

**The Role of Type III Effectors in Disrupting Guard Cell Function that Prevents
Bacterial Invasion**

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Abstract

Plants have defense mechanisms that detect bacteria and hinder their ability to invade from the leaf surface to the leaf interior. Specifically, stomata on the underside of the leaf surface can be closed by the leaf's guard cells to prevent bacterial entry. This defense response can be obtained when the plant recognizes the pathogen-associated molecule patterns (PAMPs) on bacteria. Bacteria release molecules called Type III Effectors (T3Es) that are virulence factors capable of suppressing PAMP-induced defenses. However, it is unknown whether bacteria use T3Es to perturb the function of guard cells. The purpose of this study is to test the hypothesis that two T3Es, AvrRpm1 and AvrRpt2, target stomatal immunity. Supporting this hypothesis, AvrRpm1 and AvrRpt2 are known to perturb the Arabidopsis RIN4 protein and RIN4 is known to regulate guard cell function. The bacteria under investigation is *Pseudomonas syringae* pv. tomato DC 3000 (Pto). Because Pto produces a toxin called coronatine (COR) that also targets guard cells, we will use a coronatine deficient mutant strain of Pto (Pto COR-). Using a spray inoculation method, which requires invasion through stomata prior to bacterial growth, a growth curve was obtained for Pto COR- bacteria expressing either AvrRpm1 or AvrRpt2 on Arabidopsis plants that are either *RIN4* or *rin4*. Pto carrying the empty vector (without AvrRpm1 or AvrRpt2) is used as the negative control and Pto COR+ is used as the positive control. The ability of the T3Es to help Pto COR- to grow better in *RIN4* plants is consistent with the hypothesis that they prevent stomatal defense in a RIN4-dependent manner. To further examine this hypothesis, we will directly observe stomatal defense, by measuring stomatal apertures using epidermal peels following exposure to the same set of bacterial strains.

Problem Identification and Justification

In agriculture, when plants are infected by bacteria, little can be done but to remove and destroy the infected plants in order to prevent spread of the disease. Many bacterial pathogens only cause disease after they invade the intracellular spaces of the plant tissues. Limiting the bacteria from entering the plants could limit the amount of disease. Gram negative flagellated bacteria are known to enter through the stomata in the leaves. The stomata respond to the invading bacteria, but little is known about this interaction.

Background

For many years the prevailing method for researching plant defense against foliar bacterial pathogens was to infiltrate a bacterial suspension directly into the intercellular spaces of the leaf (the apoplast). However, in nature, bacteria often infect the apoplast by migrating from the leaf surface through wounds or stomata. Plants also possess a defense mechanism against stomatal invasion; guard cells can detect bacteria and close stomata to prevent bacterial entry (Melotto 2006).

Closure of stomata can be elicited by recognition of pathogen-associated molecular patterns (PAMPs). PAMPs are conserved structural elements of microbes, for example FLG22 is a 22 amino acid fragment from bacterial flagellin that functions as a PAMP. PAMP-perception initiates signaling pathways that lead to varied defense responses. One of the responses, stomatal closure, prevents the entry of flagellated bacteria into the apoplast where they proliferate and cause disease.

Bacteria have evolved virulence factors that suppress plant defenses. One example is the deployment of toxins, *e.g.* coronatine (COR) reopens stomata to facilitate

bacterial invasion. Bacteria also release a class of virulence factors called type III effector proteins (T3Es), which are bacterial proteins that get injected into the cytosol of plant cells via the type III secretion system. A known function of T3Es is to suppress defense responses elicited by (PAMPs) when bacteria are already inside the apoplast.

Whether T3Es are injected into guard cells and, if so, whether they perturb the ability of stomata to close in response to PAMPs, is unknown. It is known that T3E molecules inhibit PAMP-induced signaling, weakening the plant's basal defense. One target of T3Es is an Arabidopsis protein called RIN4, which is a negative regulator of PAMP signaling. Overexpression of RIN4 has been shown to inhibit PAMP-induced defense signaling, independent of any T3E. (Kim 2005)

Plants have a second defense mechanism utilizing disease-resistance R proteins, which recognize T3E proteins from bacteria. R protein activation by T3Es often causes a hypersensitive response (HR), a defense response that culminates in plant cell death. However, R-proteins that do not cause cell death can still activate pathways which restrict bacterial growth. Learning about these innate defense pathways can shed light on preventing microbial invasion and preventing plant diseases that limit the quality and yield of agricultural crops.

This study looks at the effect of two unrelated T3Es on stomatal immunity in wild type Arabidopsis and in *rin4* mutant plants. The effect on RIN4 is a point of convergence for the activity of at least four sequence unrelated Type III Effectors (AvrRpm1, AvrRpt2, AvrB, and HopF2). AvrRpm1 induces phosphorylation of RIN4, while AvrRpt2 is a cysteine protease that cleaves RIN4 (Mackey 2002, Mackey 2003). Past studies have shown that RPM1 and RPS2 help guard the plant against bacteria deploying AvrRpm1 or

AvrRpt2 and induce a HR (Kim 2009). AvrRpt2 proteolytic cleavage of RIN2 causes activation of RPS2 and AvrRpm1 causes activation of RPM1; activation of either RPS2 or RPM1 initiates effector-triggered immunity defense signaling. (Kim 2009). We are interested in the virulence mechanism of AvrRpm1 and AvrRpt2. Using plants mutant in *RPM1* and *RPS2* (*rpm1rps2* double mutant) prevents the R-protein mechanisms from inducing an HR or any non-cell death defense response. Thus we can observe most clearly the virulence contribution of the T3Es. Similarly, in an *rpm1rps2rin4* triple mutant background, we can observe how interaction with RIN4 affects the virulence activity of the T3Es.

Objectives

We hypothesize that the T3Es AvrRpm1 and AvrRpt2 suppress stomatal immunity. The first objective is to observe if T3Es regulate stomatal immunity. We will measure the growth of bacteria following spray inoculation. When spray inoculated, bacteria must invade through stomata prior to proliferating in the apoplast. The second objective is to observe if T3Es affect stomatal aperture. We will measure the aperture of stomata following exposure to bacteria expressing AvrRpm1, AvrRpt2, or neither.

Materials and Methods

The bacteria used in these experiments are *Pseudomonas syringae* pv. tomato DC 3000 (Pto) and a mutant unable to produce COR (Pto COR-). In addition, we examine these strains carrying a plasmid for expression of AvrRpm1 (*e.g.* Pto (AvrRpm1) or Pto COR- (AvrRpm1)), AvrRpt2, or carrying the empty vector (without either T3E, *e.g.* Pto (EV)).

We use six-week-old *Arabidopsis* plants grown under daily photoperiods of eight hours. The *rpm1rps2* are mutant for two resistance genes, *RPM1* and *RPS2*, which normally elicit defense responses against AvRpm1 and AvrRpt2, respectively. The *rpm1rps2rin4* triple mutant plants additionally lack *RIN4*.

For the bacterial growth assay, the bacteria are streaked onto an agar plate with appropriate selective media and allowed to grow. After two days, the bacteria are removed from plates and diluted in a solution of 10 mM MgCl₂. The density of the bacterial samples is adjusted to an OD₆₀₀=1.0, which corresponds to 5x10⁸ CFU/mL. The plants are then inoculated with the bacterial suspension using a paint sprayer. The plants are placed into a growth chamber and a clear plastic lid is put over the plants in order to keep the relative humidity at 100%.

After 4 days, nine leaves are collected from each plant sample and leaf discs are taken using a number 5 cork borer. The surface of the leaves are sterilized to remove bacteria that have not invaded the leaf interior by immersing the leaf discs in 75% ethanol for 30 seconds followed by rinsing in water to remove the ethanol. The leaves are then ground to release the bacteria from the leaves. The material is serially diluted and plated onto selective agar. After allowing the bacteria to grow, the colonies are counted and growth of the bacteria is calculated.

For the stomatal aperture assay, leaf disks are removed using a number 2 cork borer and floated, abaxial down, in ddH₂O under light for one hour. The ddH₂O is removed and the disks are floated in a bacterial suspension with an OD₆₀₀=0.3 (1.5x10⁸ CFU/mL) or a solution of 10mM FLG22. After 1 and 3 hours, the leaf disks are mounted on a microscope slide, with abaxial side against the slide, using medical adhesive. The

mounted disks are scraped leaving the epidermal layer on the slide. The slides are photographed and, from the pictures, the apertures of the stomata are measured. The average aperture and standard error was calculated from 60 stomata for each treatment/time point.

Results

Preliminary data from the lab indicated that AvrRpm1 and AvrRpt2 can enhance the growth of spray-inoculated Pto. These experiments were done with wild-type Pto capable of producing COR. I have attempted to repeat this assay using Pto as well as Pto COR-. The inability of Pto COR- to produce COR limits the ability of this strain to invade through stomata. Thus, we will maximize our ability to observe a contribution of AvrRpm1 or AvrRpt2 to bacterial growth that is mediated through regulating stomatal function. Efforts to carry out this experiment are ongoing.

We have also undertaken a stomatal assay to directly observe the effect of AvrRpm1 and AvrRpt2 on stomates. Pictures were taken after one hour of exposure to the treatment and taken again after three hours. It is known that stomata close in response to PAMPs by one hour, and that virulent bacteria can reopen the stomata by three hours (Melotto 2006).

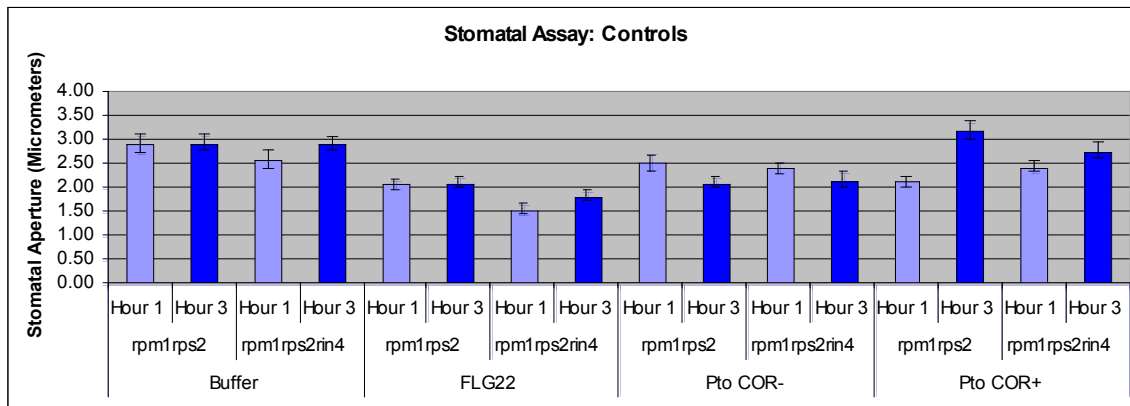


Figure 1: The Effect of Two Controls on Stomatal Aperture

Two controls were used to examine the closure of the stomata (Figure 1). The buffer is a solution of 10 mM MgCl₂. The first control is FLG22, a PAMP from the bacterial flagellin protein. As expected, relative to buffer, FLG22 causes stomata to close. The second control is Pto with and without COR. As expected, both bacteria cause stomata to close after one hour and after three hours, COR reopens stomata. It has been reported that the ability of COR to reopen stomata requires *RIN4* (Liu 2009). It is not possible from our results to confirm or refute this finding.

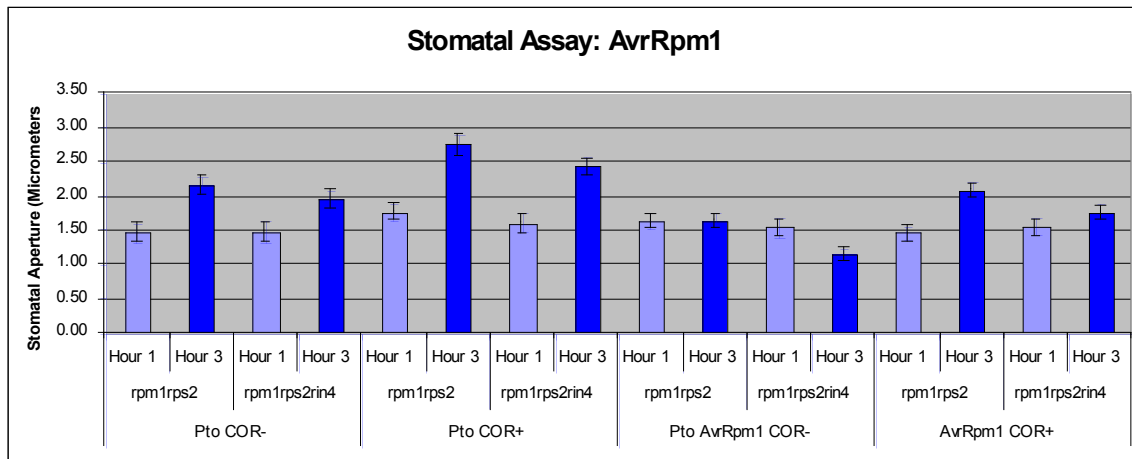


Figure 2: Comparing Effects of AvrRpm1

In our next experiment we sought to examine the effects of AvrRpm1 on stomatal aperture (Figure 2). As expected, bacteria containing COR were able to reopen the stomata. However, contrary to our hypothesis, there is no evidence that AvrRpm1 reopens stomata. The Pto COR- (AvrRpm1) strain failed to reopen stomata after three hours in both *rpm1rps2* and *rpm1rps2rin4* plants.

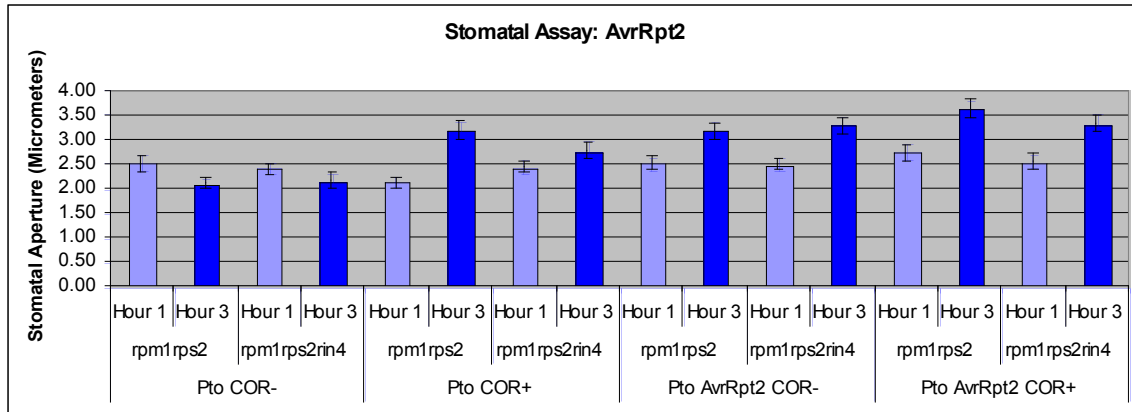


Figure 3: Comparing Effects of AvrRpt2

In another experiment we examined the effects of a second T3E, AvrRpt2 (Figure 3). These results support the hypothesis that T3Es reopen stomata. Similar to the Pto COR+ strain, it appears that Pto COR- (AvrRpt2) causes the stomata to close during the stomata in the first hour, but then reopen during, the third hour. Thus, it appears that AvrRpt2 reopens the stomata. Interestingly, the ability of AvrRpt2 to reopen stomata does not appear to depend on its ability to target RIN4, since the effect of AvrRpt2 on stomatal aperture is apparent in the *rpm1 rps2 rin4* mutant plants.

Discussion

Prior data from the lab suggests that AvrRpm1 and AvrRpt2 can promote the growth of Pto following spray inoculation onto *rpm1 rps2* Arabidopsis plants. We will repeat these experiments, including Pto COR- in addition to Pto. Following spray inoculation, bacteria must invade through stomata prior to growing to high levels inside the apoplast of the leaf. Thus, based on these preliminary data, we hypothesized that AvrRpm1 and AvrRpt2 may directly regulate stomatal aperture.

We sought to assess directly the effect of AvrRpm1 and AvrRpt2 on stomatal aperture. Our stomatal aperture assay with AvrRpm1 does not support the hypothesis that this T3E can reopen stomata. Thus, the ability of AvrRpm1 to promote the growth of

spray-inoculated Pto may be independent of reopening stomata, *e.g.* it may be manifested by function of AvrRpm1 once the bacteria are inside the leaf. However, our stomatal aperture assay with AvrRpt2 does support the hypothesis that this T3E can reopen stomatal aperture. Assuming these results are reproducible, we will next search for evidence that AvrRpt2 is delivered by the type three secretion system into guard cells.

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