure to water stress following infection of *A. thaliana* leaves (Wright and Beattie 2004). During gene-for-gene resistance triggered by a *P. syringae* effector, these cells were exposed to a rapid decrease in water potential to levels that were low enough to inhibit bacterial cell division when examined in vitro. The cells were exposed to much less severe levels of water stress during disease development than during the expression of resistance. Furthermore, the sizes of the *P. syringae* populations within leaves were strongly correlated with the water availability sensed by the bacterial cells. Collectively, these results suggest that low water availability is a major factor restricting bacterial growth during gene-for-gene mediated resistance. In this study, we test this hypothesis and explore the physiological mechanisms underlying changes in water availability to invading pathogens.

Leaf hydration represents a balance between water flowing into tissues through the xylem and water evaporating through stomata, and both of these activities can be altered during plant

defense. For example, plants respond to some vascular pathogens by plugging the xylem via the formation of tyloses or gels within the vasculature, as occurs with *Xanthomonas campestris* pv. *manihotis* on cassava (Kpemoua et al. 1996). Alternatively, water movement may be disrupted by the formation of ethylene-induced air bubbles (embolisms) in the xylem, as occurs with *Xylella fastidiosa* on grapes (Perez-Donoso et al. 2007). Defenses against nonvascular pathogens can also involve vascular disruption, although this has been reported primarily for nonbacterial pathogens and the mechanisms are unknown. For example, an HR in tomato cotyledons triggered by elicitors of the fungal pathogen *Cladosporium fulvum* involved early cell death of tissues lining the vascular system and loss of up to 80% of the initial leaf weight, suggestive of water loss, within 48 h after inoculation (Hammond-Kosack et al. 1996). Similarly, tobacco leaves challenged with the *Tobacco mosaic virus* (TMV) exhibited a reduction in transpiration preceding visible necrosis (Chaerle et al. 1999) and *Nicotiana edwardsonii* leaves infiltrated with TMV exhibited vascular restriction preceding visible necrosis (Wright et al. 2000). In contrast to gene-for-gene mediated resistance, basal resistance of *Nicotiana benthamiana* to *P. syringae* involved reduced vascular flow (Oh and Collmer 2005). Interestingly, a variety of plants, including tobacco and tomato, express genes during a resistance response that share homology with genes induced in response to desiccation (Ciccarelli and Bork 2005; Gopalan et al. 1996).

Stomatal apertures also change during plant defense. Stomata of tomato and *Commelina communis* close in response to exogenous application of fungal-derived and plant-derived elicitors, including oligogalacturonic acid and chitosan (Lee et al. 1999). Stomatal closure in *A. thaliana* in response to specific bacterial structures, designated microbial-associated molecular patterns (MAMPs), has been reported (Melotto et al. 2006) and has been interpreted as a deliberate plant defense reaction that prevents bacterial invasion (Lee et al. 1999; Melotto et al. 2006). Interestingly, the phytotoxin coronatine produced by some *P. syringae* strains as well as a compound secreted by a *Xanthomonas campestris* pv. *campestris* strain can reverse MAMP-mediated stomatal closure and, thus, increase bacterial access to the leaf interior (Gudesblat et al. 2009; Melotto et al. 2006).

The purpose of this study was to evaluate the fine-scale kinetics of water loss, vascular activity, and stomatal conductance during gene-for-gene mediated resistance using the *A. thaliana*-*P. syringae* pathosystem and to identify the impact of leaf water loss on intercellular bacterial population sizes. Our results indicate that an effector-mediated resistance response in *A. thaliana* Col-0 involves a rapid, near-complete cessation of vascular water flow into the infection site and a rapid reduction in stomatal conductance and leaf transpiration. Furthermore, preventing leaf drying completely reversed the inhibitory effect of this resistance response on bacterial growth without eliminating plant PCD. Collectively, these results indicate that leaf water loss contributes to *A. thaliana* suppression of *P. syringae* growth during gene-for-gene mediated resistance.

RESULTS

The kinetics of leaf-mass loss indicate rapid water loss during an *avrRpm1*-mediated resistance response.

Tissue desiccation following necrosis is a hallmark of gene-for-gene mediated resistance; however, the kinetics of water loss for the period preceding visible necrosis have not been reported. Vacuum-infiltration of *P. syringae* pv. *tomato* DC3000(pP_{lavrRpm1}) into *A. thaliana* ecotype Col-0 resulted in a 54% reduction in leaf fresh weight within 9 h postinoculation (hpi) (Fig. 1A). By comparison, inoculation with the

Fig. 1. Kinetics of water loss in leaves following bacterial infection. **A**, *Arabidopsis thaliana* plants were vacuum-infiltrated with buffer, DC3000(pPI), or DC3000(pP_{lavrRpm1}). **B**, Tomato cultivars Rio Grande PtoS (susceptible) or Rio Grande PtoR (resistant) plants were vacuum-infiltrated with buffer or DC3000(pPI). Leaves were excised from plants at selected timepoints and individually weighed. Leaves weighed immediately following inoculation provided the 0 h postinoculation (hpi) values. Values shown are the mean percentage change in fresh weight from the mean fresh weight at 0 hpi $\pm$ standard error ($n = 15$).

disease-causing strain DC3000(pPI) resulted in only a 14% reduction over a similar period. These values increased to 80 and 34% by 24 hpi for DC3000(pPIavrRpm1) and DC3000(pPI), respectively (Fig. 1A). In *A. thaliana* plants infected with DC3000(pPIavrRpm1), the largest decrease occurred between 6 and 9 hpi and, thus, overlapped with the first detection of macroscopic lesions, which occurred at 6 hpi. A similar difference between a resistant and susceptible response in the kinetics of water loss was observed when DC3000(pI) was infiltrated into tomato cultivars Rio Grande PtoR (resistant) and Rio Grande PtoS (susceptible), although the reductions in leaf weight were proportionally smaller (Fig. 1B).

The LI-6400 photosynthesis system is effective for near-continuous monitoring of leaf physiology following bacterial inoculation.

We investigated the possibility of using a LI-6400 photosynthesis system to capture real-time measurements of whole-leaf physiology in inoculated *A. thaliana* leaves that were still attached to plants. The LI-6400 estimates stomatal conductance and rates of transpiration and photosynthesis based on gas exchange rates for CO₂ and water, leaf temperature, leaf area, and atmospheric pressure, and the instrument can collect measurements on a nearly continuous basis. In this study, leaf physiology measurements were taken at 10-min intervals from 2 h prior to inoculation until 10 hpi. Small amounts of residual water on the leaf surfaces obstructed accurate measurements in the 30 min following inoculation by influencing the estimates of water exchange rates (Fig. 2). Although the physiological activities of individual leaves in the same treatment group showed leaf-to-leaf variability, as illustrated for stomatal conductance in leaves treated with buffer (Fig. 2A) and DC3000(pPIavrRpm1) (Fig. 2B), the kinetics of the physiological changes were generally consistent within a treatment. Some of the leaf-to-leaf variability was associated with the ambient relative humidity (RH), as illustrated in Figure 2A. To account for differences in the size and physiology of the leaves prior to treatment, estimates of stomatal conductance and rates of transpiration and photosynthesis were expressed as a per-

centage of the values that were measured just prior to inoculation. Expressing these values as “percent change” reduced the apparent leaf-to-leaf variation and better reflected the kinetic similarity among the replicate leaves within each treatment (Fig. 2).

The kinetics of the physiological changes in leaves following pathogen infiltration are complex.

Following vacuum-infiltration with the disease-inducing strain DC3000(pPI), the stomatal conductance increased between 2.5 and 5 hpi (Fig. 3A). This large increase was likely the result of coronatine, a phytotoxin that can open stomata during bacterial challenge (Melotto et al. 2006). This was confirmed by the absence of an increase in leaves infiltrated with the coronatine-deficient mutant DC3118 (Fig. 3B) (Mittal and Davis 1995; Moore et al. 1989). Interestingly, continuous monitoring of stomatal activity indicated that this coronatine-mediated increase is only transient, as a large and rapid decrease occurred between 5 and 7 hpi in the DC3000(pPI)-inoculated leaves (Fig. 3A). To evaluate whether factors secreted by the T3SS influence the kinetics of stomatal conductance, we infiltrated plants with a DC3000 mutant defective in HrcC, an outer membrane protein required for type III-mediated secretion (Yuan and He 1996). This mutant caused a large sustained increase in stomatal conductance, consistent with its ability to produce coronatine, but did not induce a subsequent decrease, as observed with the wild-type strain (Fig. 3B). This observation suggests that effectors secreted through the T3SS are responsible for the eventual decrease in conductance, although the sustained increase could be due to the previously reported overproduction of coronatine by the *hrcC* mutant (Penalzo-Vazquez et al. 2000).

The stomatal conductance of leaves infiltrated with the effector-expressing strains DC3000(pPIavrRpm1) and DC3000(pPIavrRpt2) decreased rapidly starting as early as 1 hpi (Fig. 3A). Although both strains are capable of producing coronatine, stomatal conductance did not increase after 1 hpi with either strain, suggesting that coronatine-mediated stomatal changes are suppressed during the effector-mediated resistance. The

Fig. 2. Reproducibility of real-time changes in *Arabidopsis thaliana* leaves vacuum-infiltrated with **A**, buffer or **B**, DC3000(pPIavrRpm1) cells. Single leaf measurements were taken every 10 min, using an LI-6400 photosynthesis system. Three replicate leaves, each indicated by a separate line, were vacuum-infiltrated with buffer or DC3000(pPIavrRpm1) 2 h after measurements began (arrows indicate time of inoculation). Lights were turned off at 10 h postinoculation (hpi). The replicate leaves indicated by the solid, dashed, and dotted lines were examined at ambient relative humidity levels of, respectively, 53, 61, and 75% (**A**) and 45, 70, and 75% (**B**). For both **A** and **B**, the first graph shows the conductance of the stomata to water vapor (mol H₂O/m²s), whereas the latter three show the percentage change in stomatal conductance, transpiration, and photosynthesis, respectively, relative to the values at 0 hpi.

stomatal conductance of these leaves clearly diverged from that of leaves infiltrated with DC3000(pPI) by as early as 3 hpi, with DC3000(pPI) inducing an increase and the incompatible pathogens inducing a large and sustained decrease (Fig. 3A).

Stomatal conductance is influenced by stomatal aperture, stomatal density, guard-cell size, and environmental conditions (Farquhar and Sharkey 1982). Microscopic examination of leaf surfaces confirmed that stomatal density and average guard-cell sizes were similar among the leaves in distinct treatments, and the environmental conditions were controlled by the LI-6400 photosynthesis system. Therefore, the relative stomatal conductance was likely proportional to the relative stomatal aperture for the leaves in these experiments. The increased conductance induced by coronatine-producing strains, therefore, suggests stomatal opening, as shown in a previous study (Melotto et al. 2006), whereas the decreased conductance induced by incompatible strains suggests stomatal closure. Microscopic examination indicated that stomatal widths on DC3000 (pPI*avrRpm1*)-treated leaves were reduced nearly 70% in 6 h, compared with the preinoculation values, but they did not completely close (Supplementary Fig. 1), despite the fact that the stomatal conductance decreased by as much as 90% within 8 hpi (Fig. 3).

Transpiration rates were correlated with stomatal conductance in all vacuum-infiltrated leaves, although changes in transpiration rate were attenuated relative to changes in stomatal conductance (Fig. 3A and B). Similarly, photosynthetic rates were correlated with transpiration rates. For all physiological properties examined, the kinetic events following infiltration of DC3000(pPI*avrRpt2*) were delayed approximately 2 h relative to those following infiltration with DC3000(pPI*avrRpm1*).

The kinetics of physiological changes following leaf infiltration with the nonpathogenic strain *P. fluorescens* A506 were complex and depended on RH. At a RH of 62%, the stomatal conductance of A506-treated leaves decreased within 2 hpi and then remained steady (Fig. 3B), as expected for an organism inducing only a basal defense response. Surprisingly, A506 was similar to the *hrcC* mutant in its effect on transpiration (Fig. 3B), despite that A506 does not produce coronatine and that coronatine produced by the *hrcC* mutant likely increased transpiration by inducing stomatal opening. At 12% RH, however, A506 increased stomatal conductance and did not increase transpiration (Supplementary Fig. 2A), an observation that we cannot presently explain.

Inoculation by leaf immersion shows physiological changes that occur within 1 h after inoculation.

We also measured physiological changes in leaves after inoculation by immersion, a method that mimics natural leaf invasion better than vacuum infiltration. Stomatal conductance following immersion in buffer was particularly dependent on RH; it increased within 1 hpi when the ambient RH was 45 to 50% (Fig. 3C) but decreased within 1 hpi when the ambient RH was 25 to 40%. When comparing treatments at RH levels >45% (Fig. 3C), the stomatal conductance in DC3000(pPI)-immersed leaves was significantly lower than in the buffer-immersed leaves at 1 hpi (Fig. 3C), consistent with reports of rapid MAMP-mediated stomatal closure. Similarly, plants immersed in MAMP-rich suspensions of the bacterial peptide elicitor flg22 exhibited lower stomatal conductance within 1 hpi than those immersed in buffer, confirming a role for flg22 in stomatal closure. These observations also support the possi-

Fig. 3. Physiological changes in *Arabidopsis thaliana* leaves following treatment with buffer or bacteria. **A**, Leaves were vacuum infiltrated with buffer, DC3000(pPI), DC3000(pPI*avrRpt2*), and DC3000(pPI*avrRpm1*); lines are representative of at least three replicate treated leaves. **B**, Leaves were vacuum-infiltrated with DC3000(pPI), a DC3000 *hrcC* mutant, the coronatine-deficient DC3118 mutant, and *Pseudomonas fluorescens* A506; lines are representative of at least two replicate treated leaves. **C**, Leaves were inoculated by immersion in buffer, DC3000(pPI), 20 μ M Flg22, *P. fluorescens* A506, and DC3000(pPI*avrRpm1*). Lines are representative of at least three replicate leaves. All values reflect the percentage change from the values at 0 h postinoculation.

bility that flg22 and other DC3000(pPI)-associated MAMPs prevented an increase in stomatal opening, as occurred in the buffer treatment, rather than inducing stomatal closure. Interestingly, the stomatal conductance in the DC3000(pPI*avrRpm1*)-immersed leaves was significantly lower than in the DC3000(pPI)-treated leaves at 1 hpi (Fig. 3C), suggesting that the AvrRpm1 effector affects stomatal activity directly or indirectly as early as 1 hpi. DC3000(pPI)-immersed leaves exhibited an increase in stomatal conductance between 30 min and 3 hpi that was induced by coronatine, as evidenced by its absence in leaves immersed in DC3118 (Supplementary Fig. 3). Similar to the results following infiltration of the strains, the absence of an increase in stomatal conductance in the DC3000(pPI*avrRpm1*)-immersed leaves between 1 and 2 hpi indicates that coronatine-mediated stomatal opening is suppressed during the plant response to DC3000(pPI*avrRpm1*). Similar to the results following infiltration of A506, the stomatal conductance of A506-immersed leaves was lower than the buffer-treated leaves at 1 hpi at RH levels >45% (Fig. 3C), suggestive of MAMP activity in these leaves, but was not lower in leaves at lower RH levels (25 to 30%).

The changes in transpiration and photosynthetic activity in the leaves subjected to leaf immersion were not as closely correlated with stomatal conductance as in the leaves subjected to infiltration (Fig. 3). This may be due to the presence of more bacteria in the leaf intercellular spaces following infiltration and to a greater opportunity for intercellular bacteria than surface bacteria to introduce effectors into guard cells and other leaf cells due to the absence of a cuticle on internal plant cell surfaces. Despite the attenuated effects of the immersion treatments, DC3000(pPI*avrRpm1*)-immersed leaves showed decreased transpiration and photosynthetic activity after 4 hpi, consistent with the infiltration results. The flg22-immersed leaves showed a particularly large decrease in photosynthetic activity; this decrease was not observed in leaves immersed in only 10 μ M flg22 and, thus, was likely a function of the high flg22 concentration used.

The kinetics of vascular activity changes indicate rapid and extreme vascular restriction during an AvrRpm1-mediated resistance response.

The decreased water availability sensed by intercellular bacteria during gene-for-gene mediated resistance in a previous study (Wright and Beattie 2004) could have resulted from increased water evaporation through stomata with increased stomatal apertures; however, the stomatal conductance decreased rather than increased during an effector-mediated resistance response (Fig. 3). Alternatively, decreased water availability could have resulted from a reduction in the vascular water flow to the infected region. To evaluate this, we syringe-infiltrated bacteria into *A. thaliana* leaves and then placed the petioles of detached leaves at various times into a safranin-O dye solution for 2 h. The movement of the dye through the xylem provided an excellent visual indication of vascular water flow (Fig. 4A) and indicated that vascular flow into regions infiltrated with DC3000(pPI*avrRpm1*) was almost totally halted by 3 hpi (Fig. 4A). When quantified using Adobe Photoshop, dye levels in these regions averaged only 10% of the dye levels in buffer- and DC3000(pPI)-infiltrated regions (Fig. 4B). Dye levels in DC3000(pPI)-treated regions also decreased but at a slower rate than in DC3000(pPI*avrRpm1*)-treated regions (Fig. 4A and B). Reduced vascular flow was localized to the site of bacterial infiltration (Fig. 4C), with dye levels in adjacent areas remaining virtually unchanged for at least 24 h. Infiltration with *P. fluorescens* A506, which induces basal resistance, resulted in a slight reduction of vascular water flow by 48 hpi (Fig. 4D). Strong vascular restriction was also ob-

served during gene-for-gene mediated resistance in the tomato cultivar Rio Grande PtoR when infiltrated with DC3000(pI) (Fig. 4E). Hence, reduced vascular flow occurs during a variety of microbe interactions with *A. thaliana* and tomato, with rapid and virtually complete vascular restriction occurring during gene-for-gene resistance.

Microscopic plant cell death is evident as early as 2 h after inoculation.

Although PCD is a well-established hallmark of *R* gene-mediated resistance, we evaluated the kinetics with which dead plant cells arose during the initial 24 hpi. Plant cell death increased dramatically over the first 3 h after inoculation with DC3000(pPI*avrRpm1*) (Fig. 5) and was detected as early as 2 hpi using microscopy. Plant cell death did not increase until 9 to 24 hpi in the DC3000(pPI)-treated plants (Fig. 5B). Levels of plant cell death remained relatively unchanged following infiltration with buffer or the nonpathogenic *P. fluorescens* A506 (Fig. 5B). Using microscopy, we observed that confluent regions of dead plant cells often occurred along leaf veins (Supplementary Fig. 4), implying a possible causal relationship between plant cell death and vascular restriction.

Preventing water loss from infected leaves prevents bacterial growth suppression during gene-for-gene mediated resistance.

To evaluate the impact of leaf water loss on intercellular bacterial populations, we vacuum-infiltrated *A. thaliana* with bacterial suspensions and then halted leaf drying by covering both the upper and lower leaf surfaces with a thin layer of petroleum jelly. The presence of a water-impermeable barrier prevented water loss through epidermal openings, as evidenced by the lack of significant mass loss with subsequent incubation. The petroleum jelly treatment increased the population sizes of DC3000 (pPI) by an average of 0.2 log units per square centimeter by 24 hpi (Fig. 6A) ($P = 0.03$, Student's *t*-test) and increased the population sizes of DC3000(pPI*avrRpm1*) and DC3000(pPI*avrRpt2*) by an average of 1.0 and 0.7 log units per square centimeter, respectively, by 24 hpi (Fig. 6B and C) ($P < 0.001$). In fact, the *avrRpm1*- and *avrRpt2*-expressing strains grew to levels nearly identical to those of the disease-inducing strain DC3000(pPI) in petroleum jelly-covered leaves. Interestingly, the majority of the population growth benefit in the petroleum jelly-covered leaves occurred soon after inoculation, i.e., between 4 and 8 hpi, with little significant growth benefit detected in the 8 to 24 hpi period after inoculation (Fig. 6D). Significant plant cell death had occurred by 8 hpi in DC3000(pPI*avrRpm1*)-inoculated leaves regardless of the petroleum jelly treatment (Fig. 6E), demonstrating that this treatment did not inhibit the HR. Furthermore, the observation that DC3000(pPI*avrRpm1*) grew to unrestricted levels in the petroleum jelly-treated leaves despite a high level of plant cell death supports prior evidence that plant PCD can be uncoupled from pathogen growth suppression (Clough et al. 2000; Gassmann 2005; Hinsch and Staskawicz 1996; Yu et al. 1998). We also evaluated the impact of leaf water loss on intercellular bacterial populations by enclosing the plants in humidity tents to minimize water loss through evaporation. Plants in the humidity tents maintained visible leaf water-soaking for at least 24 h and supported the establishment of significantly larger populations of both DC3000(pPI) and DC3000(pPI*avrRpm1*) by 24 hpi than plants that were outside the humidity tents (Fig. 7) ($P < 0.001$, Student's *t* test), although DC3000(pPI*avrRpm1*) did not establish populations quite as large as those of DC3000(pPI). Collectively, these results provide strong evidence that leaf water loss contributes to the restriction of *P. syringae* growth during gene-for-gene mediated resistance in *A. thaliana*.

DISCUSSION

Gene-for-gene mediated resistance can be triggered by a diverse range of organisms including viruses, bacteria, fungi, and nematodes. A major functional outcome of resistance is the limited growth and spread of a pathogen within the plant. Identifying the physiological and biochemical mechanisms by which this resistance suppresses bacterial growth has been problematic due to the co-occurrence of many changes in the plant. Although this suppression occurs within the first 24 hpi in the *A. thaliana*–*P. syringae* pathosystem (Fig. 6B and C), the majority of physiological research on resistance in this pathosystem has characterized events only after 24 hpi. This study characterized early events in gene-for-gene mediated resistance with the intention of identifying physiological changes for which the kinetics are consistent with a causal role in limiting bacterial growth. The combination of these kinetic

studies (Figs. 1 through 5) and the studies blocking leaf water loss (Figs. 6 and 7) provide strong evidence that plant-mediated drying at the infection site is an important physiological mechanism by which bacterial growth is limited during gene-for-gene mediated resistance.

These studies demonstrate that vascular restriction occurs during *avrRpm1*-induced resistance to *P. syringae*, a nonvascular bacterial pathogen, and that this restriction is virtually complete within 3 hpi. The timeline presented in Figure 8 illustrates the sequence of physiological changes that occurred within 9 h after *P. syringae* infiltration leading to resistance or disease. The vascular restriction preceded or was simultaneous to reductions in stomatal conductance, leaf transpiration, and photosynthesis, with microscopic cell death detected at 2 hpi and significant cell death observed at 3 hpi. Macroscopic lesions were first visible at 6 hpi and coincided with the onset of maximum leaf drying. In contrast to resistance, the induction

Fig. 4. A through D, Vascular activity in the infiltrated regions of *Arabidopsis thaliana* and **E,** tomato leaves. Leaves were inoculated with buffer or bacteria, as indicated. Syringe-infiltrated leaves were excised at 1, 3, 6, and 24 h after infiltration (A through C) as well as at 48 h postinoculation (hpi) (D) or at 3 hpi (E). The petioles were immersed in a 1% aqueous safranin-O dye solution for 2 h. The infiltrated zones on each leaf were indicated with a black marker on the leaf (A) or as demarcated with a white line (E). The total amount of dye in the infiltrated zones was assessed in digital scans (B and D) and was expressed as intensity, which reflects mean Photoshop luminosity units $\pm$ standard error (SE). The mean ratio of dye in the infiltrated leaf zone to that in the adjacent uninoculated region $\pm$ SE is shown in C. Values are based on five to 23 replicate leaves (B and C) and three to five replicate leaves (D).

of disease symptoms by DC3000(pPI) involved a dramatic increase in stomatal conductance and leaf transpiration preceding a rapid decline in these values starting at approximately 5 hpi. Visible necrosis was not observed until at least 24 hpi.

The kinetics described here are for events following infiltration with an inoculum density of 10^8 cells/ml. This density is commonly used in studies focused on the HR. Although delivery of cells at this high density into the apoplast induces mac-

Fig. 5. Plant cell death in the infiltrated region of *Arabidopsis thaliana* leaves inoculated with **A**, DC3000(pPIavrRpm1) or **B**, buffer, DC3000(pPI), DC3000(pPIavrRpm1), or *P. fluorescens* A506. After syringe-infiltration and excision, leaves were stained with Evans blue solution and were digitally scanned. The total amount of dye in the infiltrated zones reflected the amount of plant cell death and is expressed as mean Photoshop luminosity units $\pm$ standard error ($n = 7$ to 11).

Fig. 6. Effect of stomatal blockage on the growth of bacterial populations and plant cell death in inoculated *Arabidopsis thaliana* leaves. Plants were inoculated by vacuum-infiltration, and the surfaces were allowed to dry for 1 h. A thin layer of petroleum jelly was applied uniformly to both the upper and lower surfaces of leaves to block stomata and, thus, leaf drying. The population sizes were determined by plate counts for petroleum jelly-treated (●) and untreated (○) leaves inoculated with **A**, DC3000, **B**, DC3000(pPIavrRpm1), or **C**, DC3000(pPIavrRpt2). **D**, The rate of change in the bacterial populations between 4 and 8 h is contrasted with the rate of change between 8 and 24 h for the data shown in A through C, with DC3000(pPI) (white bars), DC3000(pPIavrRpm1) (black bars), and DC3000(pPIavrRpt2) (hatched bars). **E**, The extent of plant cell death is shown for the untreated (black bars) and petroleum jelly-treated (white bars) leaves sampled at 8 h postinoculation, as determined using Evans blue staining. Data are represented as mean $\pm$ standard error ($n = 24$ to 29 for A through D and 10 to 23 for E).

roscopic levels of plant cell death, delivery of lower densities have been verified to similarly induce PCD but on a microscopic level (Turner and Novacky 1974). The techniques for assessing vascular activity used in this study were not amenable to detecting potentially localized changes in vascular activity resulting from the infiltration of low cell densities. How-

ever, measurements of changes in leaf transpiration showed clear differences between treatments with DC3000(pPI) and DC3000(pPIavrRpm1) after inoculation by immersion (Fig. 3C), a process that introduces far fewer cells into the apoplast than vacuum infiltration.

We do not yet know the mechanism of vascular restriction. We observed that confluent regions of plant cell death often overlapped one or more vascular bundles at 3 hpi. In fact, vascular tissue may be selectively targeted for PCD during *R* gene-mediated resistance based on the observation that a lesion mimic mutant of *A. thaliana* called *vad1* (for vascular associated death) exhibits HR-like PCD preferentially in mesophyll cells lining the vascular tissue (Lorrain et al. 2004). The *Vad1* protein is constitutively expressed at very low levels in healthy plants but is highly induced in vascular tissues upon syringe-inoculation with DC3000 expressing *avrRpm1* as well as with an HR-inducing strain of *Xanthomonas campestris* pv. *campestris*. This induction was rapid, peaking at 3 hpi, and was thus consistent with the kinetics of vascular restriction in the present study. The death of vascular-associated cells, such as the bundle sheath cells, could conceivably allow air to enter the xylem and form a block, or embolism, in the water column. Such embolisms would form more easily if water flowed primarily through xylem vessels rather than the network of tracheids that are commonly present at the ends of the minor veins in leaves (Chatelet et al. 2006); these networks provide multiple pathways for water flow. Because the water movement into the areoles bound by the minor veins appeared to be stopped in response to DC3000 (pPIavrRpm1) (Fig. 4), embolism formation is unlikely to be the mechanism of vascular restriction. The formation of tyloses or gels is also unlikely to be the mechanism because of the speed

Fig. 7. Effect of continuous incubation under high relative humidity (RH) on the growth of bacterial populations in *Arabidopsis thaliana* leaves. Plants were inoculated with DC3000(pPI) or DC3000(pPIavrRpm1) by vacuum-infiltration and were placed on a laboratory bench with (high RH) or without (low RH) being enclosed in a humidity tent. The population sizes were determined at 0 and 24 h postinoculation. Data are represented as mean $\pm$ standard error ($n = 14$).

Gene-for-gene mediated resistance: Response to DC3000(pPIavrRpm1)

Disease induction: Response to DC3000(pPI)

Fig. 8. Timeline of physiological changes in *Arabidopsis thaliana* in response to *Pseudomonas syringae* pv. *tomato*. All events occurred following vacuum-infiltration, except as indicated. The inocula contained 10^8 cells/ml. ^aThe time of effector delivery is based on the work of de Torres and associates (2003). ^bReduced stomatal conductance within 1 h postinoculation (hpi) was observed only following immersion inoculation and likely reflects microbial-associated molecular pattern-mediated stomatal closure. A greater decrease in stomatal conductance with DC3000(pPIavrRpm1) than by DC3000(pPI) within 1 hpi suggests effector-related stomatal closure. ^cIn the leaves inoculated with DC3000(pPIavrRpm1), Evans blue cell staining showed substantial plant cell death by 3 hpi. ^dThe kinetics of the events following vacuum-infiltration with DC3000(pPIavrRpm1) were delayed approximately 2 h relative to those with DC3000(pPIavrRpm1). ^eNear-complete loss of vascular activity, based on the exclusion of the vascular dye Safranin-O from the infiltrated region, had occurred by 3 hpi. ^fIncreased conductance reflected coronatine-mediated stomatal opening, as reported by Melotto and associates (2006). ^gDecreased conductance appeared to reflect type III secretion system-dependent effector-mediated stomatal closure based on its absence following inoculation with DC3000 *hrcC*.

with which vascular movement into the infection site was halted, which was within 3 hpi, relative to the speed of tylosis formation, which typically is days rather than hours. In *A. thaliana*, the minor veins function as the primary sites for photoassimilate collection, with this collection mediated primarily by apoplastic phloem loading (Turgeon 2006). Because phloem loading involves sucrose-proton cotransporters, it is conceivable that the function of these cotransporters could be disrupted, such as via cell death or rapid changes in apoplastic ions, and that this could reduce xylem flow because of the coupled phloem loading and xylem movement inherent to the osmotically generated pressure-flow mechanism for movement of photoassimilates (Evert 2006).

A major finding of this work is that bacterial growth suppression in plant tissues during gene-for-gene mediated resistance could be completely reversed by halting leaf water loss; water loss was halted by blocking transpiration using either petroleum jelly or continuous incubation at high RH. This finding, in conjunction with our previous observation that *P. syringae* cells sensed inhibitory levels of water stress in resistant *A. thaliana* plants (Wright and Beattie 2004), supports the possibility that the gene-for-gene mediated resistance response suppresses the growth and spread of bacterial populations by limiting water availability to the bacteria and thus effectively starving them of water. The petroleum jelly treatment enhanced bacterial growth in the first 8 hpi, a period during which the bacteria generally exhibited little to no growth in untreated leaves. A lag preceding a period of exponential growth is characteristic of bacterial adaptation to a new environment or to environmental stress. The finding that the petroleum jelly eliminated this putative adaptation period by eliminating leaf water loss is consistent with an adaptation period necessitated by low water availability and a period of exponential growth after resumption of cellular homeostasis. Halting leaf water loss could have impacted other aspects of the resistance response that are critical to bacterial growth suppression; however, the petroleum jelly treatment did not prevent plant PCD, indicating that the signaling pathways culminating in PCD were not inhibited. Furthermore, limiting leaf water loss by incubation at high RH also reversed the bacterial growth suppression associated with resistance. Functionally, limiting water in the early hours of gene-for-gene mediated resistance may provide time for the plant to deploy other mechanisms to limit pathogen growth, spread, and damage. These other mechanisms may be causal to the smaller populations of bacteria that are ultimately established in plants exhibiting this resistance as compared with disease.

The *P. syringae*-derived phytotoxin coronatine promotes stomatal opening as reported previously (Melotto et al. 2006) and confirmed here, but this opening appears to be only transient during disease induction and is suppressed during the effector-mediated resistance. Coronatine-induced stomatal opening was demonstrated by Melotto and associates (2006), using microscopy to directly measure stomatal aperture. Here, measurements of stomatal conductance independently verified this phenomenon. Interestingly, the increase in stomatal conductance that occurred between 2.5 and 5 hpi in DC3000(pPI)-inoculated plants was followed by a large and rapid decrease between 5 and 7 hpi (Fig. 8). This stomatal closure may function to reduce the rate of water loss through the stomata and thus improve the intercellular environment as a habitat for pathogenic cells following their entry into the leaves. This function was proposed by Goel and associates (2008) to explain the increased growth of *P. syringae* in water-stressed plants in the presence of high levels of the effector HopAM1, which enhances abscisic acid-mediated stomatal closure. Our results also indicate that stomatal closure may be mediated by one or more T3SS effectors

based on the absence of a dramatic closure in plants inoculated with a T3SS-deficient *hrcC* mutant. Whether HopAM1 is involved in promoting stomatal closure under the test conditions here is unclear. During gene-for-gene resistance, the coronatine-mediated stomatal opening was greatly attenuated or did not occur. This could be due to suppression of coronatine production in *P. syringae* or to the suppression of the coronatine-mediated effects on stomatal activity. The dramatic decrease in stomatal conductance that accompanies this resistance response suggests the latter.

Leaves exposed to the MAMP flg22 exhibited reduced stomatal conductance. This is consistent with previous reports of MAMP-mediated stomatal closing (Lee et al. 1999; Melotto et al. 2006). However, based on our observation that leaf immersion in buffer promotes sustained stomatal opening, at least under conditions of ambient RH >45%, our results are also consistent with the possibility that flg22 inhibits stomatal opening rather than promotes stomatal closure. Such inhibition of stomatal opening by flg22 was recently reported by Zhang and associates (2008). When inoculated by immersion, leaves exposed to DC3000(pPIavrRpm1) consistently exhibited a lower stomatal conductance within 1 hpi than those exposed to DC3000(pPI). This result is consistent with a previous report of AvrRpt2-mediated stomatal closing (Melotto et al. 2006) and suggests that the AvrRpm1 effector, like flg22, promotes stomatal closure or inhibits stomatal opening. This rapid effect following inoculation may reflect effector entry into the guard cells themselves.

This study demonstrates the power of a real-time analysis of living leaves for characterizing leaf physiology during bacterial challenge. In particular, the stomatal conductance, leaf transpiration, and photosynthesis, as monitored using a LI-6400 photosynthesis system, were shown to be highly dynamic following *P. syringae* inoculation. Static measurements of physiological properties such as vascular activity and plant cell death were needed during the early hours of a gene-for-gene mediated resistance response to begin to relate these properties to pathogen dynamics. Such physiological data are critical to complement the accumulating wealth of knowledge on the signal transduction cascades and transcriptional changes associated with resistance and pathogenesis (Kim et al. 2008; Livaja et al. 2008; Pieterse and Dicke 2007; Thilmony et al. 2006). In particular, our demonstration that vascular activity can be strongly and rapidly altered following infection and can be altered in a highly localized manner, indicates the need to examine cellular targets influencing vascular activity and water relations within leaves for a role in plant defense against pathogens.

MATERIALS AND METHODS

Bacterial strains.

Derivatives of the rifampin-resistant (Rif^r) phytopathogen *P. syringae* pv. *tomato* DC3000 (Moore et al. 1989) were used in this study. DC3000(pPIavrRpm1) and DC3000(pPIavrRpt2) are derivatives that contain *avrRpm1* and *avrRpt2* cloned into the *Clal* site of the kanamycin-resistant (Km^r) plasmid pPI, previously called pPProIce (Wright and Beattie 2004); these genes were expressed from their native promoters. Two non-pathogenic strains were used, *Pseudomonas fluorescens* A506 (Wilson and Lindow 1993), which is resistant to Rif, and DC3000 *hrcC* (Yuan and He 1996), previously designated DC3000 *hrpH*, which is deficient in production of the T3SS and is resistant to chloramphenicol (Cm). The DC3000 mutant DC3118 (Ma et al. 1991) (Rif^r Km^r) is deficient in the production of coronatine. All strains were grown in King's B (KB) medium (King et al. 1954) at 28°C, using antibiotics at the following levels (µg/ml): Rif, 50; Km, 50; and Cm, 30.

Plant growth and inoculation.

Seeds of *A. thaliana* ecotype Col-0 and *Solanum lycopersicum* cultivars Rio Grande PtoS and Rio Grande PtoR (obtained from A. Bogdanove, Iowa State University) were grown in a 10-h light and 14-h dark cycle at 24°C and 50 to 60% relative humidity. Whole leaves of 5- to 6-week-old plants were used for all experiments. Bacterial cells grown overnight in liquid KB medium were centrifuged and resuspended in 10 mM MgSO₄ to a final inoculum density of 10⁸ cells/ml. For inoculation by vacuum infiltration, plant leaves were immersed in buffer (10 mM MgSO₄) or bacterial suspensions containing 0.01% Silwet, were exposed to a partial vacuum for 5 min, and were allowed to air dry. For inoculation by syringe infiltration, bacterial suspensions containing 0.01% Silwet were introduced into the abaxial side of leaves using a 5-ml syringe and the region of infiltration was marked with a permanent marker. For inoculation by leaf immersion, plant leaves were dipped for 5 min into buffer (10 mM MgSO₄) or bacterial suspensions containing 0.02% Silwet and were then partially dried with a fan for 3 min.

Measurements of stomatal activity, transpiration, and photosynthesis.

Single-leaf measurements were performed using a LI-6400 photosynthesis system (LI-COR Biosciences, Lincoln, NE, U.S.A.). This system was equipped with a light-emitting diode fixed on top of a 6-cm² standard chamber containing dual infrared gas analyzers. These gas analyzers were capable of measuring the difference in water vapor content (mmol H₂O/mol air) and CO₂ concentration (μmol CO₂/mol air) between the air streams entering and leaving the chamber. Gas flow was adjusted to a constant rate of 0.10 mmol/s, and chamber temperature was maintained at 23°C with a constant light level of 150 μEinsteins. The LI-6400 system uses measurements of water vapor, CO₂, atmospheric pressure (kPa), and leaf temperature (°C) to estimate values for stomatal conductance (mol H₂O/m²s), transpiration (mmol H₂O/m²s), photosynthetic rate (μmol CO₂/m²s), and relative humidity (designated as RH). For each series of measurements, plants were used after exposure to their first 2 h of light in the morning. The leaf chamber was clamped onto a single *A. thaliana* leaf, which was still attached to the plant, and measurements were taken every 10 min for 2 h. The leaf was uncoupled from the leaf chamber and was inoculated using either vacuum infiltration or immersion for 5 min and was then dried by blotting with tissue or gentle air-drying with a fan for 3 min, respectively. The leaf chamber was again clamped onto the leaf for continued monitoring. The lights were turned off at 10 hpi, and monitoring was continued for one more hour.

Characterization of vascular activity and plant cell death.

Leaves of *A. thaliana* and *S. lycopersicum* were inoculated with bacterial suspensions by syringe-infiltration using a needleless syringe applied to either side of the midrib of the abaxial surface. The inoculation zone was marked with a permanent marker. Leaves were detached at the base of the petiole at various times, designated the sampling times, and the petiole was immediately placed in an aqueous 1% (wt/vol) safranin-O solution for 2 h. Leaves were scanned using a flatbed scanner at 300 DPI, and the quantity of safranin-O dye in target regions was measured, using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, U.S.A.), by converting areas exhibiting the dye color to white and measuring the average number of white pixels against a black background of an area equal to the original leaf. To quantify dead or dying plant cells, excised whole leaves were immersed in a 1% (wt/vol) aqueous solution of Evans blue containing 0.05% Silwet. The

submerged tissues were subjected to vacuum-infiltration three times for 5-min intervals, followed by a 15-min incubation in the solution at atmospheric pressure. Leaves were rinsed three times for 5 min, using a 10 mM MgSO₄ solution containing 0.05% Silwet followed by a 15-min incubation in the solution at atmospheric pressure. Leaves were scanned using a flatbed scanner and the Evans blue dye was quantified using Adobe Photoshop as described for safranin-O.

Quantification of bacterial population sizes in planta.

Leaves were inoculated by vacuum infiltration as described above and were allowed to dry for 1 h. Leaves of selected plants were completely covered with a thin film of semimolten (40°C) petroleum jelly; this was applied to both the adaxial and abaxial sides of each leaf using a sterile cotton swab. Other leaves were inoculated but were not treated with petroleum jelly. Alternatively, plants were inoculated and the canopy of leaves in each pot was immediately enclosed in a clear plastic bag, which was secured with a rubber band around the pot base, forming a humidity tent for the enclosed plants. Leaf disks collected with a 5-mm cork borer or entire leaves were collected and homogenized in a 10 mM MgSO₄ solution. Dilutions were drop-plated onto KB medium containing appropriate antibiotics and were grown for 2 days to quantify the colony-forming units.

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LITERATURE CITED

- Century, K. S., Holub, E. B., and Staskawicz, B. J. 1995. *ndr1*, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. *Proc. Natl. Acad. Sci. U.S.A.* 92:6597-6601.
- Chaerle, L., Van Caeneghem, W., Messens, E., Lambers, H., Van Montagu, M., and Van Der Straeten, D. 1999. Presymptomatic visualization of plant-virus interactions by thermography. *Nat. Biotechnol.* 17:813-816.
- Chatelet, D. S., Matthews, M. A., and Rost, T. L. 2006. Xylem structure and connectivity in grapevine (*Vitis vinifera*) shoots provides a passive mechanism for the spread of bacteria in grape plants. *Ann. Bot.* 98:483-494.
- Ciccarelli, F. D., and Bork, P. 2005. The WHY domain mediates the response to desiccation in plants and bacteria. *Bioinformatics* 21:1304-1307.
- Clough, S. J., Fengler, K. A., Yu, I. C., Lippok, B., Smith, R. K., Jr., and Bent, A. F. 2000. The *Arabidopsis dnd1* "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. *Proc. Natl. Acad. Sci. U.S.A.* 97:9323-9328.
- Cook, A. A., and Stall, R. E. 1977. Effects of watersoaking on response to *Xanthomonas vesicatoria* in pepper leaves. *Phytopathology* 67:1101-1103.
- Dangl, J. L., and Jones, J. D. 2001. Plant pathogens and integrated defense responses to infection. *Nature* 411:826-833.
- DeRoy, S., Thilmony, R., Kwack, Y. B., Nomura, K., and He, S. Y. 2004. A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. *Proc. Natl. Acad. Sci. U.S.A.* 101:9927-9932.
- Evert, R. F. 2006. *Esau's Plant Anatomy*. 3rd edition. John Wiley & Sons, Inc., Hoboken, NJ, U.S.A.
- Farquhar, G. D., and Sharkey, T. D. 1982. Stomatal conductance and photosynthesis. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 33:317-345.

- Galan, J. E., and Wolf-Watz, H. 2006. Protein delivery into eukaryotic cells by type III secretion machines. *Nature* 444:567-573.
- Gassmann, W. 2005. Natural variation in the *Arabidopsis* response to the avirulence gene *hopPsyA* uncouples the hypersensitive response from disease resistance. *Mol. Plant-Microbe Interact.* 18:1054-1060.
- Goel, A. K., Lundberg, D., Torres, M. A., Matthews, R., Akimoto-Tomiya, C., Farmer, L., Dangl, J. L., and Grant, S. R. 2008. The *Pseudomonas syringae* type III effector HopAM1 enhances virulence on water-stressed plants. *Mol. Plant-Microbe Interact.* 21:361-370.
- Gopalan, S., Wei, W., and He, S. Y. 1996. *hrp* gene-dependent induction of *hin1*: A plant gene activated rapidly by both harpins and the *avrPto* gene-mediated signal. *Plant J.* 10:591-600.
- Gudesblat, G. E., Torres, P. S., and Vojnov, A. A. 2009. *Xanthomonas campestris* overcomes *Arabidopsis* stomatal innate immunity through a DSF cell-to-cell signal-regulated virulence factor. *Plant Physiol.* 149:1017-1027.
- Hammond-Kosack, K. E., Silverman, P., Raskin, I., and Jones, J. 1996. Race-specific elicitors of *Cladosporium fulvum* induce changes in cell morphology and the synthesis of ethylene and salicylic acid in tomato plants carrying the corresponding *cf* disease resistance gene. *Plant Physiol.* 110:1381-1394.
- Hauck, P., Thilmony, R., and He, S. Y. 2003. A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. *Proc. Natl. Acad. Sci. U.S.A.* 100:8577-8582.
- Heath, M. C. 2000. Hypersensitive response-related death. *Plant Mol. Biol.* 44:321-334.
- Hinsch, M., and Staskawicz, B. 1996. Identification of a new *Arabidopsis* disease resistance locus, *RPS4*, and cloning of the corresponding avirulence gene, *avrRps4*, from *Pseudomonas syringae* pv. *pisi*. *Mol. Plant-Microbe Interact.* 9:55-61.
- Huang, H. C., Schuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutcheson, S. W., and Collmer, A. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene-cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants. *J. Bacteriol.* 170:4748-4756.
- Jambunathan, N., Siani, J. M., and McNellis, T. W. 2001. A humidity-sensitive *Arabidopsis* copine mutant exhibits precocious cell death and increased disease resistance. *Plant Cell* 13:2225-2240.
- Jamir, Y., Guo, M., Oh, H.S., Petnicki-Ocwieja, T., Chen, S., Tang, X., Dickman, M. B., Collmer, A., and Alfano, J. R. 2004. Identification of *Pseudomonas syringae* type III effectors that can suppress programmed cell death in plants and yeast. *Plant J.* 37:554-565.
- Kang, L., Tang, X., and Mysore, K. S. 2004. *Pseudomonas* type III effector AvrPto suppresses the programmed cell death induced by two nonhost pathogens in *Nicotiana benthamiana* and tomato. *Mol. Plant-Microbe Interact.* 17:1328-1336.
- Kim, M. G., Kim, S. Y., Kim, W. Y., Mackey, D., and Lee, S. Y. 2008. Responses of *Arabidopsis thaliana* to challenge by *Pseudomonas syringae*. *Mol. Cells* 25:323-331.
- King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301-307.
- Kpemoua, K., Boher, B., Nicole, M., Calatayud, P., and Geiger, J. P. 1996. Cytochemistry of defense responses in cassava infected by *Xanthomonas campestris* pv. *manihotis*. *Can. J. Microbiol.* 42:1131-1143.
- Lee, S., Choi, H., Suh, S., Doo, I. S., Oh, K. Y., Choi, E. J., Schroeder Taylor, A. T., Low, P. S., and Lee, Y. 1999. Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato and *Commelina communis*. *Plant Physiol.* 121:147-152.
- Livaja, M., Zeidler, D., von Rad, U., and Durner, J. 2008. Transcriptional responses of *Arabidopsis thaliana* to the bacteria-derived PAMPs harpin and lipopolysaccharide. *Immunobiology* 213:161-171.
- Lorrain, S., Lina, B. Q., Auriac, M. C., Kroj, T., Saindrenan, P., Nicole, M., Balague, C., and Roby, D. 2004. Vascular associated death1, a novel gram domain-containing protein, is a regulator of cell death and defense responses in vascular tissues. *Plant Cell* 16:2217-2232.
- Ma, S. W., Morris, V. L., and Cuppels, D. A. 1991. Characterization of a DNA region required for production of the phytotoxin coronatine by *Pseudomonas syringae* pv. *tomato*. *Mol. Plant-Microbe Interact.* 4:69-74.
- Mattoo, S., Lee, Y. M., and Dixon, J. E. 2007. Interactions of bacterial effector proteins with host proteins. *Curr. Opin. Immunol.* 19:392-401.
- Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S. Y. 2006. Plant stomata function in innate immunity against bacterial invasion. *Cell* 126:969-980.
- Mittal, S., and Davis, K. R. 1995. Role of the phytotoxin coronatine in the infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. *tomato*. *Mol. Plant-Microbe Interact.* 8:165-171.
- Mittler, R., Simon, L., and Lam, E. 1997. Pathogen-induced programmed cell death in tobacco. *J. Cell Sci.* 110:1333-1344.
- Moore, R. A., Starratt, A. N., Ma, S. W., Morris, V. L., and Cuppels, D. A. 1989. Identification of a chromosomal region required for biosynthesis of the phytotoxin coronatine by *Pseudomonas syringae* pv. *tomato*. *Can. J. Microbiol.* 35:910-917.
- Morel, J. B., and Dangl, J. L. 1997. The hypersensitive response and the induction of cell death in plants. *Cell Death Differ.* 4:671-683.
- Mur, L. A. J., Kenton, P., Lloyd, A. J., Ougham, H., and Prats, E. 2008. The hypersensitive response; the centenary is upon us but how much do we know? *J. Exp. Bot.* 59:501-520.
- Oh, H. S., and Collmer, A. 2005. Basal resistance against bacteria in *Nicotiana benthamiana* leaves is accompanied by reduced vascular staining and suppressed by multiple *Pseudomonas syringae* type III secretion system effector proteins. *Plant J.* 44:348-359.
- Penalzo-Vazquez, A., Preston, G. M., Collmer, A., and Bender, C. L. 2000. Regulatory interactions between the Hrp type III protein secretion system and coronatine biosynthesis in *Pseudomonas syringae* pv. *tomato* DC3000. *Microbiology* 146:2447-2456.
- Perez-Donoso, A. G., Greve, L. C., Walton, J. H., Shackel, K. A., and Labavitch, J. M. 2007. *Xylella fastidiosa* infection and ethylene exposure result in xylem and water movement disruption in grapevine shoots. *Plant Physiol.* 143:1024-1036.
- Pieterse, C. M. J., and Dicke, M. 2007. Plant interactions with microbes and insects: From molecular mechanisms to ecology. *Trends Plant Sci.* 12:564-569.
- Stall, R. E., and Cook, A. A. 1979. Evidence that bacterial contact with the plant-cell is necessary for the hypersensitive reaction but not the susceptible reaction. *Physiol. Plant Pathol.* 14:77-84.
- Thilmony, R., Underwood, W., and He, S. Y. 2006. Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the human pathogen *Escherichia coli* O157:H7. *Plant J.* 46:34-53.
- Turgeon, R. 2006. Phloem loading: How leaves gain their independence. *Bioscience* 56:15-24.
- Turner, J. G., and Novacky, A. 1974. The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. *Phytopathology* 64:885-890.
- Wang, C., Cai, X., and Zheng, Z. 2005. High humidity represses *Cf-4/Avr4*- and *Cf-9/Avr9*-dependent hypersensitive cell death and defense gene expression. *Planta* 222:947-956.
- Weymann, K., Hunt, M., Uknes, S., Neuenschwander, U., Lawton, K., Steiner, H. Y., and Ryals, J. 1995. Suppression and restoration of lesion formation in *Arabidopsis lsd* mutants. *Plant Cell* 7:2013-2022.
- Whalen, M. C., Innes, R. W., Bent, A. F., and Staskawicz, B. J. 1991. Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* 3:49-59.
- Wilson, M., and Lindow, S. E. 1993. Interactions between the biological control agent *Pseudomonas fluorescens* A506 and *Erwinia amylovora* in pear blossoms. *Phytopathology* 83:117-123.
- Wright, C. A., and Beattie, G. A. 2004. *Pseudomonas syringae* pv. *tomato* cells encounter inhibitory levels of water stress during the hypersensitive response of *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 101:3269-3274.
- Wright, K. M., Duncan, G. H., Pradel, K. S., Carr, F., Wood, S., Oparka, K. J., and Cruz, S. S. 2000. Analysis of the *N* gene hypersensitive response induced by a fluorescently tagged tobacco mosaic virus. *Plant Physiol.* 123:1375-1386.
- Yoshioka, K., Kachroo, P., Tsui, F., Sharma, S. B., Shah, J., and Klessig, D. F. 2001. Environmentally sensitive, SA-dependent defense responses in the *cpr22* mutant of *Arabidopsis*. *Plant J.* 26:447-459.
- Young, J. M. 1974. Effect of water on bacterial multiplication in plant-tissue. *NZ J. Agric. Res.* 17:115-119.
- Yu, I. C., Parker, J., and Bent, A. F. 1998. Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis dnd1* mutant. *Proc. Natl. Acad. Sci. U.S.A.* 95:7819-7824.
- Yuan, J., and He, S. Y. 1996. The *Pseudomonas syringae* Hrp regulation and secretion system controls the production and secretion of multiple extracellular proteins. *J. Bacteriol.* 178:6399-6402.
- Zhang, W., He, S. Y., and Assmann, S. M. 2008. The plant innate immunity response in stomatal guard cells invokes G-protein-dependent ion channel regulation. *Plant J.* 56:984-996.