A Prominent Role of the Flagellin Receptor FLAGELLIN-SENSING2 in Mediating Stomatal Response to Pseudomonas syringae pv tomato DC3000 in Arabidopsis1[W][OA]

Weiqing Zeng and Sheng Yang He*

Department of Energy Plant Research Laboratory (W.Z., S.Y.H.) and Department of Plant Biology (S.Y.H.), Michigan State University, East Lansing, Michigan 48824–1312

The FLAGELLIN-SENSING2 (FLS2) receptor kinase recognizes bacterial flagellin and initiates a battery of downstream defense responses to reduce bacterial invasion through stomata in the epidermis and bacterial multiplication in the apoplast of infected plants. Recent studies have shown that during Pseudomonas syringae pv tomato (Pst) DC3000 infection of Arabidopsis (Arabidopsis thaliana), FLS2-mediated immunity is actively suppressed by effector proteins (such as AvrPto and AvrPtoB) secreted through the bacterial type III secretion system (T3SS). We provide evidence here that T3SS effector-based suppression does not appear to be sufficient to overcome FLS2-based immunity during Pst DC3000 infection, but that the phytopxin coronatine (COR) produced by Pst DC3000 also plays a critical role. COR-deficient mutants of Pst DC3000 are severely reduced in virulence when inoculated onto the leaf surface of wild-type Columbia-0 plants, but this defect was rescued almost fully in fls2 mutant plants. Although bacteria are thought to carry multiple microbe-associated molecular patterns, stomata of fls2 plants are completely unresponsive to COR-deficient mutant Pst DC3000 bacteria. The responses of fls2 plants were similar to those of the Arabidopsis G-protein alpha subunit1-3 mutant, which is defective in abscisic acid-regulated stomatal closure, but were distinct from those of the Arabidopsis non-expressor of PR genes1 mutant, which is defective in salicylic acid-dependent stomatal closure and apoplast defense. Epistasis analyses show that salicylic acid signaling acts upstream of abscisic acid signaling in bacterium-triggered stomatal closure. Taken together, these results suggest a particularly important role of FLS2-mediated resistance to COR-deficient mutant Pst DC3000 bacteria, and nonredundant roles of COR and TSS effector proteins in the suppression of FLS2-mediated resistance in the Arabidopsis—Pst DC3000 interaction.

Stomata are microscopic pores formed by pairs of guard cells in the epidermis of terrestrial plants; they are essential for CO₂ and water exchange with the environment. Plants regulate the stomatal aperture in response to changing abiotic environmental conditions (e.g. light, humidity, CO₂ concentration) to optimize CO₂ uptake and water transpiration. The molecular mechanisms underlying the stomatal regulation in response to abiotic signals are a subject of intense studies. Research in this area has uncovered many signaling components, indicating that stomatal guard cells have one of the most dynamic regulatory networks in plants (Schroeder et al., 2001; Shimazaki et al., 2007; Neill et al., 2008; Wang and Song, 2008).

Stomatal openings are also a major route of pathogen entry into the plant (Melotto et al., 2006). Accordingly, guard cells have developed mechanisms to regulate stomatal aperture in response to pathogens. Melotto and colleagues found that the bacterial pathogen Pseudomonas syringae pv tomato (Pst) strain DC3000 induces stomatal closure in Arabidopsis (Arabidopsis thaliana) within 1 h post inoculation. However, after 3 to 4 h, stomata reopen (Melotto et al., 2006). The ability of Pst DC3000 to reopen stomata is dependent on the polyketide toxin coronatine (COR), a virulence factor that had previously been shown to be important for bacterial multiplication within the mesophyll space, disease symptom development, and induction of systemic susceptibility of infected plants (Mittal and Davis, 1995; Bender et al., 1999; Budde and Ullrich, 2000; Brooks et al., 2004; Cui et al., 2005; Melotto et al., 2006b). Stomatal reopening by Pst DC3000 was also shown to be dependent on the RPM1-INTERACTING PROTEIN4 in Arabidopsis (Liu et al., 2009). Recently, another bacterial pathogen, Xanthomonas campestris pv campestris, was shown to cause stomatal closure and subsequent reopening during infection (Gudesblat...
et al., 2009). In this case, a virulence factor of smaller than 2 kD was identified, but the molecular identity of this virulence factor is not yet known. In fungal pathogens, examples of virulence factors that inhibit stomatal closure include fusicoccin (Turner and Graniti, 1969; Assmann and Schwartz, 1992; Kinoshita and Shimazaki, 2001) and oxalic acid (Guimaraes and Stotz, 2004), although their role in pathogen invasion has not been established.

Stomatal guard cells also respond to purified microbe-associated molecular patterns (MAMPs), such as chitosan, a polymer of β-1,4-glucosamine residues derived from fungal chitin (Lee et al., 1999; Amborabe et al., 2008), flg22, a 22-amino acid peptide derived from bacterial flagellin (Melotto et al., 2006; Cho et al., 2008; Desikan et al., 2008; Zhang et al., 2008), and bacterial lipopolysaccharides (LPSs; Melotto et al., 2006; Cho et al., 2008). Peptidoglycan, derived from Gram-positive bacteria, is shown to be able to induce plant innate immune responses (Gust et al., 2007; Erbs et al., 2008). However, peptidoglycan has not yet been shown to trigger stomatal responses. MAMPs are recognized by plant pattern-recognition receptors, such as Arabidopsis proteins FLAGELLIN-SENSING2 (FLS2) that recognizes bacterial flagellin (Gómez-Gómez and Boller, 2000), EF-TU RECEPTOR (EFR) that recognizes bacterial elongation factor TU (Zipfel et al., 2006), and CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) that perceives an unknown MAMP from *Pst* DC3000 (Gimenez-Ibanez et al., 2009a, 2009b). In the case of flg22-induced stomatal closure, FLS2 is required (Melotto et al., 2006). Stomata from *fls2* mutant plants, however, still respond to purified LPS (Melotto et al., 2006), illustrating both specificity in MAMP recognition by guard cells and the capacity of guard cells to recognize multiple MAMPs (Melotto et al., 2006). However, it has not been formally proven that the perception of any individual MAMPs actually contributes to stomatal closure induced by live bacteria, as bacteria produce many other factors in the context of an infection.

Studies using purified MAMPs have shown that stomatal closure in response to biotic signals requires the phytohormone abscisic acid (ABA), the guard cell-specific OPEN STOMATA1 (OST1) kinase, the production of reactive oxygen species and nitric oxide, the heterotrimeric G protein, and the regulation of K⁺ channels—all of which are hallmarks of abiotic signal-induced stomatal closure (Melotto et al., 2006; Neill et al., 2008; Zhang et al., 2008). These findings suggest that the guard cell signal transductions in response to biotic and abiotic signals share common steps. Besides shared signaling components, however, MAMP-triggered stomatal closure also requires the plant defense hormone salicylic acid (SA; Melotto et al., 2006). At present, it is not clear whether SA per se or a downstream signaling component, such as the NON-EXPRESSOR OF FR GENES1 (NPR1), is required for stomatal closure. Nor do we understand the epistatic relationship between SA and ABA signaling in the regulation of bacterium/MAMP-triggered stomatal closure.

In this study, we conducted experiments to further characterize stomatal regulation during *Pst* DC3000 infection of Arabidopsis plants. In particular, we sought to determine (1) whether the perception of well-documented MAMPs indeed contributes to stomatal closure in response to live bacteria, (2) the roles of the heterotrimeric G protein (involved in ABA signaling) and NPR1 (involved in SA signaling) in stomatal response during bacterial infection, and (3) the relationship between SA signaling and ABA signaling in regulating bacterium-triggered stomatal closure. These experiments revealed a critical role of FLS2 in mediating disease resistance against COR-deficient mutant *Pst* DC3000 bacteria.

**RESULTS**

**Restoration of Virulence of *Pst* DC3000 COR-Deficient Mutants in *fls2* Mutant Arabidopsis Plants**

Although purified bacterial MAMPs, such as flg22 and LPS, have been shown to cause stomatal closure (Melotto et al., 2006), it is not known whether they have a relevant biological role in mediating stomatal closure during actual bacterial infection. We hypothesized that if a MAMP plays a substantial role in mediating bacterium-triggered stomatal closure, the corresponding MAMP receptor mutant plants might be able to rescue the virulence defect of COR-deficient mutants, which are unable to overcome stomatal defense. To test this hypothesis, we dip inoculated two well-characterized MAMP receptor kinase mutant plants of Arabidopsis, *fls2* and *efr-1*, with COR-deficient mutants *Pst* DC3118 (Moore et al., 1989; Ma et al., 1991) and DB29 (Brooks et al., 2004) at the concentration of 1E + 8 colony-forming units (CFU)/mL (Katagiri et al., 2002). We did not observe restoration of the virulence of COR-deficient mutants in *efr-1* mutant plants. The disease symptoms and growth of *Pst* DC3118 (Fig. 1, A and B) and DB29 (Supplemental Fig. S1) were similar in *efr-1* mutant plants and in Columbia-0 (Col-0) plants. In contrast, *fls2* mutant plants showed severe disease symptoms and allowed the COR-deficient mutants to multiply to levels comparable to that of wild-type *Pst* DC3000 in wild-type Col-0 plants (Fig. 1, A and B; Supplemental Fig. S1). This observation is consistent with a study published earlier showing that *fls2* mutant plants allow spray-inoculated COR-deficient *Pst* DC3000 to multiply to a higher level (about 10-fold) compared to Col-0 plants (Nekrasov et al., 2009). This restoration of virulence of COR-deficient mutant bacteria in *fls2* plants suggests that the lack of functional FLS2 in plants could compensate for the loss of COR production in wild-type and DB29.

To further confirm our observation, we tested the Wassilewskija (Ws-0) ecotype plants. It was previously
shown that Ws-0 does not have a functional FLS2 and therefore is a natural fls2 mutant allele (Bauer et al., 2001; Gómez-Gómez and Boller, 2002; Kunze et al., 2004; Zipfel et al., 2004; Chinchilla et al., 2006). In our experiments, this ecotype behaved similarly to fls2 mutant plants that are defective in only one MAMP receptor (Fig. 1, A and B). We considered the possibility that flagellin perception by FLS2 may be the primary recognition event during the initial Pst DC3000 invasion through stomata. If so, one would expect that fls2 mutant stomata would not respond to COR-deficient mutant bacteria, even though these bacteria have the potential to release multiple MAMPs. We examined this possibility by conducting stomatal assays with live Pst DC3118 bacteria. The fls2 single mutant, the Ws-0 ecotype, and the fls2 efr-1 double-mutant plants were all impaired in the stomatal closure response to Pst DC3118, whereas efr-1 mutant plants retained the ability to close stomata in a manner similar to that of wild-type Col-0 (Fig. 2). We also tested fls2 plants with the wild-type Pst DC3000 bacteria. Again, stomata of fls2 plants did not show a closure response to the bacteria (Fig. 2C). These results confirm that FLS2 plays an essential role in mediating the stomatal closure response to Pst DC3000 bacteria in Arabidopsis.

To directly visualize the effect of the fls2 mutation on bacterial entry into Arabidopsis leaves, we quantified bacteria that are inside leaves 1 h after surface inoculation with GFP-labeled Pst DC3118 using confocal microscopy. We found that there were already about 6 times more bacteria inside fls2 leaves compared to wild-type Col-0 leaves at this early time point (Fig. 3; Supplemental Fig. S2).

**FLS2-Mediated Apoplast Defense Does Not Play a Major Role in Restricting Infection of COR-Deficient Mutant Bacteria**

When infiltrated directly into the Arabidopsis leaf apoplast (i.e. bypassing the leaf epidermis) at the inoculum concentration of 1E + 6 CFU/mL, Pst DC3000 COR-deficient mutants multiplied similarly as Pst DC3000 (Mittal and Davis, 1995; Melotto et al., 2006). Furthermore, disease symptom development in infiltration experiments with 1E + 6 CFU/mL Pst DC3000 most closely resembles symptom development in dipping experiments using 1E + 8 CFU/mL Pst DC3000. Interestingly, in infiltration experiments with lower inoculum concentrations, COR-deficient

---

**Figure 1.** fls2 and Ws-0 plants exhibit enhanced susceptibility to COR-deficient mutant bacteria. Leaf appearances (A and C) and bacterial populations (B and D) 3 d after surface inoculation with Pst DC3118 at 1E + 8 CFU/mL. Results are displayed as means of four different leaves from four different plants, with ses indicated. Statistical differences are detected with ANOVA (P = 0.0001) followed by Turkey's multiple comparison test (showing comparisons to Col-0; ***, P < 0.001) for B, and with two-tailed t test (**, P < 0.01) for D.
mutants multiplied less than wild-type Pst DC3000 in Arabidopsis (Brooks et al., 2004, 2005; Fig. 4, A and B). These results suggest that COR has roles in not only facilitating bacterial invasion through the epidermis, but also bacterial growth in the apoplast in the Pst DC3000-Arabidopsis interaction. The restoration of virulence of COR-deficient mutants of Pst DC3000 in fls2 plants, as revealed by dip inoculation (Fig. 1), could therefore result from a defect in not only FLS2-mediated defense during bacterial invasion, but also post invasion in the apoplast or a combination of both. To determine the relative contributions of these possible defects, we measured the multiplication of Pst DC3118 in fls2 plants by infiltrating bacteria directly into the Arabidopsis leaf apoplast. At the inoculum of 1E + 5 CFU/mL, fls2 and Col-0 plants showed similarly high susceptibility to Pst DC3118 (Figs. 4, C and D, and 5C). Although variations were observed in some experiments, the difference of susceptibility between fls2 and Col-0 plants was less than 5-fold. This suggests that the FLS2-mediated, post-entry defense in the apoplast does not play a major role in restricting infection by COR-deficient mutant Pst DC3118 bacteria.

Differences in FLS2- and SA-Mediated Resistance against COR-Deficient Mutant Bacteria

COR-deficient mutants of Pst DC3000 can multiply efficiently in SA-deficient nahG and eds16/sid2 plants (Brooks et al., 2005; Melotto et al., 2006). These plants are also defective in the stomatal closure response to Pst DC3000 COR-deficient mutant bacteria (Melotto et al., 2006). However, it is not known whether it is SA per se or SA signaling or both that is required for stomatal defense. Moreover, it has not been determined whether the increased susceptibility of SA-deficient plants to COR-deficient mutant bacteria results from a defect in stomatal defense, SA-mediated apoplast defense, or both. We tested the requirement of NPR1, a key regulator of SA signaling, for stomatal closure response and resistance to COR-deficient mutant bacteria. We found that npr1-1 plants were defective in the stomatal closure response to COR-deficient Pst DC3118 as well as wild-type Pst DC3000 bacteria (Fig. 5A). When dip inoculated, npr1-1 plants showed much enhanced susceptibility to Pst DC3118 (Fig. 5B). Contrary to fls2 plants, however, npr1-1 plants also

Figure 2. Stomatal closure responses to COR-deficient or wild-type bacteria. Stomatal apertures from leaf peels of Col-0, fls2, efr-1, and fls2 efr-1 plants treated with water or Pst DC3118 at 1E + 8 CFU/mL (A), Col-0 and Ws-0 plants treated with water or Pst DC3118 at 1E + 8 CFU/mL (B), and Col-0 and fls2 plants treated with water or Pst DC3000 at 1E + 8 CFU/mL (C). Results are displayed as means of 30 to 60 stomata, with sfs indicated. Statistical differences between water and bacterial treatment are detected with two-tailed t test (**, P < 0.01). In all our experiments, to preserve the stomatal aperture status in plants used for both stomatal and bacterial pathogenesis assays, we did not further treat leaf peels in any stomatal opening buffer.

Figure 3. Entry of Pst DC3118-GFP into Col-0 and fls2 leaves detected by confocal microscopy. A, A Col-0 leaf with sampling areas indicated by squares. B, Numbers of bacteria observed, showing means from six microscopic views (0.1 mm2 each) with sfs. Statistical difference is detected with a two-tailed t test (**, P < 0.01). C and D, Side views of three-dimensional models reconstructed from overlaid z-sections of GFP channel in Col-0 and fls2 leaves, respectively. The dimensions of the leaf section (in μm) of axis (x, z) are labeled. Please note that GFP-labeled bacteria (green dots) are inside the leaves.
showed higher susceptibility to DC3118 than wild-type Col-0 plants when bacteria were infiltrated directly into the leaf apoplast (Fig. 5C). These results suggest that NPR1 is involved not only in stomatal defense, but also in post-entry defense in the context of infection by COR-deficient mutant Pst DC3000 bacteria.

Similarity of FLS2-Mediated Resistance with G-Protein α-Subunit1-Mediated Resistance against COR-Deficient Mutant Bacteria

A recent study has shown that the heterotrimeric G-protein α-subunit1 (GPA1) is required for stomatal closure in response to flg22 (Zhang et al., 2008). However, whether the virulence defect of COR-deficient Pst DC3000 mutant bacteria can also be restored in the gpa1 mutant plants has not been determined. Therefore, we examined the susceptibility of the gpa1-3 mutant plants to Pst DC3118 and found that the responses of gpa1-3 mutant plants were strikingly similar to those of fls2 mutant plants (Fig. 6). First,

Figure 4. Susceptibility of plants to Pst DC3000 or Pst DC3118 when inoculated by infiltration at 1E + 5 CFU/mL. A, Leaf appearance of Col-0 plants 3 d after bacterial infiltration. B, Bacterial population in Col-0 leaves at day 0 and day 3 after infiltration. C, Leaf appearance (abaxial sides) 3 d after infiltration with Pst DC3118. D, Bacterial populations at day 0 and day 3 after infiltration with Pst DC3118. Results displayed here (B and D) are means of four different leaves from four different plants, with sse indicated. Statistical differences are detected with two-tailed t test (***, P < 0.001).

Figure 5. Role of NPR1 in plant response to COR-deficient or wild-type Pst DC3000 bacteria. A, Stomatal apertures in Col-0 and npr1-1 leaf peels incubated with Pst DC3118 or DC3000 at 1E + 8 CFU/mL. Results are displayed as means of 30 to 60 stomata with sse shown. B, Leaf appearance and bacterial populations 3 d after dip inoculation of Col-0 and npr1-1 plants with Pst DC3118 at 1E + 8 CFU/mL. C, Bacterial populations at day 3 in leaves of Col-0, npr1-1, and fls2 plants infiltrated with Pst DC3118 at 1E + 5 CFU/mL. B and C, Results are displayed as means of four different leaves from four different plants, with sse shown. Statistical differences are detected with two-tailed t test (***, P < 0.001) for A and B, and with ANOVA (P = 0.014) followed by Turkey’s multiple comparison test (showing comparisons to Col-0; *, P < 0.05) for C.
gpa1-3 mutant plants are highly susceptible to *Pst* DC3118 when dip inoculated, showing prominent disease symptoms (Fig. 6A) and allowing high levels of bacterial multiplication (Fig. 6B). Second, like *fls2* plants, *gpa1-3* plants are also defective in stomatal closure response to COR-deficient *Pst* DC3118 as well as wild-type *Pst* DC3000 bacteria (Fig. 6C). Finally, unlike *npr1-1* plants, *gpa1-3* plants did not show significantly increased susceptibility to *Pst* DC3118 compared with wild-type Col-0 plants, when bacteria were infiltrated directly into the leaf apoplast, bypassing the epidermis (Fig. 6D). These results show that, like FLS2, GPA1 contributes to Arabidopsis resistance to COR-deficient mutant *Pst* DC3000 bacteria primarily through modulating stomatal defense.

SA Acts Upstream of ABA through NPR1 in the Stomatal Closure Signaling Pathway

Our previous and current studies show an important role for ABA and SA in regulating the stomatal closure response to bacteria and MAMPs. However, the epistatic relationship between SA and ABA in such regulation is not clear. We examined this relationship and found that SA-deficient *eds5-1, eds16/sid2*, and *nahG* plants showed normal stomatal closure responses to exogenous ABA (Fig. 7A). On the other hand, exogenously applied SA could induce stomatal closure responses of SA-deficient mutant plants *eds5-1* and *sid2*, but it could not induce stomatal closure in the ABA biosynthetic mutant *aba2-1* (Fig. 7B). These results indicate that SA acts upstream of ABA in the signaling pathway, leading to bacterium-triggered stomatal closure.

Exogenous SA induced stomatal closure in wild-type Col-0 plants, but it could not induce stomatal closure in *npr1-1* plants (Fig. 7C). In contrast, exogenous ABA was still able to induce stomatal closure in *npr1-1* as well as in *fls2* plants (Fig. 7D). These results indicate that NPR1 acts downstream of SA, but upstream of ABA, in stomatal guard cell signaling.

Requirement of Distinct Signaling Components for FLS2-Mediated Stomatal Closure and Plant Growth Inhibition

In addition to mediating innate immunity, flagellin and flg22 are also able to inhibit root elongation and seedling growth (Gómez-Gómez et al., 1999). We were interested in knowing whether induction of stomatal closure and inhibition of root elongation and plant growth by FLS2 perception of flg22 share the same components. SA-deficient, ABA-deficient, *fls2*, and *gpa1-3* mutant plants were examined for their responses to flg22-mediated inhibition of root elongation. As expected, root elongation of wild-type Col-0 plants, but not the *fls2* mutant plants or the Ws-0 ecotype plants, was...
inhibited by the presence of peptide flg22 (Fig. 8). However, inhibition of root elongation was also observed for all the other mutants tested, including eds5-1 (defective in SA biosynthesis), aba2-1 (defective in ABA biosynthesis), ost1-2 (defective in ABA signaling), and gpa1-3 (defective in heterotrimeric G-protein function; Fig. 8). Thus, FLS2 perception of flg22 appears to initiate multiple and distinct signaling pathways. Whereas the stomatal closure pathway involves SA and ABA, the pathway leading to the inhibition of root elongation does not. However, the inhibition of root elongation by flg22 in gpa1-3 plants was not as dramatic as in other mutants. We further tested gpa1-3 and Col-0 plants with lower concentrations of the flg22 peptide. Again, inhibition of root elongation by flg22 in gpa1-3 plants was not as much as in Col-0 (Supplemental Fig. S3). Therefore, GPA1 not only participates in stomatal closure response, but also seems to play some role in flg22-induced root growth inhibition.

DISCUSSION

Previous studies have shown that both wild-type and flagellin-lacking Pst DC3000, when infiltrated into the apoplast, elicit the expression of largely the same set of MAMP-responsive genes in Arabidopsis (Thilmony et al., 2006), and that fls2 mutant plants still respond to infiltration with MAMPs released from Pst DC3000 lysates (Zipfel et al., 2004). Both findings suggest that Pst DC3000 produces MAMPs other than flagellin, and that Arabidopsis leaf cells (at least the mesophyll cells inside the apoplast) are capable of perceiving multiple MAMPs. It has also been shown that stomatal guard cells of Arabidopsis can respond to more than one MAMP (Melotto et al., 2006; Cho et al., 2008; Desikan et al., 2008; Gudesblat et al., 2009). Therefore, it was surprising to observe that (1) stomata in fsl2 plants are completely defective in response to Pst DC3000 and its COR-deficient mutants (Fig. 2), as if Pst DC3000 bacteria carry only a single MAMP—flagellin, and (2) Pst DC3000 COR-deficient mutant bacteria are able to multiply aggressively and cause disease symptoms in fsl2 mutant plants when surface inoculated (Fig. 1). These observations seem to contradict the idea that multiple MAMP-receptor interactions are at work in the Arabidopsis-Pst DC3000 interaction and suggest that not all potential MAMPs from a given pathogen are presented simultaneously to all plant cell types during the course of an infection. As shown in this study, FLS2-mediated resistance against COR-deficient Pst DC3000 mutant bacteria seems to be manifested mainly at the level of epidermal stomatal defense. It may be that for bacteria to invade through stomata, they must be living and they must possess flagella. At this early stage of infection, other MAMPs (particularly those that are likely released from dead bacteria) may not be present at sufficient concentrations near guard cells to affect stomatal movements. At subsequent stages of infection within the plant apoplast, on the other hand, many different MAMPs, derived from live or dead bacteria, may accumulate to sufficient concentrations near mesophyll cells, which could mask the unique role of FLS2-mediated resistance in the apoplast.

Alternatively, Pst DC3000 releases multiple MAMPs during infection, but most Pst DC3000-derived MAMPs may not be as potent as flagellin in eliciting defense responses in Arabidopsis. This possibility is
supported by earlier observations that, at the same concentration (1 μM), flg22 (derived from the conserved N terminus of eubacterial flagellins; Felix et al., 1999) is more potent than elf18 (derived from Escherichia coli; Kunze et al., 2004) to induce the oxidative burst in Arabidopsis leaves (Zipfel et al., 2006), and elf26 peptides from Agrobacterium tumefaciens and Erwinia amylovora are 50 times more potent to induce medium alkalinization of Arabidopsis cell culture than elf26 from Pst DC3000 (Kunze et al., 2004). Consistent with this observation, our stomata assays showed that the E. coli-derived elf18 is much more potent than the corresponding peptide from Pst in inducing stomata closure in Arabidopsis Col-0 leaf epidermis (Supplemental Fig. S4). This observation might explain why fls2 mutant plants are unable to close stomata in response to Pst DC3118 or DC3000 (Fig. 2), but still respond to E. coli (Melotto et al., 2006). Interestingly, when inoculated by spraying with Pst DC3000 at very high inoculums (1E + 9 CFU/mL), efr-1 mutant plants showed higher susceptibility than wild-type Col-0 plants (Saijo et al., 2009). On the other hand, Nekrasov and colleagues (2009) showed that the efr-1 mutant plants were not more susceptible than wild-type Col-0 plants to wild-type Pst DC3000 or COR-deficient mutant bacteria when spray inoculated at the concentration of 1E + 7 CFU/mL. Similar susceptibilities of efr-1 and Col-0 plants to spray inoculation with 1E + 7 CFU/mL of Pst DC3000 or COR-deficient mutant bacteria were also observed by Li et al. (2009). It seems that only at the high inoculums of 1E + 9 CFU/mL can the concentration of ET-Tu derived from Pst DC3000 bacteria reach a high enough level that the unique contribution of the EFR signaling pathway can be detected in Arabidopsis. Interestingly, flagellin perception has been shown to be critical for Pst DC3000 infection of Nicotiana benthamiana, likely because this plant lacks a functional EFR (Kvitko et al., 2009).

Although we focused on COR-deficient Pst DC3000 mutant bacteria in this study, several studies have shown a role of FLS2-mediated immunity in resistance to wild-type Pst DC3000 (Zipfel et al., 2004; Zhang et al., 2007; Xiang et al., 2008; Clay et al., 2009). However the degrees of enhanced susceptibility of fls2 mutant plants to wild-type Pst DC3000 are generally subtle and variable, ranging from approximately 2- to 10-fold (Zipfel et al., 2004; Sun et al., 2006; Heese et al., 2007; Göhre et al., 2008; Xiang et al., 2008; Clay et al., 2009; Gimenez-Ibanez et al., 2009a; Nekrasov et al., 2009). This variability likely reflects different plant growth and experimental conditions used in these studies. In our own experiments, although significantly enhanced disease symptoms in fls2 mutant plants are observed consistently compared to wild-type Col-0 plants following dip inoculation, the enhanced Pst DC3000 multiplication in fls2 plants was small and variable in different experiments. Results from a representative experiment (about a 3-fold difference at day 2 after inoculation) are shown in Supplemental Figure S5. Under the same conditions, however, the enhanced multiplication of COR-deficient mutant Pst DC3118 was dramatic, measuring 100-fold or higher (Fig. 1). A prevailing explanation for the relatively small contribution of FLS2 to Arabidopsis resistance to Pst DC3000 is that this pathogen produces two type III secretion system (T3SS) effectors, AvrPto and AvrPtoB, that suppress the function of FLS2 and related receptor kinases, such as BAK1 and CERK1 (Shan et al., 2008; Xiang et al., 2008; Gimenez-Ibanez et al., 2009a). The striking FLS2-dependent virulence defect of Pst DC3000 COR-deficient mutants observed in this study, however, suggests that AvrPto and AvrPtoB are not sufficient to suppress FLS2-mediated immunity in the absence of COR. Instead, COR and T3SS effectors are both required for completely disabling the FLS2-mediated resistance mechanism during Pst DC3000 infection of Arabidopsis.

While the requirement of both COR and T3SS effectors for suppressing FLS2-mediated resistance highlights the prominent role of FLS2 in disease resistance, it raises a conceptual issue regarding the functional relationship between various types of virulence factors. As a foliar pathogen, Pst DC3000 encounters different host cell types in a temporal and spatial manner. When inoculated onto the plant surface, Pst DC3000 bacteria first make contact and interact with the epidermal cells (including stomatal guard cells) before they enter the
plant apoplast and interact with internal mesophyll cells. Because COR is a small, diffusible molecule, it has the potential to reach many host cells from the site of production. In contrast, T3SS effectors seem to be delivered into only those host cells that are in direct contact with bacteria. With different delivery mechanisms, the two classes of bacterial virulence factors may not reach the same host cells at the same time during every stage of infection. This might explain why \textit{Pst} DC3000 requires both COR and T3SS effectors AvrPto and AvrPtoB to suppress FLS2-mediated resistance during a presumably multistage infection process that involves distinct host cell types. As already discussed, FLS2-mediated resistance against \textit{Pst} DC3000 COR-deficient mutant bacteria appears to be manifested mainly at the epidermis. This observation implies that T3SS effectors such as AvrPto and AvrPtoB are not able to substitute for COR in suppressing FLS2-mediated stomatal closure (and possibly other defenses) in the epidermis. Unlike the diffusible COR, AvrPto and AvrPtoB are perhaps not delivered into stomatal guard cells and other epidermal cells in sufficient amounts during early stages of infection. On the other hand, the lack of a major contribution of FLS2-mediated resistance against \textit{Pst} DC3000 COR-deficient mutant bacteria in the apoplast suggests that the role of COR in suppressing FLS2-mediated resistance in this space is likely redundant to and masked by that of effector proteins such as AvrPto and AvrPtoB.

Extending the previous finding of the important role of SA and ABA in bacterium- and MAMP-triggered stomatal closure, this study demonstrates the involvement of two signaling components, NPR1 and GPA1, and defines the epistatic relationship between SA and ABA signaling in this process (Fig. 7). Although the involvement of NPR1 and GPA1 in bacterium-triggered stomatal closure is not entirely surprising, the demonstrated requirement of NPR1 does expand the role of this key SA signaling regulator to an important plant cell type (the guard cell) and raises a pertinent issue about the function of NPR1. NPR1 is best known for controlling the expression of SA-responsive genes (Wang et al., 2005), and a recent study shows that MAMP signaling later leads to expression of SA response genes in \textit{Arabidopsis} leaves (Tsuda et al., 2008). Therefore, the requirement of NPR1 for bacterium-triggered stomatal closure could imply an involvement of NPR1-controlled gene expression in stomatal regulation. We examined NPR1-regulated genes from the publicly available microarray data (Blanco et al., 2005; Wang et al., 2005; Tsuda et al., 2008; Blanco et al., 2009), but could not identify any genes that are known to be involved in stomatal regulation. However, these gene expression analyses were performed many hours after chemical or pathogen treatments of whole leaves, which are composed mainly of mesophyll cells. Further experiments will be necessary to determine whether there are NPR1-regulated genes in stomatal guard cells within a shorter duration (e.g. minutes) of treatments with bacteria or MAMPS. Until such genes are identified and confirmed to be required for bacterium-triggered stomatal closure, it remains possible that the \textit{npr1} mutant guard cells may be altered in some signaling/metabolic networks that affect stomatal closure independent of gene expression. The epistasis analyses performed in this study place the action of NPR1 between downstream of SA and upstream of ABA (Fig. 7), presenting a clearer picture of the signal transduction events leading to the stomatal closure induced by \textit{Pst} DC3000.

Recent studies have shown that COR mimics the active form of the plant hormone jasmonate (JA), jasmonoyl Ile, and directly targets the JA receptor complexes (composed of the COI1 F-box protein and JAZ repressor proteins) to activate JA signaling in \textit{Arabidopsis} (Thines et al., 2007; Katsir et al., 2008; Melotto et al., 2008a; Browse, 2009; Fonseca et al., 2009). A recent study shows that JA can effectively suppress flg22-induced callose deposition in mesophyll cell walls in \textit{Arabidopsis} leaves (Clay et al., 2009). Thus, by activating JA signaling, bacteria can suppress not only FLS2-mediated stomatal closure, but also FLS2-mediated mesophyll cell defenses in the leaf. An important direction of future research on the virulence action of COR will be to elucidate how activation of JA signaling inhibits FLS2 signaling in plants in both stomatal guard cells and mesophyll cells.

**MATERIALS AND METHODS**

**Plant Materials**

\textit{Arabidopsis (Arabidopsis thaliana)} wild-type (Col-0, Ws-0, and Landsberg erecta [Ler]) and mutant plants were grown in growth chambers under a day/night cycle of 16 h/8 h at 20°C, with a light intensity of 80 to 100 μE m$^{-2}$ s$^{-1}$. Plants were used for experiments when they were about 5 weeks old.

The \textit{fls2} mutant was originally identified as Salk$_{093905}$ with a T-DNA insertion in At5g6330 (Heese et al., 2007) and is from Dr. Scott Peck (University of Missouri, Columbia, MO). The \textit{acr} mutant was initially identified as Salk$_{044334}$ with a T-DNA insertion in At5g20480 (Zipfel et al., 2006) and is from Dr. Thomas Boller (University of Basel, Basel). The \textit{bsf1} double mutant is also from Dr. Thomas Boller. The \textit{npr1} mutant is from Dr. Ximin Dong (Duke University, Durham, NC), and the \textit{gun1-3} mutant is from Dr. Sarah M. Assmann (Pennsylvania State University, University Park, PA). The \textit{ eds1-1} mutant was purchased from the \textit{Arabidopsis Biological Resource Center} (CS775 for gene At4g39030). The \textit{sid2} mutant is from Dr. Mary Wildermuth (University of California, Berkeley, CA), and the \textit{nahG} transgenic plant was purchased from Novartis. The \textit{ahs2-1} mutant is from Dr. Jan A.D. Zeefaat (Michigan State University, East Lansing, MI), and the \textit{ sos1-2} mutant is from Dr. Anna-Chiara Mustilli (Centre National de la Recherche Scientifique, Gil-sur-Yvette, France).

**Stomatal Assays**

Leaf peels were collected from the abaxial side of mature leaves from 5-week-old plants and placed in 250 to 300 μL of distilled, deionized water, bacteria resuspended in distilled, deionized water buffer (25 mM MES, 10 mM KCl, pH 6.15), or buffer containing chemicals that were preplaced on glass slides in square petri dishes with lids on. To preserve the stomatal aperture status in plants used for both stomatal and bacterial pathogenesis assays, we did not further treat leaf peels in any stomatal opening buffer. The petri dishes were left in the growth chamber in which plants were grown for an hour before being observed under a light microscope. Images of leaf peels were randomly taken and there were at least 30 stomata recorded for each sample treatment. Stomatal apertures were measured from these images with Adobe Photoshop.
Photoshop. Bacteria were used at 1E + 8 CFU/mL, (OD\textsubscript{600} = 0.2), flg22 (EZBiolab) at 10 \mu M, ABA (Sigma) at 10 \mu M, and SA (Sigma) at 20 \mu M.

**Confocal Microscopy**

Whole leaves were detached from 5-week-old plants and incubated with Pst DC3118-GFP suspension at 1E + 8 CFU/mL for 1 h. Leaves were then rinsed in sterile water twice, each in 300 mL water for 2 min. Six leaf sections were excised from each leaf (see Fig. 3A for locations) and observed under an Olympus Fluoview FV1000 confocal microscope. For each microscopic view, 16 to 20 z-sections of 3 \mu m each were taken and overlaid for bacterial counting.

**Pathogen Infection Assays**

Bacteria were streaked from a freezer stock onto low-salt Luria-Bertani plates (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L) with appropriate antibiotics. A Luria-Bertani liquid culture of 10 mL was started from plates that had been stored at -4 \degree C for less than 2 weeks. After this small culture was grown for 12 h at 28 \degree C, a subculture was made using 1:100 dilution and was incubated further at 28 \degree C. When the OD\textsubscript{600} reached 0.8 to 1.0, bacterial cells were collected and resuspended in distilled, deionized water to OD\textsubscript{600} of 0.2.

For dip inoculation, Silwet L-77 was added to a final concentration of 0.03%. Five-week-old plants grown in meshed pots were dipped upside down in the bacterial solution for a few seconds and returned to a growth room in a covered tray. Disease symptoms were recorded by camera, and bacterial populations were monitored by serial-dilution assays (Katagiri et al., 2002).

For infiltration inoculation, bacteria concentration was adjusted with distilled, deionized water according to individual experiments. After hand infiltration with a blunt-end syringe, plants were left in open trays to dry for about 2 h before being covered. The day 0 population was sampled at 2 h after infiltration. Bacteria populations were monitored in the same way as described for dip inoculation.

**Root Elongation Assays**

Seeds were sterilized in 30% bleach solution with 0.01% Tween 20 for 20 min at room temperature on a shaker, and rinsed with sterile distilled, deionized water five times. Sterilized seeds were resuspended in sterilized 0.1% agarose solution and transferred into the wells of 96-well plates with deionized water five times. Sterilized seeds were resuspended in sterilized distilled, deionized water according to individual experiments. After hand infiltration with a blunt-end syringe, plants were left in open trays to dry for about 2 h before being covered. The day 0 population data was sampled at 2 h after infiltration. Bacteria populations were monitored in the same way as described for dip inoculation.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Bacterial populations 3 d after dip inoculation with DB29 at 1E + 8 CFU/mL.

**Supplemental Figure S2.** Entry of Pst DC3118-GFP into Col-0 and fsl2 leaves detected by confocal microscopy.

**Supplemental Figure S3.** Root elongation response of gpa1-3 and Col-0 plants to flg22.

**Supplemental Figure S4.** Stomata apertures in Col-0 leaf peels treated with flg22 and elf18 from E. coli and Pst DC3000.

**Supplemental Figure S5.** Responses of Col-0 and fsl2 plants after dip inoculation with Pst DC3000 at 1E + 8 CFU/mL.

**ACKNOWLEDGMENTS**

We thank Cyril Zipfel for elf18 peptides, Kelly Hannon for her assistance with root measurements, Christy Mceey and Melinda Frame for their help with confocal microscopy, Karen Bird for editing, and Marlene Cameron for figure formatting. We thank William Underwood, Alexandre Brutus, Maeli Melotto, and other members of the S.Y.H. lab for their comments on the manuscript.

Received March 30, 2010; accepted May 10, 2010; published May 10, 2010.

**LITERATURE CITED**


Role of FLAGELLIN-SENSING2 in Stomatal Immune Response


Zeng and He