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Plant Stomata Function in Innate Immunity against Bacterial Invasion

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SUMMARY

Microbial entry into host tissue is a critical first step in causing infection in animals and plants. In plants, it has been assumed that microscopic surface openings, such as stomata, serve as passive ports of bacterial entry during infection. Surprisingly, we found that stomatal closure is part of a plant innate immune response to restrict bacterial invasion. Stomatal guard cells of *Arabidopsis* perceive bacterial surface molecules, which requires the FLS2 receptor, production of nitric oxide, and the guard-cell-specific OST1 kinase. To circumvent this innate immune response, plant pathogenic bacteria have evolved specific virulence factors to effectively cause stomatal reopening as an important pathogenesis strategy. We provide evidence that supports a model in which stomata, as part of an integral innate immune system, act as a barrier against bacterial infection.

INTRODUCTION

The phyllosphere of terrestrial plants provides one of the most important niches for microbial inhabitation (Upper and Hirano, 1999; Lindow and Brandl, 2003). Numerous bacteria, including plant and human pathogens, can survive and even proliferate on the plant surface as epiphytes. To initiate pathogenesis, plant pathogenic bacteria must first enter plant tissues. Unlike fungal pathogens, bacteria lack the ability to directly penetrate the plant epidermis; they rely entirely on natural openings or accidental wounds to enter internal tissues. The molecular mechanism by which bacteria enter through natural openings is not known, but it has been widely assumed that these openings are passive ports for bacterial entry.

Pseudomonas syringae has been used as a model for the discovery of many fundamental mechanisms underlying host-bacterium interactions (Dangl and Jones 2001; Katagiri et al., 2002; Ausubel, 2005; Chisholm et al., 2006). *P. syringae* strains collectively infect hundreds of taxonomically diverse plant species and cause disease symptoms ranging from leaf spots to stem cankers. To date, studies on the virulence of *P. syringae* and other plant pathogenic bacteria have focused mainly on the interaction after bacteria have entered the plant tissues. This focus is in part because of the widespread use of inoculation procedures that artificially deliver bacteria directly underneath the epidermis. Emerging evidence suggests that such inoculation procedures may have prevented the discovery of important mechanisms involved in the early stages of host-pathogen interactions. For example, recent studies suggest that pathogen-associated molecular pattern (PAMP)-induced basal defense, which is analogous to innate immunity in animals (Gomez-Gomez and Boller, 2002; Takeda et al., 2003), acts early during bacterial infection of plants. It was shown that lipopolysaccharide (LPS)-triggered nitric oxide (NO) production and flagellin perception by its receptor FLS2 contribute to *Arabidopsis* resistance to *P. syringae* pv. *tomato* strain DC3000 (hereafter referred to as *Pst* DC3000) (Zipfel et al., 2004; Zeidler et al., 2004; Kim et al., 2005). The FLS2-mediated resistance, however, was effective against bacteria that had been inoculated onto the leaf surface, which mimics natural infection, but not when bacteria had been artificially infiltrated into the leaf intercellular space (Zipfel et al., 2004; Kim et al., 2005). The precise mechanism by which PAMP-induced innate immunity limits bacterial infection on the leaf surface has not been elucidated.

To successfully colonize plants, *P. syringae* and other plant pathogenic bacteria have evolved a variety of virulence factors to subvert host defenses or to obtain nutrients (Abramovitch and Martin, 2004; Nomura et al., 2005). One such virulence factor is the *hrp*-gene-encoded type III secretion system (TTSS; Buttner and Bonas, 2002; Staskawicz et al., 2001; Alfano and Collmer, 2004; Mudgett, 2005; He et al., 2004). The TTSS is used by bacteria to inject a large number of virulence effector proteins into the host cell (Collmer et al., 2002; Greenberg and Vinatzer, 2003; Chang et al., 2005; Nomura and He, 2005). The TTSS alone, however, does not appear to be sufficient for bacteria to cause disease. *P. syringae* strains, for example, also produce a variety of phytotoxins, which are

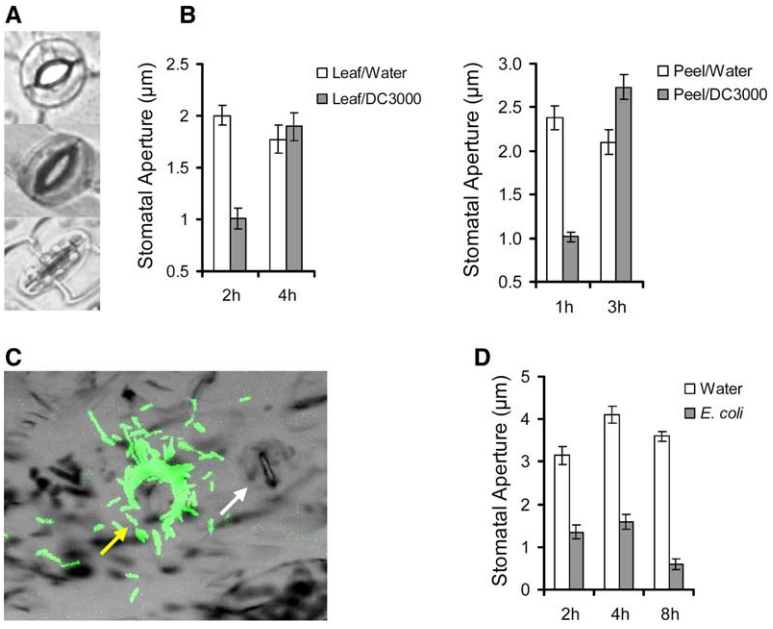

Figure 1. Bacteria and PAMPs Trigger Stomatal Closure

(A) Open (top and middle panels) and closed stomata (bottom panel).

(B) Stomatal aperture in intact leaves (left panel) or epidermal peels (right panel) of Col-0 plants exposed to water (white bars) or *Pst* DC3000 (gray bars). In this and all other figures, results are shown as mean (n = 60 stomata) ± SEM unless otherwise noted.

(C) A confocal microscopic image of GFP-labeled *Pst* DC3000 cells (green) on an epidermal peel showing that bacteria are localized around an open stoma (yellow arrow), but not around the adjacent closed stoma (white arrow).

(D) Stomatal aperture in epidermal peels of Col-0 plants exposed to water (white bars) or *E. coli* O157:H7 (gray bars).

necessary for full virulence in the host plants (Bender et al., 1999). *Pst* DC3000, used in this study, produces a polyketide toxin, coronatine (COR; Ma et al., 1991; Bender et al., 1999). COR is an important virulence factor for *Pst* DC3000 infection in *Arabidopsis* and tomato plants (Ma et al., 1991; Mittal and Davis, 1995; Brooks et al., 2004; Cui et al., 2005).

In this study, we discovered that stomata in the *Arabidopsis* leaf epidermis have an unexpected function as innate immunity gates to actively prevent bacteria from entering the plant leaf. We show that the innate immunity function of stomata is an important target of virulence factors produced by the plant pathogen *Pst* DC3000, but not those produced by the human pathogen *Escherichia coli* O157:H7. These results uncover an important evolutionary battle in plant-pathogen interactions and have broad implications in the study of not only bacterial pathogenesis and stomatal biology but also molecular ecology of bacterial diseases.

RESULTS

Plant and Human Pathogenic Bacteria Induce Stomatal Closure

Opening and closing of stomata are controlled by environmental factors such as light, humidity, and CO_2 concentration (Schroeder et al., 2001; Fan et al., 2004). Under our growth conditions, *Arabidopsis* plants exposed to light for at least 3 hr had a ratio of 70%–80% open stomata to 20%–30% closed stomata in their leaves. To investigate whether plant stomata respond to live bacteria, we first incubated *Arabidopsis* leaves (ecotype Col-0) with *Pst* DC3000, a virulent pathogen of *Arabidopsis* (Whalen et al., 1991; Katagiri et al., 2002). Within the first 2 hr of incubation, we observed a marked reduction in the number of open stomata (to circa 30%; data not shown). The reduction in the percentage of open stomata was correlated with a decrease in the average width of the stomatal aperture (Figure 1B, left). In contrast, the number of open stomata (data not shown) and the average width of the stomatal aperture remained virtually the same in leaves incubated with water. Because standard protocols for measuring stomatal responses involve the use of epidermal peels (Schroeder et al., 2001; Coursol et al., 2003; Peiter et al., 2005), mainly for better microscopic recording, we also incubated epidermal peels with *Pst* DC3000 and monitored stomatal response. We observed a remarkable ability of *Pst* DC3000 bacteria to selectively move toward open stomata (Figure 1C). We did not observe such bacterial behavior around closed stomata. Within 1 hr of incubation with bacteria, the average width of the stomatal aperture (Figure 1B, right) decreased drastically in epidermal peels, whereas it did not decrease in epidermal peels incubated with water. Interestingly, the *Pst* DC3000-induced closing of stomata was transient. After 3 hr incubation, the average width of the stomatal aperture (Figure 1B) had reverted to the pre-bacterial treatment state.

In addition to plant pathogenic bacteria, the phyllosphere is also colonized by other microbes, including human pathogenic bacteria, which are especially relevant in an agricultural setting (Beuchat, 2002; Naimi et al., 2003; Lindow and Brandl, 2003). To determine whether stomata have developed an innate ability to respond to different bacteria, we incubated leaf epidermal peels with *E. coli* O157:H7, a human pathogenic bacterium commonly associated with vegetable-based food poisoning (Park et al., 2001). Again, we observed stomatal closure within 2 hr of incubation (Figure 1D). However, the *E. coli* O157:H7-induced closure persisted for the duration of the entire experiment (8 hr). These results demonstrate

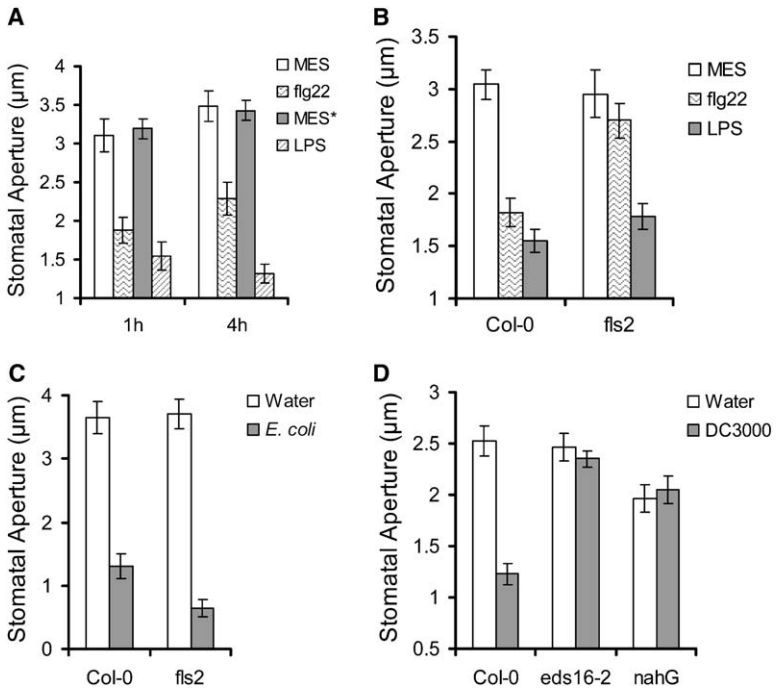

Figure 2. Involvement of the FLS2 Receptor and Salicylic Acid in PAMP-Induced Stomatal Closure

(A) Stomatal aperture in epidermal peels of Col-0 plants exposed to MES buffer (white and gray bars), 5 μM flg22 (wavy bars), or 100 ng/μl LPS (diagonal bars). The MES and MES* bars correspond to the controls for the flg22 and LPS treatments, respectively.

(B) Stomatal aperture in epidermal peels of wild type (Col-0) and *fls2* mutant SAIL_691C4 plants 4 hr after incubation with MES buffer (white bars), 5 μM flg22 (wavy bars), or 100 ng/μl LPS (gray bars).

(C) Stomatal responses in epidermal peels of wild-type (Col-0) and *fls2* mutant SALK_line 93905 plants to 1 × 10^8 cfu/ml *E. coli* O157:H7. Stomatal responses were recorded 4 hr after exposure to bacteria.

(D) Stomatal responses in wild-type Col-0, *eds16-2* mutant, and *nahG* transgenic plants to 1 × 10^8 cfu/ml *Pst* DC3000 after 1 hr of incubation.

that (1) stomata actively close as an initial response to both plant and human pathogenic bacteria, (2) *Pst* DC3000 has evolved a mechanism (or mechanisms) to reopen stomata 3 hr after incubation with plant leaves or epidermal peels, and (3) stomata in leaves and epidermal peels respond similarly to bacteria.

To determine the minimal *Pst* DC3000 concentration needed to induce the stomatal response in *Arabidopsis*, we performed a serial dilution experiment. We found that the inoculum concentration of 1 × 10^7 cfu/ml is sufficient to induce closure at 1 hr and reopening at 3 hr, but reproducible stomatal response was no longer observed at 1 × 10^6 cfu/ml of *Pst* DC3000 (see Figure S1 in the Supplemental Data available with this article online). Consistent with this observation, confocal micrographs of GFP-expressing *Pst* DC3000 on the leaf surface immediately after dip inoculation showed that the leaf surface was evenly covered with bacterial cells at 1 × 10^7 cfu/ml (Figure S2). In contrast, at 1 × 10^6 cfu/ml bacterial suspension, very few and dispersed bacterial cells were detected on the leaf surface (Figure S2).

Involvement of Conserved Bacterial Surface PAMPs in Triggering Stomatal Closure in a Salicylic-Acid-Dependent Manner

The ability of both human and plant pathogenic bacteria to induce stomatal closure within the first hour of contact with plant tissue suggests that guard cells, which form stomata, can sense conserved bacterial molecules. PAMPs are such molecules, and they are best known for their ability to stimulate innate immunity in plants and animals (Gomez-Gomez and Boller, 2000; Takeda et al., 2003). Here we show that both flg22 (a biologically active peptide derived from flagellin; Asai et al., 2002; Zipfel et al., 2004) and LPS (Zeidler et al., 2004) cause dramatic stomatal closure in the wild-type Col-0 plant (Figure 2A). The flg22 peptide failed to induce the closure of stomata in epidermal peels of the *Arabidopsis fls2* flagellin receptor mutant (Gomez-Gomez and Boller, 2000), whereas LPS still induced the closing of stomata in *fls2* epidermal peels (Figure 2B). Live *E. coli* O157:H7 bacteria were also able to induce stomatal closure in *fls2* epidermal peels (Figure 2C). These results suggest that guard-cell perception of flg22 requires the FLS2 receptor but that FLS2 is only one of probably several receptors that enable guard cells to sense multiple PAMPs displayed on the bacterial surface. The involvement of PAMPs and FLS2 in stomatal closure was the first clue that stomatal closure is an integral part of the *Arabidopsis* innate immune system.

The innate immune response in the *Arabidopsis* leaf can be activated via a salicylic acid (SA)-independent (Hauck et al., 2003; Zipfel et al., 2004) or SA-dependent mechanism (DebRoy et al., 2004). To investigate the SA dependence of bacterium-induced stomatal defense, we examined stomatal responses in SA-deficient *nahG* transgenic plants (Delaney et al., 1994) and SA-biosynthetic mutant *eds16-2* plants (Wildermuth et al., 2001). We found that the ability of stomata to close in response to bacteria and LPS was compromised in these plants (Figure 2D and Figure S3A). This result demonstrates that defense through stomatal closure is an integral part of the SA-regulated innate immune system.

We also studied whether stomatal responses differ between incompatible and compatible interactions based on the presence or absence of a resistance gene-*avr* gene interaction. Specifically, we examined the stomatal

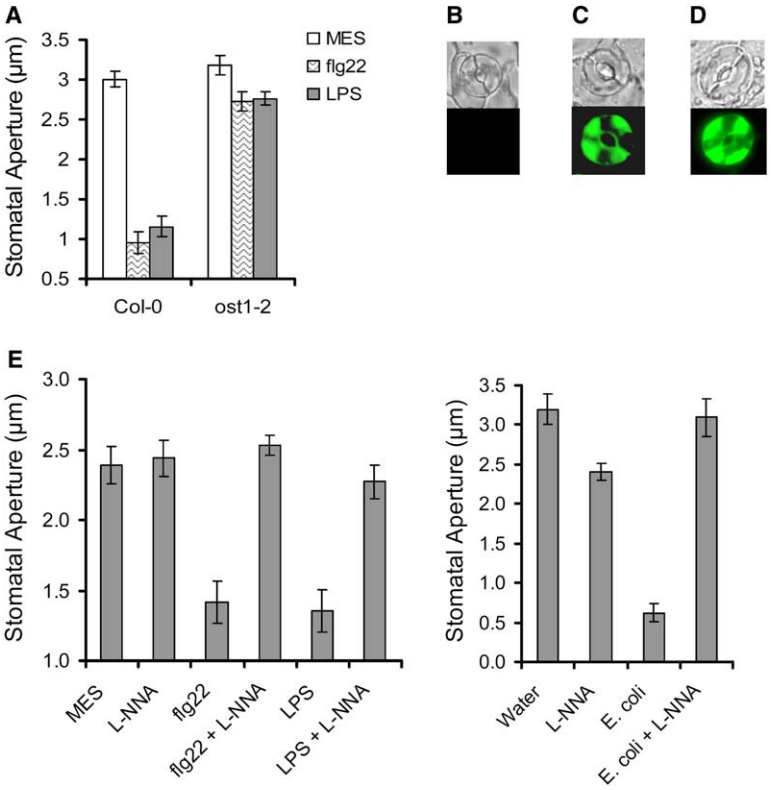

Figure 3. Involvement of the ABA Signaling Components in PAMP-Induced Stomatal Closure

(A) Stomatal aperture in *Arabidopsis ost1-2* mutant plants 2 hr after incubation.

(B–D) NO production in guard cells of Col-0 treated with MES buffer (B), 5 μM flg22 peptide (C), or 100 ng/μl LPS (D). NO production was not observed in the control treatment; therefore, only a black screen was seen under the fluorescence microscope.

(E) Effect of the NOS inhibitor L-NNA (0.2 mM) on stomatal closure when coincubated with PAMPs (5 μM flg22 or 100 ng/μl LPS) or 1×10^8 cfu/ml *E. coli* for 2 hr.

responses of wild-type Col-0 plants to two bacteria: *Pst* DC3000 (representing a susceptible interaction) and *Pst* DC3000/*avrRpt2* (representing a resistant interaction; Whalen et al., 1991). Like *Pst* DC3000, the avirulent strain *Pst* DC3000/*avrRpt2* caused stomatal closure within 1 hr. However, in all three independent experiments, the avirulent strain was less effective in reopening stomata than the virulent strain at 3 hr after incubation (Figure S3B). This result suggests that the gene-for-gene resistance mediated by *avrRpt2*/*RPS2* has a positive effect on promoting stomatal closure.

PAMP-Induced Stomatal Closure Is Mechanistically Linked to Abscisic-Acid Signaling in Guard Cells

Stomatal closure during abiotic stresses (e.g., drought) is well studied and requires the plant hormone abscisic acid (ABA) and several downstream signal transduction components, such as the guard-cell-specific OST1 kinase, nitric oxide (NO), and H_2O_2 (Schroeder et al., 2001; Fan et al., 2004). To determine whether PAMP-induced stomatal closure requires components of the ABA signal transduction pathway, we examined the stomatal response to bacterial PAMPs in the *ost1* kinase mutant (Mustilli et al., 2002) or ABA-deficient *aba3-1* mutant (Leon-Kloosterziel et al., 1996) plants. Neither flg22 nor LPS could induce stomatal closure in *ost1-2* (Figure 3A) or *aba3-1* mutant plants (Figure S3C). Furthermore, both flg22 and LPS rapidly (within 10 min) induced the production of NO in guard cells of wild-type stomata that subsequently closed (Figures 3C and 3D). In addition, Nω-nitro-L-arginine (L-NNA), an inhibitor of nitric oxide synthase (NOS), effectively prevented flg22-, LPS-, and *E. coli* O157:H7-induced stomatal closure (Figure 3E), suggesting that NO is required for PAMPs and bacteria to close stomata. Taken together, these results establish a mechanistic connection between PAMP-induced stomatal closure and ABA signaling pathways in the guard cell.

We also investigated the role of ABA in *Pst* DC3000- and *Pst* DC3000/*avrRpt2*-induced stomatal closure by examining the stomatal response in the ABA-deficient *Arabidopsis* mutant *aba3-1*. We found that the stomata of the ABA-deficient *aba3-1* plants were greatly compromised in the ability to respond to either *Pst* DC3000 or *Pst* DC3000/*avrRpt2* bacteria compared with those of Col-0 plants (Figure S3B). These results suggest that ABA biosynthesis is also required for stomatal closure in response to these bacteria.

Identification of *Pst* DC3000 Virulence Factors that Disable Stomatal Defense

As shown in Figure 1, a striking difference between stomatal response to the human pathogen *E. coli* O157:H7 and to the plant pathogen *Pst* DC3000 is that stomata reopen after approximately 3 hr of incubation with *Pst* DC3000, but not with *E. coli* O157:H7. We suspected that *Pst* DC3000, but not *E. coli* O157:H7, has evolved a natural virulence mechanism (or mechanisms) that can counter PAMP-induced stomatal closure. *Pst* DC3000 contains

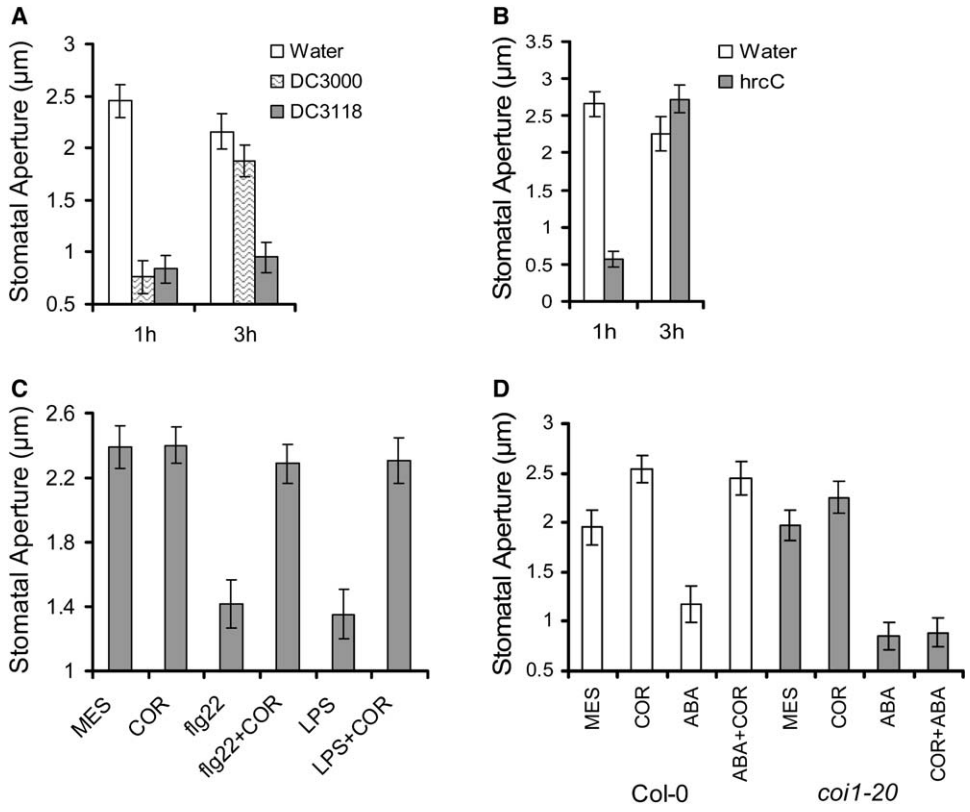

Figure 4. COR Disables Stomatal Defense in a COI1-Dependent Manner

(A) Col-0 leaves were exposed to water (white bars), *Pst* DC3000 (wavy bars), or the *cor* mutant *Pst* DC3118 (gray bars). Bacterial concentration used was 1×10^8 cfu/ml.

(B) Col-0 leaves were exposed to water (white bars) or 1×10^8 cfu/ml TTSS-defective nonpolar *hrcC* mutant.

(C) Stomatal responses to 0.5 ng/μl COR, 5 μM flg22, 5 μM flg22 + 0.5 ng/μl COR, 100 ng/μl LPS, or 100 ng/μl LPS + 0.5 ng/μl COR, in wild-type Col-0 plants after 3 hr of treatment.

(D) Stomatal response in epidermal peels of wild-type Col-0 and *coi1-20* mutant plants. 0.5 ng/μl COR and 10 μM ABA were used. For COR + ABA experiments, epidermal peels were preincubated with 0.5 ng/μl COR for 30 min. The COR solution was then replaced with the 0.5 ng/μl COR + 10 μM ABA solution. Note that COR fails to prevent ABA-induced stomatal closure in the *coi1-20* epidermis.

two well-characterized virulence factors: TTSS and the phytotoxin COR (Nomura et al., 2005). We examined stomatal responses to mutants of *Pst* DC3000 that were either COR deficient (*cor*$^-$; Ma et al., 1991) or TTSS defective (nonpolar *hrcC*$^-$; Penaloza-Vazquez et al., 2000). In contrast to wild-type *Pst* DC3000, the *cor* mutant could not reopen closed stomata (Figure 4A), thus behaving similarly to *E. coli* O157:H7 (Figure 1D). The *hrcC* mutant, however, was not affected in the ability to reopen stomata (Figure 4B). These results show that the virulence factor responsible for suppressing stomatal defense is coronatine.

The requirement of COR for reopening stomata was intriguing because *cor* mutants have been known for a long time to be greatly reduced in virulence compared with the wild-type bacterium when inoculated onto the leaf surface, a procedure that mimics natural infection. However, if *cor* mutants are infiltrated directly into the apoplast, bypassing the epidermis, they multiply similarly to wild-type bacteria (Mittal and Davis, 1995; Brooks et al., 2004). This observation led Mittal and Davis (1995) to hypothesize a decade ago that COR suppresses an early defense in *Arabidopsis*. The nature of this early defense has remained elusive. We investigated the possibility that COR could interfere with PAMP-induced stomatal closure. Indeed, at a concentration as low as 0.5 ng/μl, COR counteracted flg22- and LPS-induced stomatal closure (Figure 4C).

COR Inhibits PAMP-Induced ABA Signaling in the Guard Cell

To further define the virulence action of COR in PAMP/bacterium-triggered stomatal defense, we analyzed several key steps of stomatal closure in *Arabidopsis*. During abiotic stress, ABA increase and NO production represent two critical early events in the guard cell (Schroeder et al., 2001; Fan et al., 2004). We found that COR effectively inhibited ABA-induced stomatal closure (Figure 4D

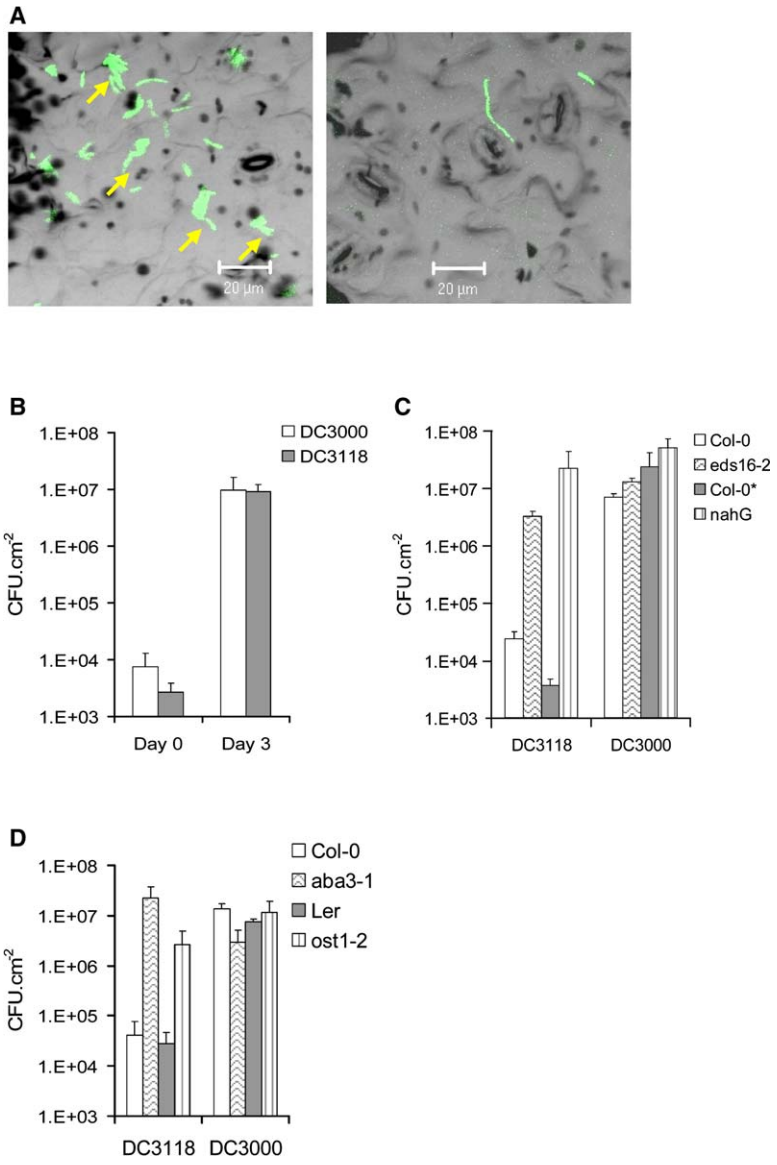

Figure 5. The Functional Role of Stomatal Closure in Bacterial Resistance

(A) Bacterial suspensions (1×10^7 cfu/ml) of the GFP-labeled strains *Pst* DC3000 (left panel) and *Pst* DC3118 (right panel) were placed in contact with the cuticle of Col-0 epidermal peels. Microscopic images are representative of the relative number of bacterial cells that passed through stomatal openings after 3 hr incubation. Bacterial clusters formed by *Pst* DC3000 on the upper side of the epidermis are indicated by yellow arrows.

(B) Suspensions (1×10^6 cfu/ml) of the wild-type *Pst* DC3000 (white bars) or the *cor* mutant *Pst* DC3118 (gray bars) were vacuum infiltrated into Col-0 plants. Bacterial growth was assessed 3 days after inoculation. Results in (B)–(D) are shown as mean (n = 6) + SD.

(C and D) Wild-type Col-0 and SA-deficient *eds16-2* mutant and *nahG* transgenic plants (C) and wild-type Col-0 and ABA-deficient *aba3-1* plants and wild-type Landsberg *erecta* (Ler) and *ost1-2* mutant plants (D) were dipped into suspensions (1×10^8 cfu/ml) of *Pst* DC3118 or *Pst* DC3000. The Col-0 and Col-0* bars in (C) represent the controls for *eds16-2* and *nahG* plants, respectively, as these plants were inoculated on different days. Bacterial growth was assessed 3 days after inoculation.

and Figure S5). However, COR could not prevent ABA-induced closure of stomata (Figure 4D), and COR-producing *Pst* DC3000 could not efficiently reopen stomata (Figure S4) in the COR-insensitive *Arabidopsis coi1* mutants (Xie et al., 1998; Kloek et al., 2001). Taken together, these results suggest that COR counteracts PAMP-induced stomatal closure downstream of ABA and that the COI1 (a subunit of an E3 ubiquitin ligase) dependent proteolysis (Xie et al., 1998) is necessary for COR-mediated blockage of the PAMP signal transduction pathway in the guard cell.

COR did not inhibit the production of NO in response to PAMPs or ABA (Figure S6). This result suggests that COR acts downstream or independent of NO production to reverse the effects of PAMPs on the ABA signal transduction pathway.

Demonstration of a Biologically Relevant Role for COR-Mediated Suppression of Stomatal Defense in Bacterial Infection

A critical question is whether the observed bacterium- and PAMP-induced stomatal closure results in effective restriction of bacterial entry through the epidermis. To address this question directly, we placed GFP-labeled DC3000 and *cor* mutant bacteria underneath an epidermal peel (the cuticle side was in contact with the bacterial suspension) and monitored the ability of bacteria to reach the upper side. After 3 hr incubation, numerous *Pst* DC3000 cells were found on the upper surface of the peel and formed characteristic clusters (Figure 5A), but the upper surface of peels treated with the *cor* mutant was virtually devoid of bacterial clusters, except for certain restricted regions where a few *cor* mutant bacteria could be found

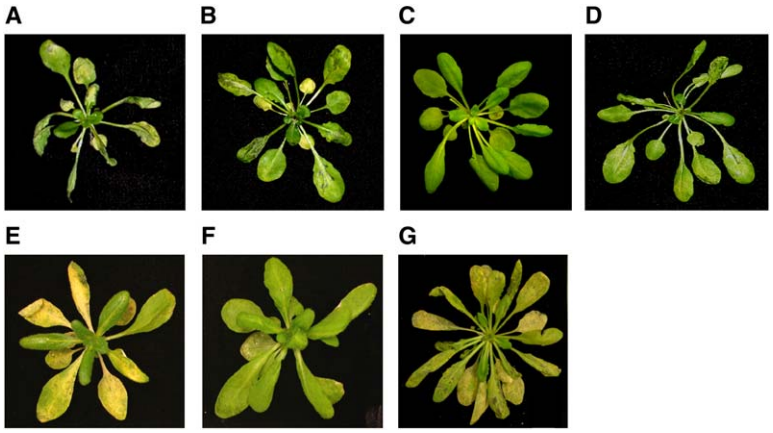

Figure 6. Disease Symptoms in *Arabidopsis* Genotypes Caused by Wild-Type *Pst* DC3000 and the *cor* Mutant *Pst* DC3118

Pictures were taken 3 days after inoculation.

(A–D) Col-0 plants were vacuum infiltrated (A and B; 1 × 10^6 cfu/ml) or dip inoculated (C and D; 1 × 10^8 cfu/ml) with *Pst* DC3118 (A and C) or *Pst* DC3000 (B and D).

(E–G) *ost1-2* mutant (E), wild-type Ler (F), and *nahG* transgenic (G) plants were dip inoculated with 1 × 10^8 cfu/ml *Pst* DC3118.

(Figure 5A). This experiment provides direct evidence that PAMP-induced stomatal closure effectively blocks the passage of *cor* mutant bacteria through the epidermis, whereas *Pst* DC3000 has evolved a virulence strategy to overcome this restriction.

We further tested the biological importance of stomatal defense in bacterial infection of whole plants by examining the ability of the *cor* mutant to infect *Arabidopsis aba3-1*, *ost1-2*, and *eds16-2* mutants and *nahG* transgenic plants, which are defective in bacterium/PAMP-induced stomatal closure (Figure 2D, Figure 3A, and Figure S3). In control experiments, *Pst* DC3000 and the *cor* mutant multiplied similarly and efficiently in wild-type Col-0 leaves when infiltrated directly into the apoplast (Figure 5B) and caused typical disease symptoms, including necrosis and chlorosis (Figures 6A and 6B). This result confirms that the *cor* mutant is not defective in virulence once inside the host. In contrast, when bacteria were applied to the leaf surface, the multiplication of the *cor* mutant in wild-type Col-0 and Landsberg *erecta* leaves was greatly reduced (100- to 1000-fold in different experiments) compared with that of *Pst* DC3000 (Figure 5C), and no disease symptoms were observed (Figures 6C and 6F). Remarkably, in surface-inoculated *aba3-1*, *ost1*, *eds16*, and *nahG* leaves, the *cor* mutant multiplied to levels similar to that reached by the wild-type bacterium *Pst* DC3000 at day 3 and caused disease symptoms (Figures 5C and 5D and Figures 6E and 6G). These results suggest that suppression of stomatal defense is the primary function of COR in local leaves and that the COR-mediated suppression of stomatal defense is critical for *Pst* DC3000 infection of host plants. Our results also provide an explanation for the basis of the enhanced susceptibility of the *eds16-2* mutant and *nahG* transgenic plants to *cor* mutant bacteria observed recently (Brooks et al., 2005).

DISCUSSION

Stomata represent one of the most important cell types in plants. These microscopic pores in the epidermis allow plants to conduct water transpiration and gas exchange necessary for photosynthesis, which are critical for the remarkable success of land plants on Earth. The presence of numerous pores on the plant surface, however, also presents opportunities for nondiscriminative entry of diverse microbes into the plant, which could have important consequences in host-microbe coevolution and microbial ecology both on the surface and inside of the plant. The results presented in this paper challenge the common assumption that bacteria can freely enter the plant through stomata on the leaf surface. We found that, in *Arabidopsis*, stomata function as innate immunity gates to actively prevent bacterial entry.

The Importance of Stomatal Defense in Bacterial Disease

The importance of stomatal defense in bacterial disease is illustrated in several ways. First, stomata close as an initial response to both human and plant pathogenic bacteria (Figure 1). This observation suggests that plants have developed an innate ability to sense the danger of potential bacterial invasion and have evolved a mechanism to close a major port of bacterial entry into the plant. Second, stomatal defense is under the control of the defense-signaling molecule SA (Figure 2). Because SA plays a central role in host defense against many pathogens, including *P. syringae*, the regulation of stomatal defense by SA suggests that stomatal defense is an integral part of the SA-regulated innate immune system. Third, plant pathogens have evolved specific virulence factors to suppress stomatal defense, as demonstrated for *Pst* DC3000 in this study, further suggesting that overcoming stomatal defense must be critically important in bacterial infection in nature. Overall, our study not only uncovers an evolutionarily important function for stomata but also identifies a major biologically relevant contribution of innate immunity to plant disease resistance. Specifically, we provide direct evidence that stomatal defense against bacterial invasion is an important function of innate immunity in plants.

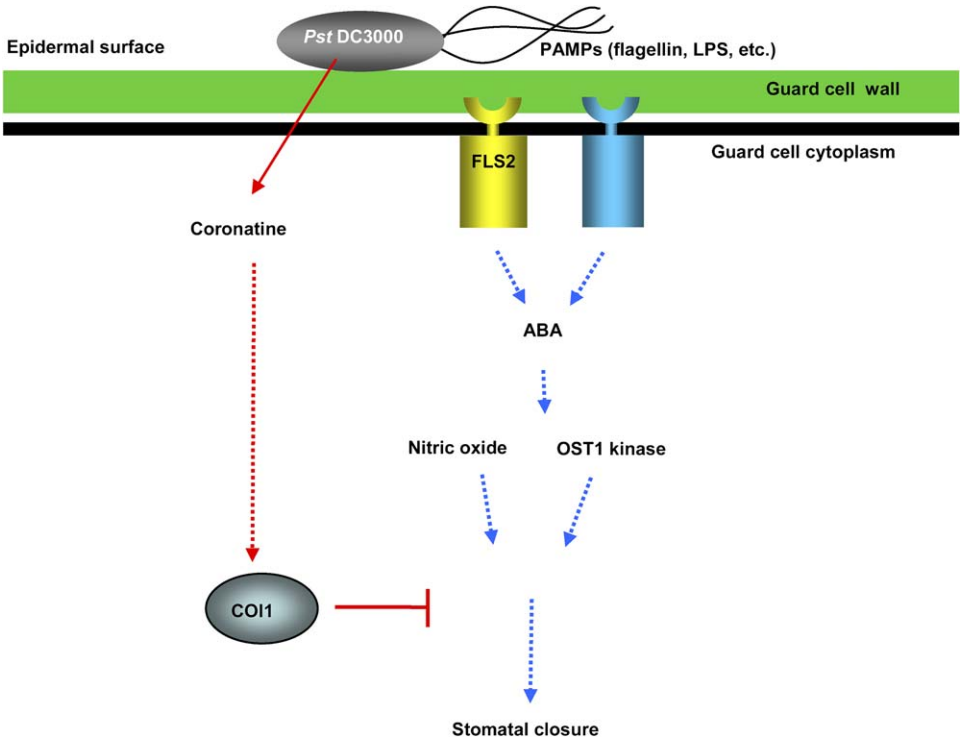

Figure 7. Model Depicting Bacterium- and PAMP-Induced Stomatal Closure in the *Arabidopsis* Guard Cell

Only those signal transduction components that are relevant to this study are shown. Our results suggest that PAMPs displayed on the surface of either a plant or human pathogenic bacterium are perceived by receptors (the FLS2 receptor is shown in yellow; another hypothetical receptor is in blue) in the stomatal guard cell. PAMP perception is mechanistically linked to ABA-regulated stomatal closure. The virulent *Arabidopsis* pathogen *Pst* DC3000 secretes the virulence factor coronatine, which functions downstream or independent of NO production to interfere with stomatal closure. The action of coronatine is dependent on COI1, a subunit of an E3 ubiquitin ligase also involved in the signaling of the plant defense hormone jasmonic acid. Both *FLS2* and *COI1* genes are expressed in guard cells (Figure S7).

Integration of Abiotic and Biotic Signals in Stomatal Guard Cells

Using a combination of plant mutants, chemical dyes, and inhibitors, we showed that several key steps involved in guard-cell sensing of abiotic stresses—including ABA synthesis, NO production, and the OST1 kinase—are required for PAMP/bacterium-induced stomatal closure (Figure 3, Figure 7, and Figure S3). Thus, stomatal guard cells have evolved a way of integrating abiotic and biotic signals to adapt to the multiple functions of stomata in controlling water loss, preventing microbial invasion, and maintaining the ability to conduct gas exchange essential for photosynthesis. The mechanistic linkage between biotic and abiotic regulation of stomatal functions has significant ramifications for future research on stomatal biology. Our results suggest that a comprehensive understanding of stomatal function and physiology will require elucidation of signal transduction pathways underlying not only abiotic stresses but also biotic stresses in the guard cells.

Coronatine Is a Suppressor of Stomatal Defense

One of the most intriguing results from this study is that plant pathogens have evolved virulence factors to suppress the innate immunity function of stomata. We have shown that, in *Pst* DC3000, COR is both necessary and sufficient to suppress stomatal defense in *Arabidopsis*. In support of this conclusion, the *cor* mutant is completely defective in suppressing stomatal defense (Figure 4A), and purified COR efficiently blocks PAMP-induced stomatal closure (Figure 4C). COR appears to act downstream or independent of NO production to reverse the effects of PAMPs on the ABA signal transduction pathway (Figure 7 and Figure S6). The demonstrated role of COR in disabling stomatal defense solves a decade-long puzzle that has existed since Mittal and Davis (1995) first observed that a COR-defective mutant could not cause disease when inoculated onto the leaf surface but caused wild-type infection if infiltrated directly into the apoplast, bypassing the epidermis.

Stomatal Defense and Bacterial Disease Ecology

The discovery of stomatal defense against bacterial invasion and the functional connection between COR and jasmonates should have significant implications in future research to understand the molecular bases of several mysterious phenomena in bacterial disease ecology. Many strains of *P. syringae* and other plant pathogenic bacteria can live and proliferate on the plant surface as

epiphytes for an extended period without causing disease (Lindow and Brandl, 2003). It has long been recognized that heavy rains (large rain drops) are associated with the transition from epiphytic growth to endophytic parasitism and frequently cause *P. syringae* disease outbreaks in the field (Upper and Hirano, 1999). Intense rains not only increase humidity but may also activate the wound response, a combination of which could favor stomatal opening and could therefore facilitate transition from epiphytic growth to endophytic parasitism. A recent study has suggested an antagonistic interaction between ABA signaling and jasmonate signaling during wound response in *Arabidopsis* leaves (Anderson et al., 2004). Based on structural similarities and the induction of analogous biological responses in plants, COR has been proposed to function as a molecular mimic of jasmonates and to activate the jasmonic acid (JA) signaling pathway (Bender et al., 1999; Zhao et al., 2003). However, Suhita and coworkers (2004) showed that application of methyl JA caused stomatal closure, suggesting that, unlike COR, exogenous methyl JA may not antagonize ABA-induced stomatal closure.

We used 10^8 cfu/ml (OD_{600} = 0.2) in most of our experiments because this standard inoculum concentration is necessary to reproducibly and uniformly induce disease in *Arabidopsis* leaves by the surface inoculation method (Katagiri et al., 2002; Brooks et al., 2004; Zipfel et al., 2004). In nature, epiphytic populations of bacteria are expected to be highly variable even on the same leaf due to tremendous spatial heterogeneity of nutrient availability and/or leaf surface topology (Lindow and Brandl, 2003). The total epiphytic bacterial population on the leaf surface can be very high, up to 10^8 cfu/g fresh weight (Kinkel et al., 2000; Lindow and Brandl, 2003). Even when the total number of bacteria is low on a leaf, bacterial concentrations at specific sites can be high, especially in aggregates (Lindow and Brandl, 2003), which could contribute to the observed discrete infection sites/lesions on the same leaf in the field (in contrast to the uniform infection of entire leaves in the laboratory).

Possible Stomatal Defense and Counterdefense in Other Plant-Pathogen Interactions

The importance and bacterial suppression of stomatal defense are likely beyond the *Arabidopsis-Pst* DC3000 interaction analyzed in this study. First, at least five *P. syringae* pathovars are known to produce coronatine, and they infect diverse plants (Bender et al., 1999). We found that *Pst* DC3000 also modulates stomatal response in another host plant, tomato (Figure S8), and that another COR-producing strain, *P. syringae* pv. *maculicola* ES4326, reopens stomata in *Arabidopsis* (Figure S9A). Second, LPS induces stomatal closure in tomato (Figure S10). Third, *P. syringae* pv. *tabaci*, which does not produce coronatine, induces the closure of stomata initially and then reopens stomata 3 hr after incubation in the host plant tobacco (Figure S9B). These results raise the exciting possibility that stomatal defense and bacterial suppression are common phenomena in plant-bacterium interactions and that coronatine is likely only one of the virulence factors used by bacteria to counter stomatal defense.

It would be interesting to investigate in the future whether stomatal defense also restricts the invasion of microorganisms other than bacteria. Although many fungi can directly penetrate the epidermis to enter the internal tissues, it has been shown that components of fungal cell walls (such as chitosan) can induce stomatal closure (Lee et al., 1999). The biological relevance of chitosan-induced stomatal closure to fungal invasion is not known. A previous study showed that a fungal elicitor, Avr9, modulates K^+ currents in tobacco guard cells in a *Cf9* disease-resistance-gene-dependent manner (Blatt et al., 1999). The physiological function of Avr9/Cf9-mediated ion fluxes in the guard cell is not yet clear. However, in light of our results, it would be interesting to determine whether the guard cell is a physiological target of the Avr9/Cf9 interaction and whether this interaction has an active role in promoting stomatal defense against fungal entry into the host tissue. Besides restricting pathogen invasion, regulation of stomatal opening/closure is probably also important for the development of wilting-disease symptoms and/or controlling pathogen release and reinfection from infected tissues.

Concluding Remarks

Our study uncovers a novel and crucial early battleground in host-bacterium interactions in the phyllosphere. The innate immune function of stomata has evaded discovery for a long time, presumably because of the widespread use of unnatural inoculation procedures to study bacterial infection in the laboratory. Because stomata are found in all vascular plants, we suggest that PAMP-induced stomatal closure is a widespread defense in vascular plants against invasion by the potentially vast number of bacteria to which plants are exposed in nature. To be a successful foliar pathogen, a bacterium must either evolve virulence factors to overcome stomatal defense or, if the bacterium has not evolved a specific virulence factor, rely on environmental conditions (e.g, heavy rains, frost damage, or accidental wounding) under which stomata may not effectively respond to PAMPs. Suppression of stomatal defense is likely a key adaptation for the transition from an epiphytic lifestyle to endophytic parasitism that is characteristic of numerous bacterial diseases in plants. The discovery of host-bacterium battles at stomata therefore represents a significant conceptual advance in our understanding of not only bacterial pathogenesis and stomatal biology but also microbial ecology of plant and human pathogenic bacteria in the phyllosphere.

EXPERIMENTAL PROCEDURES

All experiments reported here were repeated at least three times with similar results.

Plant Material

Arabidopsis plants (ecotypes Col-0 and Landsberg *erecta* [Ler] and mutant lines derived from these ecotypes as indicated in the figures)

were grown in controlled growth chambers at 22°C with a 12 hr photoperiod under light intensity of 100 μE/m^2/s. For all experiments, 5- to 6-week-old plants were used. *coi1-1* and *coi1-20* mutant plants were selected from heterozygote populations using CAPS markers (Xie et al., 1998) and root sensitivity to MeJA (Kloek et al., 2001), respectively. Two independent T-DNA lines of the *fls2* mutant (SAIL_691C4 and SALK_line 93905) were used for the experiments, and they showed similar responses to treatments. Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were grown in a greenhouse with controlled temperature of 22°C–25°C and natural light. Fully expanded leaves of 6- to 8-week-old tobacco plants were used for the experiments. Tomato seedlings (*Solanum lycopersicum* cv. Castlemart) were grown in Jiffy peat pots (Hummert International) in a growth chamber maintained under 17 hr of light (200 μE/m^2/s) at 28°C and 7 hr of dark at 18°C. Ten- to twenty-day-old tomato seedlings were used for all experiments.

Chemicals

Purified chemicals were used at the following concentrations: 10 μM abscisic acid (ABA, Sigma), 0.2 mM L-NNA (Nω-nitro-L-arginine, Sigma), 0.5 ng/μl coronatine (COR, purchased from C. Bender, Oklahoma State University), 100 ng/μl lipopolysaccharide (LPS from *P. aeruginosa*, Sigma), 5 μM flg22 peptide (Alpha Diagnostics, Inc.). Chemicals were diluted in MES (2-(*N*-morpholino)-ethanesulfonic acid) buffer (25 mM MES-KOH [pH 6.15] and 10 mM KCl), except for LPS solution, which also contained 0.25 mM $MgCl_2$ and 0.1 mM $CaCl_2$. Ultrapure LPS preparations from *E. coli* O55:B5 (Sigma) and *Salmonella minnesota* R595 (Re) (Calbiochem) were also tested with similar results. Concentrations of LPS, flg22, and COR were chosen based on previous studies (Zeidler et al., 2004; Zhao et al., 2003; Zipfel et al., 2004) and dose-response experiments (Figures S5 and S11).

Assessment of Response of Stomata to Treatments

To assure that most stomata were open before beginning experiments, we kept plants under light (100 μE/m^2/s) for at least 3 hr. Fully expanded young leaves were immersed in water or bacterial suspension (10^8 cfu/ml in water). At various time points, epidermis of three leaves was peeled off and immediately observed under a microscope (Zeiss Axiophot D-7082 photomicroscope with A3 fluorescence cube or laser scanning confocal microscope). Alternatively, epidermis was peeled from fully expanded leaves and placed on glass slides with the cuticle side in contact with water, MES buffer (25 mM MES-KOH [pH 6.15] and 10 mM KCl), chemical solutions in MES buffer, or bacterial suspensions in water. At various time points, pictures were taken of random regions. The width of the stomatal aperture was measured using the software Image-Pro version 4.5 for Windows (Cybernetics, Inc.). We found that stomata in intact leaves and epidermal peels responded similarly to various treatments. All stomatal aperture results reported here were from blind experiments in which genotypes and treatments were unknown to the experimenters who measured stomatal responses until the completion of experiments.

Detection of Nitric Oxide Production in Guard Cells

Epidermal peels of *Arabidopsis* plants were preincubated for 3 hr in MES buffer (25 mM MES-KOH [pH 6.15] and 10 mM KCl), soaked in 15 μM DAF-2 DA (4,5-diaminofluorescein diacetate, Sigma) diluted in MES buffer for 20 min, washed three times in MES buffer, and then incubated with chemicals or bacterial suspensions. To assess the COR effect on PAMP-induced NO production, peels were incubated with COR for 30 min prior to addition of purified PAMPs. MES buffer was used as control for purified chemicals, and water was the control for bacterial suspensions. Photographs of guard cells were taken with a digital camera attached to a fluorescence microscope equipped with a 502-530 band-pass filter.

Bacterial Growth Assay

Pst DC3000 and mutant derivatives were cultured at 30°C in Luria-Bertani (LB; Sambrook et al., 1989) medium supplemented with appropriate antibiotics until an OD_{600} of 0.8 was reached. Bacteria were collected by centrifugation and resuspended in water to the final concentration of 10^8 cfu/ml containing 0.05% Silwet L-77 (OSi Specialties). *Arabidopsis* plants were dipped in bacterial suspension and kept under high humidity until disease symptoms developed. Some plants were vacuum infiltrated with bacterial suspension at a concentration of 10^6 cfu/ml containing 0.004% Silwet L-77. Infiltrated plants were left to dry and then covered until completion of the experiment. Bacterial population in the plant apoplast was determined as previously described (Katagiri et al., 2002).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and 11 figures and can be found with this article online at http://www.cell.com/cgi/content/full/126/5/969/DC1/.

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