Pseudomonas syringae Evades Host Immunity by Degrading Flagellin Monomers with Alkaline Protease AprA

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Bacterial flagellin molecules are strong inducers of innate immune responses in both mammals and plants. The opportunistic pathogen *Pseudomonas aeruginosa* secretes an alkaline protease called AprA that degrades flagellin monomers. Here, we show that AprA is widespread among a wide variety of bacterial species. In addition, we investigated the role of AprA in virulence of the bacterial plant pathogen *P. syringae* pv. *tomato* DC3000. The AprA-deficient DC3000 Δ*aprA* knockout mutant was significantly less virulent on both tomato and *Arabidopsis thaliana*. Moreover, infiltration of *A. thaliana* Col-0 leaves with DC3000 Δ*aprA* evoked a significantly higher level of expression of the defense-related genes *FRK1* and *PR-1* than did wild-type DC3000. In the flagellin receptor mutant *fls2*, pathogen virulence and defense-related gene activation did not differ between DC3000 and DC3000 Δ*aprA*. Together, these results suggest that AprA of DC3000 is important for evasion of recognition by the FLS2 receptor, allowing wild-type DC3000 to be more virulent on its host plant than AprA-deficient DC3000 Δ*aprA*. To provide further evidence for the role of DC3000 AprA in host immune evasion, we overexpressed the AprA inhibitory peptide AprI of DC3000 in *A. thaliana* to counteract the immune evasive capacity of DC3000 AprA. Ectopic expression of *aprI* in *A. thaliana* resulted in an enhanced level of resistance against wild-type DC3000, while the already elevated level of resistance against DC3000 Δ*aprA* remained unchanged. Together, these results indicate that evasion of host immunity by the alkaline protease AprA is important for full virulence of strain DC3000 and likely acts by preventing flagellin monomers from being recognized by its cognate immune receptor.

For both plants and animals, distinguishing self from nonself is important for the activation of innate immune responses upon pathogen attack. In mammals and plants, microorganisms are recognized by pattern-recognition receptors (PRR)

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that detect common, highly conserved microbe-associated molecular patterns (MAMPs) such as lipopolysaccharides (LPS), peptidoglycans, chitin, elongation factor Tu (EF-Tu), and flagellin (Boller and Felix 2009; Pel and Pieterse 2013). Recognition of these conserved non-self molecules enables host cells to detect millions of microorganisms with a relatively small number of receptors and mediates a first line of defense against most of the nonadapted pathogens (Bardoel and Van Strijp 2011; Jones and Dangl 2006).

Flagellin is a highly conserved MAMP that can be recognized by both animal and plant cells. This protein forms the major part of the bacterial flagellum that enables bacterial motility. Each flagellum consists of thousands of flagellin molecules (Chevance and Hughes 2008; Gomez-Gomez and Boller 2002; Ramos et al. 2004; Samatey et al. 2001). Flagellin monomers can surround the bacteria due to spills during flagellum construction or due to damaging of the flagellar filaments (Gomez-Gomez and Boller 2002; Komoriya et al. 1999). It is these monomers that are recognized by mammalian and plant cells (Felix et al. 1999; Wyant et al. 1999). In mammals, the PRR Toll-like receptor 5 (TLR5) is required for detection of bacterial flagellin whereas, in plants, flagellin-sensitive 2 (FLS2) is responsible for flagellin recognition (Gomez-Gomez and Boller 2000; Hayashi et al. 2001). Both TLR5 and FLS2 recognize conserved parts of the flagellin molecule. However, the TLR5 recognition site was mapped to a cluster of 13 amino acids on both the C and N terminus of the flagellin monomers, while FLS2 recognizes a 22-amino-acid sequence in the N terminus of the protein (Felix et al. 1999; Smith et al. 2003). After binding of flagellin to TLR5 or FLS2, downstream signaling is triggered, leading to immune responses that are aimed at stopping the pathogen from entering the host tissue (Asai et al. 2002; Ronald and Beutler 2010).

For pathogen survival, suppression and evasion of host immune responses is of utmost importance. Many bacterial pathogens possess a type III secretion system that allows them to transfer proteins directly into host cells. These proteins are called effectors and generally contribute to virulence by suppressing host defense responses (Boller and He 2009). Besides suppression of host immunity, evasion of host immunity is an important virulence strategy as well. For example, the fungal plant pathogen *Cladosporium fulvum* secretes a LysM domain-containing protein, called extracellular protein 6 (Ecp6). The LysM domains bind fungal chitin oligosaccharides, thereby preventing recognition of chitin by the host cell and avoiding

activation of MAMP-triggered immune responses (Bolton et al. 2008; De Jonge et al. 2010).

The opportunistic pathogen *Pseudomonas aeruginosa* secretes an alkaline protease, designated AprA, which belongs to the serralysin family of the zinc metalloproteases (Miyoshi and Shinoda 2000). This protease is secreted by a type I secretion system and has been associated with virulence (Liehl et al. 2006; Parmely et al. 1990; Tommassen et al. 1992). Recently, we demonstrated that AprA actively degrades flagellin monomers (Bardoel et al. 2011). We hypothesized that AprA-mediated degradation of spilled flagellin monomers could be a bacterial strategy to evade host immunity. Here, we show that the bacterial alkaline protease AprA is widespread among many human- and plant-pathogenic bacteria, including the bacterial plant pathogen *P. syringae* pv. *tomato* DC3000. *P. syringae* pv. *tomato* is the causal agent of bacterial speck disease on a wide range of economically important crops and is rated as the number one bacterial pathogen based on scientific and economic importance (Mansfield et al. 2012). We provide evidence that the AprA protease of DC3000 plays an important role in the evasion of host immunity and is required for full virulence of DC3000 on both *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*). Finally, we demonstrate that transgenic *A. thaliana* plants ectopically expressing the AprA inhibitor AprI of DC3000 show a significantly higher level of resistance to DC3000 infections, with which a novel tool is provided to protect plants against bacterial pathogens.

RESULTS

### AprA is widespread among highly divergent pathogenic bacteria.

The *aprA* gene encodes a 50-kDa zinc metalloprotease and, in *P. aeruginosa*, this gene is present in the same operon as the AprA inhibitor-encoding gene *aprI* and the type I secretion system encoding genes *aprD*, *aprE*, and *aprF* (Stover et al. 2000). A similar operon has been described for the plant pathogen *Dickeya dadantii* (Duong et al. 1992; Guzzo et al. 1991), and the human pathogen *Serratia marcescens* has also been shown to possess *aprA* and *aprI* (Létoffé et al. 1989). Also, in the bacterial plant pathogen *P. syringae* pv. *tomato* DC3000, an operon containing *aprA*, *aprI*, *aprD*, *aprE*, and *aprF* can be identified (Fig. 1A) (Buell et al. 2003). The presence of an *aprA* homolog in these different γ-proteobacteria led to the hypothesis that this protease might be evolutionarily conserved.

To identify AprA homologs in other bacterial species, a protein BLAST followed by selection for serralysin-specific motifs was performed. This led to the identification of 134 AprA protein homologs from 102 different bacterial strains, of which many are pathogens on plants or mammals (Supplementary Table S1). Surprisingly, AprA is not limited to a specific group of bacteria but can be found in highly divergent bacterial species. Although most bacteria with an AprA homolog are α-proteobacteria or γ-proteobacteria, AprA homologs of β-proteobacteria, cyanobacteria, and chlorobi also were identified.

For these AprA homologs to degrade flagellin monomers, as has been shown for AprA of *P. aeruginosa* (Bardoel et al. 2011), they need to be secreted. For *P. aeruginosa* and *D. dadantii*, it has been shown that this secretion depends on the type I secretion system that is encoded by *aprD*, *aprE*, and *aprF*, which are present in the same operon as *aprA* (Guzzo et al. 1991; Létoffé et al. 1990). To check whether the identified AprA homologs of the other 102 species are located in similar operons, the eight genes upstream and downstream of *aprA* were checked for the presence of protease inhibitors or genes involved in protein secretion. From the 53 bacterial species in the list of which the genomes have been sequenced and annotated, 28 had genes involved in protein secretion and 23 had genes encoding a protease inhibitor in the direct vicinity of *aprA* (Fig. 1B; Supplementary Fig. S1). We hypothesize that the *aprA* genes that are surrounded by both protease inhibitor and secretion system genes are likely to code for a protease with a function similar to that of AprA from *P. aeruginosa*.

AprA of *P. syringae* pv. *tomato* DC3000 is able to cleave flagellin.

Based on the operon structure of the *aprA*-containing operon, we postulated that DC3000 should be able to cleave flagellin molecules. To check this, the DC3000 *aprA* gene was fused to a His-tag and cloned into *Escherichia coli*, after which His-tagged AprA protein was purified. Subsequently, *P. aeruginosa* flagellin monomers were treated in vitro for 30 min with purified His-tagged DC3000 AprA and assessed for integrity by Western blot analysis. Both N-terminal and C-terminal His-tagged DC3000 AprA were able to cleave flagellin monomers in a way similar to *P. aeruginosa* AprA (Fig. 2) (Bardoel et al. 2011). To investigate whether DC3000 AprI was able to inhibit protease activity of DC3000 AprA, purified His-tagged DC3000 AprI was tested. Indeed, DC3000 AprI strongly inhibited the DC3000 AprA-mediated degradation of flagellin. Interestingly,
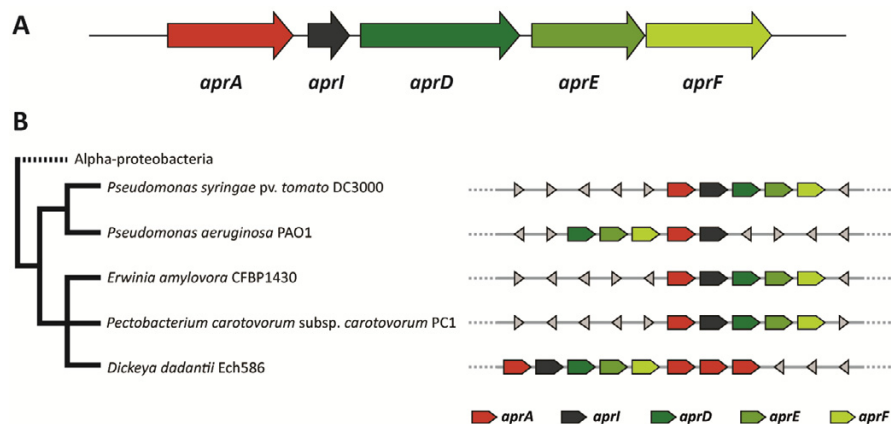
Fig. 1. AprA is present in highly divergent pathogenic bacteria. **A,** Schematic representation of the *aprA* operon in the genome of *Pseudomonas syringae* pv. *tomato* DC3000. In addition to *aprA*, the *aprI* gene encoding the AprA inhibitor AprI and the type I secretion system genes *aprD*, *aprE*, and *aprF* are present. **B,** Genes surrounding the *aprA* homologs in the genomes of DC3000 (NP_793115), *P. aeruginosa* PAO1 (NP_249940), *Erwinia amylovora* CFBP1430 (YP_003532934), *Pectobacterium carotovorum* subsp. *carotovorum* PC1 (YP_003017132), and *Dickeya dadantii* Ech586 (YP_003333619). Genes that encode AprA homologs, AprI-like protease inhibitors, or type I secretion proteins are depicted in different colors. All other genes are shown in gray. The phylogenetic tree represents species taxonomy.

purified AprI from *P. aeruginosa* did not inhibit DC3000 AprA activity, confirming previous observations that AprI activity is highly specific (Bardoel et al. 2012). The fact that both AprA from *P. aeruginosa* and AprA from DC3000 are able to cleave flagellin monomers suggests that this is a common feature for AprA homologs and may represent a conserved function of this protein in host immune evasion by bacterial pathogens.

## AprA is an important virulence factor for *P. syringae* pv. *tomato* DC3000 on tomato.

Because bacterial flagellin monomers are a substrate for DC3000 AprA, we hypothesized that AprA may function in the evasion of pathogen detection by the host plant. *P. syringae* pv. *tomato* DC3000 is the causal agent of bacterial speck disease on tomato (Preston 2000). To test the role of DC3000 AprA in pathogen virulence, a DC3000 Δ*aprA* knockout mutant was constructed by exchanging the wild-type *aprA* gene with a dysfunctional Δ*aprA* gene in which a carbenicillin (carb) resistance cassette was inserted by homologous recombination (Supplementary Fig. S2A). In addition, the DC3000 Δ*aprA* mutant was complemented with the AprA coding region under control of the constitutive *nptII* promoter (designated Δ*aprA*+pN). The genotypes were confirmed using polymerase chain reaction (PCR). To test for AprA protease production, secretion, and activity, the different DC3000 strains were grown on tryptic soy agar containing milk powder. In this protease activity assay, wild-type DC3000 produced a halo and the formation of this halo could be blocked by the addition of DC3000 AprI, indicating that this halo is AprA dependent. DC3000 Δ*aprA* did not produce a halo in this protease activity assay, indicating that it does not produce active AprA. Halo production was restored to wild-type levels in the complementation DC3000 strain Δ*aprA*+pN. This also demonstrates that the production of the type I secretion system proteins AprD, AprE, and AprF, of which the corresponding coding genes are located downstream of *aprA* in the same operon, was not affected in the DC3000 Δ*aprA* knockout mutant.

To test the role of DC3000 AprA during bacterial infection of tomato, leaflets of 5-week-old tomato plants ('Moneymaker') were inoculated with DC3000 or DC3000 Δ*aprA* and symptom development and bacterial growth was monitored. Symptoms on two opposing tomato leaflets 7 days after inoculation with DC3000 (left leaflet) or DC3000 Δ*aprA* (right leaflet) are shown in Figure 3A. Inoculation with DC3000 Δ*aprA* resulted in the development of less-severe symptoms, which was indicated by a reduced amount of cell death and chlorosis compared with that observed in leaflets that were inoculated with DC3000. In addition, growth of DC3000 Δ*aprA* was significantly reduced compared with that of wild-type DC3000 (Fig. 3B). At 4 days after infection, a fourfold difference in bacterial growth could be observed, while this difference increased to almost 10-fold on day 7 after inoculation (Fig. 3B). These results indicate that the AprA protease is an important virulence factor for DC3000 during pathogenesis on tomato.

Inactivation of flagellin by AprA is important for bacterial virulence on *A. thaliana*.

Flagellin is a ubiquitous MAMP of bacterial pathogens that is recognized by the FLS2 receptor to confer basal immunity in plants (Ronald and Beutler 2010; Zipfel et al. 2004). In *A. thaliana*, FLS2-mediated closure of stomata is an early immune response that is effective when DC3000 enters the leaves via these natural openings, such as when plants are dip inoculated (Zeng and He 2010; Zipfel et al. 2004). Previously, it was shown that, upon pressure infiltration, growth of DC3000 in mutant *fls2* plants does not differ from that in wild-type plants (Zipfel et al. 2004), suggesting that flagellin recognition does not contribute

to the activation of post-invasion immune responses triggered by wild-type DC3000. We hypothesized that this is caused by DC3000 AprA activity that degrades elicitor-active flagellin, resulting in a redundant role for FLS2 in the inhibition of DC3000 growth once the pathogen is in the intercellular spaces. We reasoned that, if DC3000 Δ*aprA* lacks the flagellin-degrading activity of AprA, it should evoke enhanced FLS2-dependent immune responses, resulting in inhibition of pathogen growth in pathogen-infiltrated leaves. To test this hypothesis, we pressure infiltrated Col-0 and *fls2* with wild-type DC3000 and mutant DC3000 Δ*aprA*. As expected, no significant differences in growth of wild-type DC3000 could be observed in Col-0 and *fls2* plants (Fig. 3C), confirming previous findings (Zipfel et al. 2004). Interestingly, infiltration of Col-0 plants with DC3000 Δ*aprA* resulted in a significantly lower bacterial growth than in plants infiltrated with the wild-type DC3000 strain (Fig. 3C), possibly because the AprA-deficient DC3000 Δ*aprA* strain evoked a stronger defense response than wild-type DC3000. In the flagellin receptor mutant *fls2*, no significant difference could be observed between growth of DC3000 andvDC3000 Δ*aprA*. These results suggest that the reduced growth of DC3000 Δ*aprA* in Col-0 is at least partly mediated through flagellin recognition by the plant and, thus, that AprA in wild-type DC3000 is involved in evasion of recognition by the FLS2 receptor when colonizing the intercellular spaces. Although no significant differences could be observed between *fls2* plants inoculated with DC3000 and DC3000 Δ*aprA*, a small difference in growth between both *P. syringae* pv. *tomato* strains was consistently observed. Additionally, the difference in growth of DC3000 Δ*aprA* in Col-0 versus *fls2* was relatively minor. This might be caused by the fact that DC3000 produces multiple MAMPs (e.g., EF-Tu) (Kunze et al. 2004), which may also be sensitive to degradation by AprA. The complemented strain *P. syringae* pv. *tomato* DC3000 Δ*aprA*+pN, which produced similar amounts of AprA in the protease activity plate assay, displayed a similar level of virulence as wild-type DC3000 (Fig. 3D), indicating that AprA function in this strain was restored. Together, these results suggest that AprA is important for full virulence of DC3000 on *A. thaliana*.
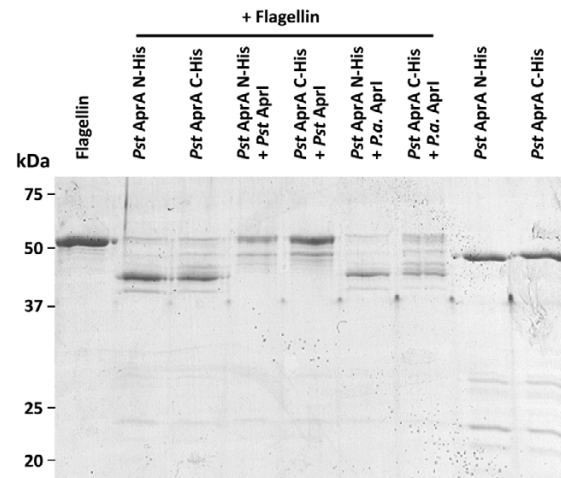
Fig. 2. Protease activity of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) AprA on bacterial flagellin. Flagellin at 250 μg ml⁻¹ was treated for 30 min with DC3000 AprA at 10 μg ml⁻¹ fused to an N- or C-terminal His-tag. The AprA inhibitor AprI from DC3000 or *P. aeruginosa* (*Pa*) was added (10 μg ml⁻¹) simultaneously with AprA when indicated. AprA protease activity on flagellin was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Coomassie staining. Untreated monomeric *P. aeruginosa* flagellin (250 μg ml⁻¹) runs at 52 kDa in lane 1. In lanes 8 and 9, purified DC3000 AprA N-His (250 μg ml⁻¹) and DC3000 AprA C-His (250 μg ml⁻¹) are loaded as controls.

AprA delays the activation of plant defense responses.

When bacteria effectively degrade their own spill of flagellin monomers by producing AprA, they can escape detection by the host immune system. Following this rationale, we hypothesized that DC3000 Δ*aprA* would be better recognized by *A. thaliana* than wild-type DC3000, and that this difference would at least partly be mediated by FLS2. To test this, Col-0 was pressure-infiltrated with wild-type DC3000 or mutant DC3000 Δ*aprA*, after which we monitored the expression of the flagellin-responsive defense-related genes *FRK1* and *PR-1* in both wild-type Col-0 and mutant *fls2* plants. The MAMP-responsive gene *FRK1* was activated in Col-0 plants by wild-type DC3000 (Fig. 4A). However, *FRK1* was activated to a significantly higher level upon inoculation by mutant DC3000 Δ*aprA*. When *fls2* mutant plants were inoculated with these bacterial strains, this difference in *FRK1* gene activation was not observed. In Col-0, the defense-related gene *PR-1* was also activated to a significantly higher level by DC3000 Δ*aprA* compared with DC3000, whereas this difference was not observed in mutant *fls2* (Fig. 4B). These results corroborate the findings shown in Figure 3 and point to a role for DC3000 AprA in reducing the activation of host defenses that are mediated via the flagellin receptor FLS2.

Ectopic expression of the bacterial AprA inhibitor AprI in *A. thaliana* confers enhanced disease resistance against *P. syringae* pv. *tomato* DC3000.

To further validate the importance of AprA for full virulence of DC3000, we constructed transgenic *A. thaliana* lines over-expressing the bacterial AprA inhibitor AprI. Purified DC3000 AprI is able to inactivate DC3000 AprA activity in vitro and in vivo (Fig. 2). Therefore, we hypothesized that overexpression of DC3000 AprI in planta would inactivate DC3000 AprA and subsequently lead to a stronger recognition by the host and, thus, enhanced disease resistance. To test this, we constructed transgenic *A. thaliana* plants in which DC3000 AprI was overexpressed and targeted to the plant apoplast. Using the constitutively expressed *Cauliflower mosaic virus 35S* promoter, two different constructs were made, one encoding AprI with its native bacterial signal peptide (SP) (designated *35Sp:aprI* [SP AprI]) and one with the SP of PR-1 from *A. thaliana* (designated *35Sp:aprI* [SP PR-1]). Wild-type *A. thaliana* and AprI-expressing *A. thaliana* lines were inoculated with DC3000 and DC3000 Δ*aprA*. DC3000 Δ*aprA* performed significantly less well on Col-0 than DC3000 (Fig. 5), confirming the findings presented in Figure 3. Interestingly, the growth rate of DC3000 on the AprI-overexpressing lines was significantly reduced and reached a level similar to that of DC3000 Δ*aprA*. In the AprI-overexpressing plants, growth of DC3000 Δ*aprA* was similar to that in Col-0 and no difference in growth could be observed between DC3000 and DC3000 Δ*aprA*, suggesting that plant-produced AprI does not directly act on bacterial growth but via an effect on bacterially produced AprA. These results show that ectopic expression of bacterial *aprI* in *A. thaliana* affects virulence of AprA-producing DC3000 but not that of mutant DC3000 Δ*aprA*. This further strengthens our notion that AprA is an important virulence factor involved in evasion of host immunity. These results also indicate that production of the
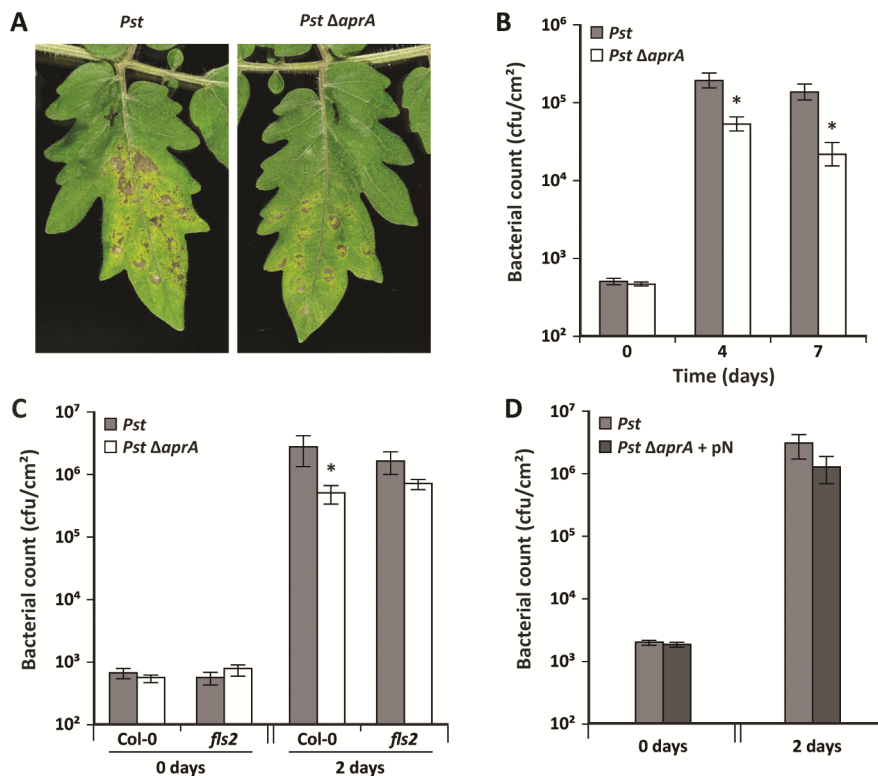
Fig. 3. AprA is important for full virulence of *Pseudomonas syringae* pv. *tomato* DC3000. **A,** Bacterial speck disease symptoms on tomato caused by DC3000 and DC3000 Δ*aprA*, 7 days postinoculation. Five-week-old 'Moneymaker' tomato plants were pressure infiltrated with DC3000 or DC3000 Δ*aprA* in two opposing leaflets of the same tomato leaf. **B,** Growth of DC3000 and DC3000 Δ*aprA* in tomato leaves. The number of bacteria in the intercellular space of pressure-infiltrated tomato leaves was determined directly after and 4 and 7 days after inoculation. Asterisks indicate statistically significant differences (Student's *t* test, $P < 0.05$). **C,** Effect of AprA on bacterial proliferation in *Arabidopsis thaliana* wild-type Col-0 and the flagellin receptor mutant *fls2*. Leaves of Col-0 and *fls2* were pressure-infiltrated with DC3000 or DC3000 Δ*aprA*. Bacterial counts were determined directly after inoculation and two days later. An asterisk indicates a statistically significant difference between DC3000 and DC3000 Δ*aprA* (Kruskal-Wallis test followed by pairwise comparisons; $P < 0.05$). **D,** Complementation of DC3000 Δ*aprA*. Col-0 plants were inoculated with DC3000 or DC3000 Δ*aprA* mutant bacteria complemented with *aprA* under control of the constitutive *nptII* promoter (pN). Bacterial proliferation was determined 2 days after inoculation. No significant differences could be observed (analysis of variance followed by Fisher's least significant difference test; $P < 0.05$). Experiments were repeated with similar results.

AprA inhibitor AprI by plants is an effective strategy to suppress bacterial infections.

DISCUSSION

Many bacteria produce and secrete proteases. The proteases produced by pathogenic bacteria are often associated with bacterial virulence, especially the zinc metalloproteases (Miyoshi and Shinoda 2000). A famous example of this is the zinc metalloprotease anthrax toxin lethal factor produced by *Bacillus anthracis*, of which the protease catalytic site is required for its lethal activity (Klimpel et al. 1994). Also, *P. aeruginosa* secretes several proteases that are involved in bacterial virulence, one of which is AprA. AprA has been shown to degrade the human cytokines interferon γ and tumor necrosis factor α that are important for host immune activation (Parmely et al. 1990). Furthermore, AprA of *P. aeruginosa* has been shown to degrade monomeric flagellin molecules that are recognized by the immune receptors TLR5 and FLS2 in human and plant cells, respectively (Bardoel et al. 2011). During growth in their hosts, flagellin monomers can surround the bacteria due to spills during flagellum construction or due to damaging of the flagellar filaments (Gomez-Gomez and Boller 2002; Komoriya et al. 1999). Hence, degradation of flagellin by bacterial AprA would be an effective mechanism of bacterial pathogens to evade flagellin-mediated recognition by the host immune system. Here, we demonstrated that AprA is, indeed, important for full virulence of the bacterial plant pathogen *P. syringae* pv. *tomato* DC3000. The Δ*aprA* mutant of DC3000 showed reduced growth in tomato and *A. thaliana* (Fig. 3) and triggered a significantly stronger expression of the defense-related genes *FRK1* and *PR-1* compared with wild-type DC3000 bacteria (Fig. 4). The fact that this reduction in pathogen growth and enhanced defense-related gene expression was not observed in the flagellin receptor mutant *fls2* suggests that flagellin is an important target of AprA in this immune evasive process.

When *A. thaliana* wild-type or *fls2* mutant plants were inoculated with wild-type DC3000 using pressure infiltration, no differences in bacterial growth were found (Fig. 3C), confirming previous findings (Zipfel et al. 2004). We hypothesized that this may be caused by inactivation of flagellin by DC3000 AprA in the intercellular space, rendering the role of FLS2 in pathogen detection in this stage of the infection to be redundant. When DC3000 Δ*aprA* was pressure infiltrated in Col-0, the pathogen grew significantly less well compared with wild-type DC3000; whereas, in mutant *fls2*, no significant difference in growth between DC3000 and DC3000 Δ*aprA* could be observed (Fig. 3C). This suggests that DC3000 Δ*aprA* is detected in the intercellular space in an FLS2-dependent manner, while wild-type DC3000 is not, pointing to a role for AprA in the evasion of FLS2-mediated pathogen detection. Despite the fact that growth of DC3000 and DC3000 Δ*aprA* did not differ significantly on mutant *fls2* plants, DC3000 Δ*aprA* consistently showed a small reduction in growth on *fls2* compared with wild-type DC3000. Also, the difference in growth of DC3000 Δ*aprA* on Col-0 versus *fls2* was relatively minor (Fig. 3C). Because DC3000 produces multiple proteinaceous MAMPs (e.g., EF-Tu) (Kunze et al. 2004), we hypothesize that degradation of one or more of these MAMPs by AprA may also contribute to the observed AprA-mediated effects on DC3000 virulence.

Escaping recognition is important for microbes with a pathogenic lifestyle and, because MAMPs such as bacterial flagellin are recognized by host PRR, they are under selective pressure (Pel and Pieterse 2013). However, changes in the conserved part of flagellin that is recognized by the FLS2 receptor result in loss of bacterial mobility (Smith et al. 2003); hence, it is not illogical that bacteria evolved other means by which they can escape from flagellin-mediated recognition by the host. AprA is able to degrade spilled flagellin molecules, which prevents recognition by the host and leads to enhanced bacterial virulence without affecting the bacterial mobility (Bardoel et al.
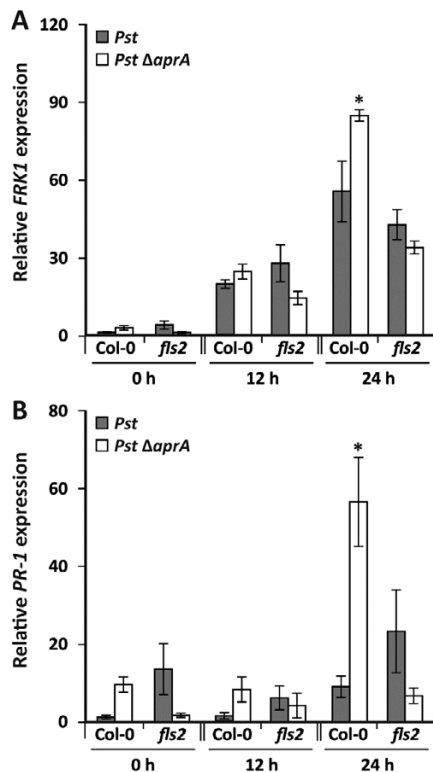
Fig. 4. Role of DC3000 AprA in evasion of defense-related gene activation in *Arabidopsis thaliana*. Quantitative reverse-transcriptase polymerase chain reaction analysis of the defense-related genes **A,** *FRK1* and **B,** *PR-1* in 5-week-old Col-0 and *fls2* plants that were inoculated with DC3000 or DC3000 Δ*aprA*. Leaf tissue was harvested for RNA analysis at 0 and 24 h after inoculation. Gene expression levels are relative to the expression at $t = 0$ in Col-0. Asterisks indicate statistically significant differences (analysis of variance followed by Fisher's least significant difference test; $P < 0.05$). Experiments were repeated with similar results.
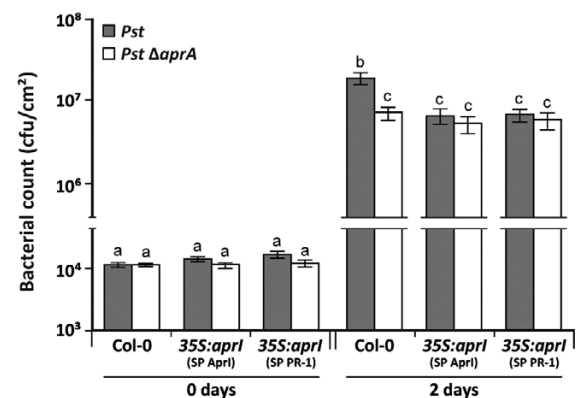
Fig. 5. Effect of ectopic expression of AprA inhibitor AprI in *Arabidopsis thaliana* on disease resistance. Data represent growth of *Pseudomonas syringae* pv. *tomato* DC3000 in leaves of transgenic *35Sp:aprI* (SP AprI) and *35Sp:aprI* (SP PR-1) *A. thaliana* plants that overexpress the *aprI* gene that encodes the AprA inhibitor AprI of DC3000 with its own signal peptide (SP AprI) or with the signal peptide of PR-1 (SP PR-1). Leaves of Col-0- and *aprI*-overexpressing plants were inoculated with DC3000 or DC3000 Δ*aprA*. Bacterial counts were determined directly after pressure infiltration and 2 days later. Different letters indicate statistically significant differences (Kruskal-Wallis test followed by pairwise comparisons; $P < 0.05$). Experiments were repeated with similar results.

2011). Therefore, this active immune evasive strategy may be widespread among bacteria. This is supported by the fact that bacterial pathogens from many different taxonomic groups possess homologous *aprA* genes (Fig. 1). In addition to bacterial pathogens, many nonpathogenic bacteria also were shown to possess homologs of *aprA* (e.g., the biological control strain *P. fluorescens* SBW25 and the nitrogen-fixing *Ensifer meliloti* 1021 that live in close contact with host organisms) (Galibert et al. 2001; Silby et al. 2009). This suggests that AprA-mediated immune evasion might also play a role in the interaction between plants and beneficial bacteria.

The AprA inhibitor gene *aprI* is encoded by the same operon as *aprA* in the majority of the bacterial species in which an *aprA* homolog was identified (Fig. 1B). Based on its SP, AprI is predicted to be transported to the periplasmic space and, for *D. dadantii*, this cellular localization has been confirmed (Létoffé et al. 1990). However, AprA is transported over the two bacterial membranes at once by the type I secretion system. Therefore, AprI is theoretically never in contact with AprA and, thus, cannot bind this protease to inhibit its function. Hence, the function of AprI for the bacterium remains unclear. Because AprA is important for full virulence of DC3000 on both *A. thaliana* and tomato and possibly for many plant–pathogen interactions with AprA producing bacterial pathogens, we hypothesized that AprI could serve as an important tool to enhance the level of resistance in plants to these bacterial pathogens. Indeed, production of AprI by transgenic *A. thaliana* plants reduced growth of DC3000 in the intercellular space but not that of DC3000 Δ*aprA* (Fig. 5), confirming our notion that AprA is important in preventing pathogen detection by the plant immune system.

Inactivating bacterial AprA by AprI appears to be a good strategy to enhance the level of disease resistance against AprA-producing pathogens. Whether AprI can be successfully used against different bacterial infections depends largely on the specificity of this protease inhibitor. It has been shown that AprI of *D. dadantii* can not only inhibit the three proteases produced by itself but also inhibit AprA of *S. marcescens*, which is an opportunistic pathogen on humans (Létoffé et al. 1989). However, AprI of DC3000 cannot inhibit AprA of *P. aeruginosa* and vice versa (Fig. 2) but the exchange of only a few residues of AprI from DC3000 and *P. aeruginosa* can abolish the observed specificity (Bardoel et al. 2012). Hence, developing AprI-based strategies may provide novel tools to protect plant and mammalian hosts against infection by AprA-producing bacterial pathogens.

MATERIALS AND METHODS

Bacterial strains and plasmids used.

The following bacterial strains and plasmids were used: *P. syringae* pv. *tomato* DC3000 (50 µg of rifampicin per milliliter [rif[50]]) (Whalen et al. 1991), DC3000 Δ*aprA* (rif[50], 100 µg of carbenicillin per milliliter [carb[100]]), DC3000 Δ*aprA* + pBBR1MCS-5_*aprAp*:*aprA* (rif[50], carb[100], 50 µg of gentamicin per milliliter [gen[50]]), DC3000 Δ*aprA* + pBBR1MCS-5_*nptIIp*:*aprA* (rif[50], carb[100], gen[50]), *Escherichia coli* Rosetta gami (DE3) pLysS (30 µg of chloramphenicol per milliliter, 50 µg of streptomycin per milliliter [str[50]], 10 µg of tetracycline per milliliter) (Novagen, Darmstadt, Germany), *E. coli* DH5α (Bethesda Research Laboratories 1986), *E. coli* S17-1 λpir (Herrero et al. 1990), *E. coli* HB101 pRK2073 (50 µg of spectinomycin per milliliter) (Boyer and Roulland-Dussoix 1969; Figurski and Helsinki 1979), *Agrobacterium tumefaciens* EHA105 pJIC.SaRep, pUC4K (100 µg of ampicillin per milliliter, [kan[25]]) (Vieira and Messing 1982), pCR-BluntII-TOPO (kan[25]; Life Technologies, Carlsbad, CA, U.S.A.), pKNG101 (str[50]) (Kaniga et al. 1991) and pBBR1MCS-5 (gen[50]) (Kovach

et al. 1994), pFAST-R02 and pFAST-R05 (Shimada et al. 2010), and pENTR⁄D-TOPO (Life Technologies).

Identification of AprA homologs.

To identify homologs of DC3000 AprA (National Center for Biotechnology Information NP_249940), a pBLAST (Altschul et al. 1990) with AprA from D 100 µg/ml ampicillinC3000 against the RefSeq database was performed. The best thousand hits were checked for the presence of the motifs HEXXHXXG XXH and SXMSY (Miyoshi and Shinoda 2000). For the available genomes the eight genes up- and downstream of the AprA homolog were checked, using MGV2 (Kerkhoven et al. 2004) or manually, for the presence of serralysin-like proteins, protease inhibitors, and genes encoding proteins involved in protein secretion.

AprA and AprI protein purification and flagellin degradation.

The *aprA* and *aprI* genes from DC3000 were cloned into the plasmid pET302 and pRSETB, respectively, and transformed to *E. coli* Rosetta gami (DE3) pLysS (Novagen), as described (Bardoel et al. 2012). The recombinant proteins were purified using a His-trap column under denaturing (AprA) or native (AprI) conditions. AprA was renatured, as described previously (Bardoel et al. 2011), and proteins were dialyzed against phosphate-buffered saline (PBS). AprI from *P. aeruginosa* was produced as described (Bardoel et al. 2011). Recombinant flagellin from *P. aeruginosa* was purified as described (Bardoel et al. 2011). Flagellin (250 µg ml⁻¹) was treated with DC3000 AprA at 10 µg ml⁻¹ in PBS for 30 min at 37°C with or without AprI at 10 µg ml⁻¹.

Construction of the *P. syringae* pv. *tomato* DC3000 Δ*aprA* knockout mutant.

The *aprA* gene was isolated and cloned into pCR-BluntII-TOPO (Life Technologies). An amp/carb resistance cassette was isolated from pUC4K (Taylor and Rose 1988) and was then cloned into the *aprA* gene in pCR-BluntII-TOPO. The *aprA* gene with the amp/carb resistance cassette inserted was then cloned from pCR-BluntII-TOPO to pKNG101 (Kaniga et al. 1991) and the resulting plasmid was introduced into *E. coli* S17-1 λpir. Next, the pKNG101 plasmid was introduced into DC3000 using triparental mating to create DC3000 Δ*aprA*. This was confirmed by PCR. For complementation of *aprA* in DC3000 Δ*aprA*, the coding sequence of *aprA* was cloned by PCR (Phusion, Bioké, Leiden, The Netherlands) from DC3000 genomic DNA. The resulting product was cloned into the broad-host-range vector pBBR1MCS-5. The *nptIIp*:*aprA* complementation construct was created by insertion of the *nptII* promoter fragment, obtained from pUC4KIXX. DC3000 Δ*aprA* was transformed with these plasmids by triparental mating.

Assessment of protease activity.

Overnight cultures of wild-type DC3000 and mutant DC3000 Δ*aprA* (in King's B [KB] medium at 28°C) were centrifuged at 4,000 rpm and resuspended in 10 mM MgSO₄ to an optical density at 600 nm (OD₆₀₀) = 0.01 (1 × 10⁷ CFU ml⁻¹). Of each strain, a 1-µl droplet of the bacterial suspension was placed on tryptic soy agar containing skimmed milk powder (Elk, Campina, Amersfoort, The Netherlands) at 0.01 g ml⁻¹. Where indicated, a 50-µl droplet of a 20-µg ml⁻¹ AprI solution was placed preceding bacterial inoculation. Subsequently, the bacteria were grown at 16°C for 4 days.

Cultivation of plants.

Arabidopsis thaliana seed (Col-0 or *fls2* [Col-0] SALK_141277; Shan et al. 2008) were sown on quartz sand. Two-

week-old seedlings were transferred to 60-ml pots containing a sand-potting soil mixture that had been autoclaved twice for 20 min with a 24-h interval (Van Wees et al. 2013). Plants were cultivated in a growth chamber with a cycle of 10-h days (100 μE m^{-2} s^{-1} at 21°C) and 14-h nights (20°C) at 70% relative air humidity (RH). For the tomato experiments, *Solanum lycopersicum* 'Moneymaker' was used. Seed were sown in 500-ml pots containing potting soil and placed in a growth chamber with a cycle of 16-h days (200 μE m^{-2} s^{-1} at 21°C) and 8-h nights (20°C) at 70% RH. Plants were watered regularly and supplied with modified half-strength Hoagland nutrient solution once a week (Hoagland and Arnon 1938).

Construction of AprI-overexpressing *A. thaliana* plants.

DC3000 *aprI* was isolated and cloned into pENTR⁄D-TOPO vector generating pENTR_D-TOPO-*aprI*-stop. Similarly, pENTR_D-TOPO-*aprI*-fusion was generated, allowing in-frame fusion of *aprI* to *GFP*. Both constructs with the native SP of AprI and constructs with *A. thaliana* PR-1 SP (SP PR-1; GenBank At2G14610) replacing the native SP were made. The SP PR-1 was amplified by PCR from *A. thaliana* Col-0 and fused in frame with the mature AprI protein-coding region in plasmids pENTR_D-TOPO-AprI-stop and pENTR_D-TOPO-AprI-fusion. Adapted *aprI* sequences were transferred to pFAST-R02 and pFAST-R05 vectors (Shimada et al. 2010). Plasmids were transferred to *Agrobacterium tumefaciens* using electroporation. *A. thaliana* Col-0 plants were transformed with *Agrobacterium tumefaciens* EHA105 (with pJIC.SaRep plasmid) using the floral dip method (Clough and Bent 1998). Transformed seed were selected based on red fluorescent protein expression using a fluorescence stereomicroscope (MZ16FA; Leica, Wetzlar, Germany).

Bacterial infections.

P. syringae pv. *tomato* DC3000 strains were grown for 24 h in liquid KB medium (King et al. 1954), supplemented with the appropriate antibiotics. Bacteria were collected and resuspended in 10 mM $MgSO_4$ to the proper OD_{600}. For DC3000 infiltration assays in *A. thaliana* ($OD_{600} = 0.0005$), leaves of 4-week-old plants were pressure infiltrated using a syringe without a needle (Van Wees et al. 2013). Plants were placed at 100% RH. Leaf discs were taken within 2 h after inoculation ($t = 0$; $n = 4$) and 2 days after inoculation ($t = 2$; $n = 8$). Subsequently, the leaf discs were ground and 30-μl aliquots of dilutions were plated onto KB plates containing rifampicin at 50 μg ml^{-1}. After 40 to 48 h of incubation at 28°C, bacterial colonies were counted. Infiltration assays with tomato plants were performed as described for *A. thaliana*, with some modifications. Opposing leaflets of the same leaf of 5-week-old plants were infiltrated with different DC3000 strains and, per plant, two leaves (four leaflets) were infiltrated. Leaf discs were collected within 2 h after inoculation ($t = 0$; $n = 3$), 4 days after inoculation ($t = 4$; $n = 3$), and 7 days after inoculation ($t = 7$; $n = 3$). For gene expression analysis, plants were dip inoculated with a bacterial suspension ($OD_{600} = 0.025$) containing 0.015% (vol/vol) Silwet L-77 (Van Meeuwen Chemicals, Weesp, The Netherlands). Leaf material was harvested directly after inoculation ($t = 0$; $n = 3$) and 24 h after inoculation ($t = 24$; $n = 3$).

Gene expression analysis.

Total RNA from leaf material was isolated as described by Van Wees and associates (1999). Fermentas RevertAid H minus reverse transcriptase (Fermentas, St. Leon-Rot, Germany) was used for cDNA synthesis according to the manufacturer's instructions. Quantitative real-time PCR of the defense-related genes *FRK1* (At2G19190) and *PR-1* (At2G14610) was performed as described by Verhage and associates (2011). The following primers were used: *FRK1* forward 5′-TTT CAA CAG TTG TCG CTG GA-3′, *FRK1* reverse 5′-AGC TTG CAA TAG CAG GTT GG-3′, *PR-1* forward 5′-CTC GGA GCT ACG CAG AAC AAC T-3′, *PR-1* reverse 5′-TTC TCG CTA ACC CAC ATG TTC A-3′, *PP2AA3* (AT1G13320) forward 5′-TAA CGT GGC CAA AAT GAT GC-3′, and *PP2AA3* reverse 5′-GTT CTC CAC AAC CGC TTG GT-3′. The expression of *PP2AA3* (At1G13320) was used for normalization (Czechowski et al. 2005; Hong et al. 2010).

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