Amino Acid Sequence of Bacterial Microbe-Associated Molecular Pattern flg22 Is Required for Virulence

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Flagellin proteins derived from *Pseudomonas syringae* pv. *tabaci* 6605 and flg22_{Pa} (QRLSTGSRINSAKDDAAGLQIA), one of the microbe-associated molecular patterns (MAMP) in bacterial flagellin, induce cell death and growth inhibition in *Arabidopsis thaliana*. To examine the importance of aspartic acid (D) at position 43 from the N-terminus of a flagellin in its elicitor activity, D43 was replaced with valine (V) and alanine (A) in *P. syringae* pv. *tabaci* flagellin and flg22_{Pa}. The abilities of flagellins from *P. syringae* pv. *tabaci* D43V and D43A to induce cell death and growth inhibition were reduced, whereas the abilities of flg22_{Pa}D43V and flg22_{Pa}D43A were abolished. These results indicate that D43 is important for elicitor activity in *P. syringae* pv. *tabaci*. When tobacco plants were inoculated with each bacterium by the spray method, both *P. syringae* pv. *tabaci* D43V and D43A mutants had remarkably reduced ability to cause disease symptoms. Both mutants had reduced or no swimming and swarming motilities and adhesion ability. In *P. syringae* pv. *tabaci* D43V, little flagellin protein was detected and few flagella were observed by electron microscopy. These results indicate that mutant flagella are unstable and that flagellar motility is impaired. Thus, the amino acid residue required for MAMP activity is important for the intrinsic flagellar function.

Additional keywords: flagellin glycosylation, FLS2, PAMPs.

Plants have evolved two types of defense systems against invasion by pathogens (Bent and Mackey 2007). One of the systems is based on the gene-for-gene theory and occurs as a result of the direct or indirect interactions between avirulence (Avr) proteins produced by pathogens and resistance (R) gene products of host plants. Another is an innate immune system triggered by recognition of the molecules present in microbes independently of incompatible race-cultivar interactions. These molecules were originally termed elicitors and recently are called pathogen-associated molecular patterns (PAMPs). PAMPs include fungal chitin and ergosterol, oomycete-derived β -glucans and transglutaminase, bacterial lipopolysaccharide (LPS), cold-shock protein, elongation factor Tu, and flagellin (Bittel and Robatzek 2007; Nürnberger et al. 2004). Because PAMPs do not always originate from pathogenic microbes,

they should be designated microbe-associated molecular patterns (MAMPs). The molecules comprising MAMPs are abundant in microbes. Thus, most MAMPs recognized by plants correspond to molecules that are essential for microbial life but do not necessarily play a role in pathogenicity (Bittel and Robatzek 2007).

The best-characterized set of a MAMP and MAMP receptor is flg22 in flagellin and FLS2 in *Arabidopsis thaliana*. Flagellin constitutes the flagellar filament and is the most abundant extracellular protein in bacteria. Felix and associates (1999) found an elicitor activity in the N-terminal fragment of flagellin and synthesized a 22-amino-acid peptide (flg22) spanning the conserved domain in gram-negative bacteria based on the sequence of *Pseudomonas aeruginosa* (Fig. 1). Flg22 stimulates defense responses such as alkalinization of the extracellular medium, generation of reactive oxygen species (ROS), pathogenesis-related (PR) gene expression, callose deposition, and growth inhibition in *Arabidopsis* and tomato (Felix et al. 1999; Gómez-Gómez et al. 1999). Functional dissection of flg22 revealed that flg15, the C-terminal part of flg22, was the smallest and most active peptide in tomato but not in *Arabidopsis* (Felix et al. 1999). Furthermore, several structural analogues of flg15 with replacements of a single amino acid residue with alanine revealed that the sequence Asp-Asp-Ala (43rd to 45th from the N-terminus) in the middle of flg15 is important for alkalinization-inducing activity in suspension-cultured tomato cells (Felix et al. 1999). Perception of flg22 is mediated by its receptor molecule FLS2 in *Arabidopsis* (Chinchilla et al. 2006; Gómez-Gómez and Boller 2000). FLS2 has an extracellular leucine-rich repeat (LRR) domain and an intracellular protein kinase domain, one of the typical MAMP recognition receptors. In the recognition of flagellin, the first step is binding the N-terminus of flg15 to the receptor, and the second step is activation of responses from the C-terminus (Meindl et al. 2000). The Asp-Asp-Ala sequence was also shown to be indispensable for binding activity (Meindl et al. 2000).

Recently, it was reported that an amino acid sequence for flg22 in *Xanthomonas campestris* pv. *campestris* is important for the defense response in *Arabidopsis* (Sun et al. 2006). Bacterial strains such as *X. campestris* pv. *campestris* B305, the flagellin of which has D43, elicited *Arabidopsis* defense responses, whereas strains such as *X. campestris* pv. *campestris* B186 with V43 did not. Thus, inoculation of *Arabidopsis* Col-0 plants with these *X. campestris* pv. *campestris* strains by vacuum infiltration resulted in severe disease symptoms in B186-inoculated leaves but not in B305-inoculated leaves. Sun and associates (2006) investigated indispensable amino acid residues for flagellin-triggered defense responses in *Arabidop-*

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sis using various kinds of recombinant flagellin polypeptides and found that a single amino acid, D43, was a key determinant of elicitor activity. However, bacterial propagation of B305 and B186 is independent of the presence of *FLS2*. Namely, B186 propagated well in both Col-0 and the *fls2* mutant to the same extent, whereas B305 propagated less well in both Col-0 and the *fls2* mutant, also to the same extent. As one of the explanations of this phenomenon, Sun and associates (2006) hypothesized that *X. campestris* pv. *campestris* bacteria do not produce enough flagellin monomers in leaves to induce the host defense response. Indeed, the motility of all *X. campestris* pv. *campestris* strains used in this study was very low compared with that of *P. syringae* pv. *tomato*. Therefore, it was difficult to evaluate the importance of D43 in flagellin in interactions between plants and motility-impaired bacteria.

We have also found elicitor activity in flagellins from *P. syringae* pv. *tabaci* 6605, pv. *glycinea* race 4, and pv. *tomato* DC3000 (Taguchi et al. 2003b). Furthermore, we found that the flagellins of *P. syringae* pathovars are glycoproteins, and that the glycosylation of flagellin affects its motility, virulence, and ability to induce the hypersensitive response (HR) (Taguchi et al. 2003a, 2006; Takeuchi et al. 2003). Flagellins from *P. syringae* induce HR in nonhost plants (Taguchi et al. 2003b, 2006). However, the amino acid sequences of flagellins from *P. syringae* pathovars are very similar. In particular, the flagellins from *P. syringae* pvs. *tabaci* and *glycinea* have identical amino acid sequences, although each flagellin possesses a distinct elicitor activity on tobacco and soybean (Taguchi et al. 2003b, 2006). These results indicate that glycosylation may determine the species-specific ability to induce HR. The importance of flagellin glycosylation was also supported by a study using recombinant whole-flagellin proteins expressed in *Escherichia coli*. All recombinant flagellins lost their specificity for HR induction (Taguchi et al. 2003b), indicating that they also lost pathovar-specific post-translational modification. Furthermore, a study using recombinant flagellin polypeptides indicated that the N-terminal domain including the conserved flg22 is required for flagellin-induced hypersensitive cell death in *Arabidopsis* (Naito et al. 2007). To evaluate the significance of the conserved amino acids in flg22 for induction of defense response and virulence, the study of recombinant flagellins is not sufficient. It is necessary to examine the effect of amino acid substitutions in flg22 in the bacteria in which flagella are important for virulence. Because *P. syringae* pv. *tabaci* 6605 is highly motile (Taguchi et al. 2006) and we had previously

confirmed that flagellar motility is required for virulence by analysis of the flagella-defective mutants $\Delta fliC$ and $\Delta fliD$ (Ichinose et al. 2003), this pathogen provides a suitable model for the analysis.

Flagellin from *P. syringae* pv. *tabaci* 6605 retains D43 in the flg22 region (Fig. 1). To examine the importance of D43 in the elicitor activity of flagellin, we generated mutant strains in which the 43rd residue (aspartic acid) was replaced with valine (D43V) or alanine (D43A) residues in the flagellin in *P. syringae* pv. *tabaci* 6605 and investigated the virulence of these mutants. We also introduced the mutation into L36 and K37, in which amino acid residues are different between *P. aeruginosa* and *P. syringae* pv. *tabaci*. Based on the results obtained with mutant strains, we discuss the MAMP activity of flagellin and its relationship to the intrinsic function of the flagellum.

RESULTS

Generation of D43-substituted mutants in *P. syringae* pv. *tabaci*.

To investigate the importance of D43 in the flg22 region, the genetic code for D43 (GAT) was converted to valine (GTT) and alanine (GCT). The site-specific mutations of the *fliC* gene in the resultant strains were confirmed by polymerase chain reaction (PCR) and sequencing. Then, the mutated *fliC* gene was introduced into *P. syringae* pv. *tabaci* by conjugation and homologous recombination, as below. The reproduction rate of the mutants did not differ from that of the wild-type strain (Supplementary Fig. 1).

Effect of D43 substitution on induction of cell death in *Arabidopsis* leaves.

To analyze the effect of the amino acid substitution, we assayed cell death in *Arabidopsis* leaves using purified flagellins and flg22 oligopeptides. The left halves of *A. thaliana* ecotype Col-0 leaves were infiltrated with flagellins from *P. syringae* pv. *tabaci* wild type, D43V and D43A mutants, flg22 of *P. aeruginosa* (flg22_{Pa}) and *P. syringae* pv. *tabaci* (flg22_{Pta}), and flg22_{Pta} derivatives (flg22_{Pta}D43V and flg22_{Pta}D43A) by a syringe-infiltration method. After 24 h of treatment with flagellin or flg22, the dead cells in the treated leaves were stained with trypan blue as described below. Areas infiltrated with flg22_{Pa} and flg22_{Pta} at concentrations of 0.1 and 2 μ M were stained with trypan blue, indicating cell death, but the infiltration with mutated oligopeptides flg22_{Pta}D43V and flg22_{Pta}D43A, even at the concentration of 2 μ M, did not cause staining (Fig. 2). On the other hand, treatment with flagellin from *P. syringae* pv. *tabaci* wild-type strain caused dark staining even at 0.1 μ M, indicating much cell death, and treatment with flagellin from *P. syringae* pv. *tabaci* D43V and D43A mutants caused light staining, indicating a low level of cell death at 2 μ M and no staining at 0.1 μ M (Fig. 2). These results suggest that the D43 residue in flg22 is important for the induction of not only seedling growth inhibition and *PR-I* gene expression, as previously reported by Sun and associates (2006), but also cell death.

Growth inhibition by flagellin and flg22 and their derivatives in *Arabidopsis* seedlings.

To examine the effect of the amino acid substitution, we performed a growth inhibition assay on *Arabidopsis* seedlings using flagellin proteins and flg22 oligopeptides. Five-day-old *Arabidopsis* seedlings were treated with 0.1 μ M flagellin or flg22 for 7 days at 24°C, and the seedlings were weighed. The inhibitory effect of flagellins and flg22 on *Arabidopsis* growth was prominent in the treatment with flagellin from *P. syringae* pv. *tabaci* wild-type strain (Fig. 3A and B). The treatments of *Arabidopsis* with the wild-type flagellin, flg22_{Pa} and flg22_{Pta},

	30	43	51
flg15 _{Pa} :		RINS	AKDDAAGLQIA
flg22 _{Pa} :	QRLSTG	SR INS	AKDDAAGLQIA
flg22 _{Pta} L43S:	TRLSSG	SKINS	AKDDAAGLQIA
flg22 _{Pta} K37R:	TRLSSGL	RINS	AKDDAAGLQIA
flg22 _{Pta} :	TRLSSGL	K INS	AKDDAAGLQIA
flg22 _{Pta} D43A:	TRLSSGL	KINS	A DAAGLQIA
flg22 _{Pta} D43V:	TRLSSGL	KINS	V DAAGLQIA
flg22 _{XccB305} :	QRLSSGL	RINS	AKDDAAGLAIS
flg22 _{XccB186} :	QQLSSG	KRITS	ASVDAAGLAIS

Fig. 1. Flg22 and flg22-derived amino acid sequences in gram-negative bacteria. Pta: *Pseudomonas syringae* pv. *tabaci* 6605, Xcc: *Xanthomonas campestris* pv. *campestris*, Pa: *Pseudomonas aeruginosa*. The 43rd amino acid residue of each and 36th and 37th amino acid residues in flg22_{Pa} and flg22_{Pta} are indicated by a bold letter.

strongly inhibited growth, whereas the mutated flg22, flg22_{Pta}D43V and flg22_{Pta}D43A, had no inhibitory activity. The mutated flagellins from D43V and D43A allowed root elongation in water, although the fresh weight of the seedlings was slightly less than that of control seedlings. Comparison of the results of treatments with flg22_{Pa} and flg22_{Pta} indicates that

flg22_{Pa} inhibited *Arabidopsis* growth more effectively. Treatments with flagellins from *P. syringae* pv. *tabaci* wild-type strain, D43-substituted mutant strains, and flg22 oligopeptides with or without D43 substitutions on the *Arabidopsis* fls2 mutant produced no growth inhibition, indicating that the *Arabidopsis* response was dependent on *FLS2* (Fig. 3C and D).

Fig. 2. Flagellin- and flg22-induced cell death in *Arabidopsis thaliana*. The left halves of leaves of *Arabidopsis* ecotype Col-0 were infiltrated with flagellins from *Pseudomonas syringae* pv. *tabaci* wild-type (WT), D43V and D43A mutants, and flg22 derivatives by the infiltration method, and incubated at 22°C for 24 h. Then, the leaves were stained by trypan blue. The concentration of flagellins and flg22 derivatives was 2 μM in the upper panels and 0.1 μM in the lower panels. Photos are representative results obtained from two to four independent experiments. The syringe marks are sometimes stained artificially as dark spots.

Fig. 3. Flagellin- and flg22-induced growth inhibition in *Arabidopsis thaliana*. *Arabidopsis* seedlings of **A** and **B**, Col-0 wild type (WT) and **C** and **D**, Col-0 *fls2* mutant were incubated in Murashige-Skoog liquid medium with 0.1 μM flagellin or flg22 for 7 days. As a control, water was used instead of flagellin or flg22. After 7 days of incubation, representative seedlings were photographed (**A** and **C**). The white bar indicates 1 cm. Fresh weights (means with standard error) of 30 independent seedlings were measured (**C** and **D**).

Flagellin- and flg22-induced activation of senescence-induced receptor-like kinase promoter in *A. thaliana*.

The flg22-induced receptor-like kinase 1 (*FRK1*; At2g19190) is reported to be one of the *Arabidopsis* genes that responds strongly to treatment with flg22 (Asai et al. 2002). Because the expression of *FRK1* is strongly induced during leaf senescence, *FRK1* is also designated *SIRK* (senescence-induced receptor-like kinase) (Robatzek and Somssich 2002). The leaves of transgenic *Arabidopsis* plants expressing the β -glucuronidase (GUS) reporter gene (*uidA* from *E. coli*) under the control of an *AtSIRK* promoter were infiltrated with 0.1 μ M flagellin or flg22. After 24 h of incubation at 22°C, GUS activity in the protein extracts was fluorometrically measured. The wild-type flagellin was the strongest inducer of GUS activity (9 nmol of 4-methylumbelliferone [4-MU] produced per milligram of protein per minute) (Fig. 4A). Infiltration of leaves with water and two mutated flg22, flg22_{Pta}D43V and flg22_{Pta}D43A, did not induce any GUS activity, whereas the leaves infiltrated with mutated flagellins D43V and D43A and flg22 from *P. syringae* pv. *tabaci* and *P. aeruginosa* induced low levels of GUS activity. Among the treatments with flagellins and flg22, the induction of GUS activity by flg22_{Pta}D43V and flg22_{Pta}D43A was lower than that by the other treatments (Fig. 4A). Interestingly we observed that the higher the concentration (2 μ M) of flagellins D43V and D43A used, the higher the GUS activity appeared to be. However the mutated flg22 peptides, flg22_{Pta}D43V and flg22_{Pta}D43A, did not induce GUS activity even at high concentrations (2 and 10 μ M). On the other hand, dose-dependent GUS activity was observed at the concentrations of flg22_{Pa} and flg22_{Pta} between 0.1 and 10 μ M. This suggests that the biologically relevant flagellin protein may interact with the FLS2 receptor in ways that flg22 does not. The histochemical staining of GUS expression in infiltrated leaf disks was consistent with the fluorometric measurement (Fig. 4). Flagellin from *P. syringae* pv. *tabaci* wild-type strain caused dark staining indicating strong expression of the *SIRK* promoter, whereas treatment with water, flg22_{Pta}D43V, and flg22_{Pta}D43A caused no staining, indi-

cating that flg22_{Pta}D43V and flg22_{Pta}D43A are not active elicitors. Furthermore, treatments with flagellins with D43V, D43A, and flg22 from *P. syringae* pv. *tabaci* and *P. aeruginosa* induced light staining, indicating that the elicitor activities of these flagellins and flg22 were lower than that of the wild-type flagellin (Fig. 4B).

Loss of virulence in D43V- and D43A-substituted *P. syringae* pv. *tabaci* mutants.

The evidence that D43 is important for the elicitor activity of flagellin and flg22 led us to speculate that D43V- and D43A-substituted mutants might cause more severe disease symptoms in their host tobacco plant. To investigate the effects of the replacements D43V and D43A on virulence, tobacco leaves were inoculated with *P. syringae* pv. *tabaci* wild-type, D43V, D43A, and Δ *fliC* mutants by the spray method. The wild type caused typical wildfire disease in tobacco, whereas the D43V and D43A mutants did not cause severe disease symptoms (Fig. 5A). Further, we observed that the Δ *fliC* mutant did not cause any visible change. The reduction of virulence in D43V and D43A mutants was also observed by the infiltration-inoculation method (Fig. 5C). The bacterial propagation of both *P. syringae* pv. *tabaci* D43A and D43V mutant strains was reduced compared with that of wild-type propagation but higher than that of the Δ *fliC* mutant in the spray-inoculated tobacco leaves (Fig. 5B). However, the reduction of bacterial growth of D43V and D43A mutants was not as obvious in the infiltrated tobacco leaves (Fig. 5D).

D43 is important for bacterial motility and adhesion in *P. syringae* pv. *tabaci*.

Flagellar motility-defective mutants such as the Δ *fliC* and Δ *fliD* mutants of *P. syringae* pv. *tabaci* are known to have no or reduced virulence on tobacco plants (Ichinose et al. 2003). Furthermore, Δ *fgt1* (Δ *orf1*) and Δ *fgt2* (Δ *orf2*) flagellin-glycosylation-defective mutants were impaired in motility, virulence, and adhesion ability (Taguchi et al. 2006). The flagellar swimming and swarming motilities (Fig. 6A and B) and adhesion

Fig. 4. Flagellin- and flg22-induced activation of senescence-induced receptor-like kinase (*SIRK*) promoter in *Arabidopsis thaliana*. β -Glucuronidase (GUS) activity derived from *Arabidopsis* *SIRK* promoter in response to flagellin and flg22 was measured after 24 h of treatment. **A**, Flagellin proteins and flg22 peptides were infiltrated into the leaves at 0.1 μ M (white boxes), 2 μ M (gray boxes), and 10 μ M (black boxes), and GUS activity was fluorometrically measured. **B**, Leaves infiltrated with 0.1 μ M flagellin or flg22 were histochemically stained. Experiments using five leaf disks were repeated five times. Photos depict representative results.

ability (Fig. 6C) of the D43-substituted mutants were compared with those of the wild-type strain. D43V was defective in all three assays and D43A was also significantly defective but more functional.

Effect of D43 substitution on flagella formation.

To investigate flagella formation, we first detected flagellin protein from the cells of bacteria cultured overnight. Bacteria were harvested by centrifugation and total proteins corresponding to approximately 4×10^8 bacterial cells were dissolved and subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Flagellin protein was detected by Western blotting analysis using a flagellin-specific antibody (Fig. 7A). Although the amounts of flagellin protein in *P. syringae* pv. *tabaci* wild type and D43A mutant were nearly the same, that in the D43V mutant was less. Electron microscopic observation revealed that *P. syringae* pv. *tabaci* wild-type (Fig. 7B) and D43A mutant strains (Fig. 7D) retained three to five long flagella. However, many cells of

D43V mutant did not possess flagella and some cells possessed only one to two short flagella per cell (Fig. 7C).

Effects of L36 and K37 substitutions on elicitor activity, virulence, and motilities.

The study using the mutant strains D43V and D43A demonstrated that D43, an important epitope for elicitor activity in flg22, is required for both motility and virulence. To confirm this conclusion, we newly introduced the mutation into L36 and K37 and generated the mutant strains L36S and K37R. Because the 36th and 37th amino acid residues are Leu-Lys in *P. syringae* pv. *tabaci* and Ser-Arg in *P. aeruginosa*, we expected that the substitution of amino acid residues in the flg22 region of *P. syringae* pv. *tabaci* and *P. aeruginosa* would not affect their motility, virulence, or elicitor activity in purified flagellin. Although the ability of the flagellins from the L36S mutant to induce GUS activity was a little weaker than that of the wild-type flagellin, they still retained remarkable elicitor activity (Fig. 8A). Further, the elicitor activity of the

Fig. 5. Loss of virulence in *Pseudomonas syringae* pv. *tabaci* D43V and D43A mutants in tobacco. Tobacco leaves were **A** and **B**, spray inoculated with 2×10^8 CFU/ml or **C** and **D**, infiltration inoculated with 2×10^5 CFU/ml and incubated at 23°C for **A**, 11 and **C**, 10 days. Bacterial populations were measured at 1, 5, and 8 days post inoculation (dpi) by the **B**, spray method or **D**, infiltration method. Bacterial strains are *P. syringae* pv. *tabaci* wild-type (WT), D43V, D43A, and $\Delta fliC$ mutant strains. The bars represent standard deviations for at least three independent experiments.

flagellin from the K37R mutant was at a level similar to that of the wild-type strain. The mutant strains L36S and K37R also caused severe disease symptoms on tobacco leaves (Fig. 8B) and showed strong motility in swimming (Fig. 8C) and swarming (Fig. 8D) tests. These results confirmed that the mutant strains with motility, such as L36S and K37R, are virulent on tobacco leaves and that the flagellin purified from these mutants actively induces a defense response, whereas the mutant strains without any or with lower motility, such as D43V and D43A, are less virulent on tobacco, and flagellin purified from these mutants is less active in inducing a defense response.

DISCUSSION

In this article, we report that the amino acid residue in flagellin required for MAMP activity is important for the intrinsic function of the flagellum in *P. syringae* pv. *tabaci* 6605. The amino acid sequence of the best-known MAMP, flg22_{pas} is

widely conserved in many gram-negative bacteria (Felix et al. 1999). However, the amino acid sequence of the flg22 region in some bacterial species is less conserved, and some, such as *Agrobacterium tumefaciens*, *Rhizobium meliloti* (Felix et al. 1999), and *Ralstonia solanacearum* (Pfund et al. 2004), do not show any elicitor activity. The importance of the flg22 region of *X. campestris* pv. *campestris* in the elicitor activity was further investigated using recombinant flagellin polypeptides. Thus, recombinant flagellin from *X. campestris* pv. *campestris* strain B305 possessing D43 elicited a defense response in *Arabidopsis* but that from *X. campestris* pv. *campestris* strain B186 possessing V43 did not (Sun et al. 2006). These results indicate that the flg22 region is a typical MAMP and that D43 is important for recognition by *Arabidopsis*.

In the case of *P. syringae*, we have found strong elicitor activity in purified flagellin proteins from *P. syringae* pv. *tabaci* 6605, *P. syringae* pv. *glycinea* race 4, and *P. syringae* pv. *tomato* DC3000 (Taguchi et al. 2003b). The flg22 regions

Fig. 6. Bacterial motility and adhesion ability. Bacterial cell densities were adjusted to an optical density at 660 nm (OD₆₆₀) of 0.3 with MMF medium, and 3- μ l aliquots were inoculated on the center of MMF (0.25% agar) and SWM agar (0.5% agar) plates for swimming and swarming assays, respectively. The photographs were taken 2 and 1 days after inoculation for **A**, swimming and **B**, swarming assays, respectively. The bar in **A** is 1 cm. **C**, Adhesion ability. Each experiment was repeated three times, and typical results were shown in **A** and **B**. In **C**, values represent means and standard errors ($P < 0.05$).

Fig. 7. Detection of flagellin from bacterial bodies and electron microscopic observation of flagella. An overnight culture of each bacterium in Luria-Bertani supplemented with 10 mM MgCl₂ was subcultured in fresh MMF medium. Bacterial bodies were harvested from 1 ml of bacterial culture that was adjusted to an optical density at 660 nm of 0.2 and suspended in 20 μ l of phosphate-buffered saline with Tween. **A**, Total proteins of the bacterial bodies were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and flagellin protein was detected by anti-flagellin antibody. Each bacterium was harvested from the overnight culture in MMF medium and bacterial cells were observed by electron microscopy for the *Pseudomonas syringae* pv. *tabaci* **B**, wild type (WT); **C**, D43V mutant; and **D**, D43A mutant. The bar indicates 1 μ m.

of flagellin in these *P. syringae* strains are identical. However, there is a difference of four amino acids between *P. aeruginosa* and *P. syringae* (Fig. 1). We further found that the elicitor activity of flagellin from *P. syringae* was localized in the N-terminal region containing flg22 by using recombinant flagellin polypeptides (Naito et al. 2007). However, flagellins from *P. syringae* are glycosylated (Taguchi et al. 2003a), and glycosylation of flagellin affects its elicitor activity (Taguchi et al. 2006). Therefore, to properly evaluate the importance of flg22 in the elicitor activity and bacterial virulence, it is necessary to investigate elicitor activity using not only chemically synthesized oligopeptides and recombinant polypeptides but also purified and glycosylated flagellins from *P. syringae*.

Compared with the strong abilities of the wild-type flagellin to induce HR cell death (Fig. 2), inhibit seedling growth (Fig. 3), and activate the *SIRK* promoter (Fig. 4), flagellins purified from *P. syringae* pv. *tabaci* D43A and D43V had remarkably reduced abilities, indicating that D43 is an important residue for the elicitor activity of flagellin, as previously reported by Sun and associates (2006). When we investigated these activities in different flg22 oligopeptides, both flg22_{pta}D43A and flg22_{pta}D43V had completely lost these abilities. Because flg22_{pta} and flg22_{pa} retained the elicitor activity, we verified that the flg22 region is a major MAMP. However, flagellins from *P. syringae* pv. *tabaci* D43A and D43V showed a weak but significant level of activities at 0.1 μ M concentration. Furthermore, the elicitor activity of flagellins from *P. syringae* pv. *tabaci* D43A and D43V increased in a dose-dependent manner (Fig. 4A). To check for concomitant contamination of the flagellin preparation with other HR-inducing elicitors, we prepared flagellin from a flagellin-defective mutant, Δ *flhC* (Ichinose et al. 2003), using the same method we used to purify flagellin

from the wild-type strain. However, the flagellin preparation from the Δ *flhC* mutant did not show any elicitor activity in *Arabidopsis* Col-0 (data not shown). These results indicate that the outside region of D43 in purified flagellin possesses another epitope that contributes to elicitor activity.

Previously, we found that nonglycosylated flagellin from *P. syringae* pv. *tabaci* 6605 Δ *fgt1* mutant strain had a reduced ability to induce HR in nonhost soybean cells (Taguchi et al. 2006), indicating that glycosylation of flagellin may affect the elicitor activity of flagellin. On the other hand, the elicitor activity of flagellin and flg22 was completely abolished in the *Arabidopsis* *fls2* mutant (Fig. 3C and D), indicating that *Arabidopsis* requires FLS2 in response not only to flg22 but also to flagellin protein. It is still unclear how the glycan of flagellin is involved in the elicitor activity. As we observed in Figure 3, elicitor activity of the glycosylated D43A and D43V flagellins also requires FLS2, which indicates a possible interaction between FLS2 and the outside region of D43 in flagellin.

Although both *P. syringae* pv. *tabaci* mutants D43A and D43V had reduced virulence in both the spray inoculation and infiltration inoculation methods (Fig. 5A and C), we observed a reduction of bacterial population in the mutants in the spray-inoculation method (Fig. 5B) but not obviously in the infiltration-inoculation method (Fig. 5D). Previously, we observed similar phenomena; flagellin glycosylation-defective mutants in *P. syringae* pv. *tabaci* are swarming defective but a reduction of bacterial propagation in the mutant strains was not obvious in the infiltration experiment (Taguchi et al. 2006). The importance of flagellar motility in bacterial virulence was also reported in many other species: *Erwinia carotovora* subsp. *carotovora* (Hossain et al. 2005), *R. solanacearum* (Tans-Kersten et al. 2004), and several pathovars of *P. syringae*

Fig. 8. Effects of L36 and K37 substitutions on elicitor activity, motility, and virulence. **A**, Flagellin proteins from *Pseudomonas syringae* pv. *tabaci* wild type (black box) and L36S- and K37R-substituted mutant strains (white boxes) were infiltrated into the leaves at 0.1 μ M, and senescence-induced receptor-like kinase promoter-derived β -glucuronidase activity was measured fluorometrically after 24 h of treatment. **B**, A virulence assay. Tobacco leaves were infiltration-inoculated with bacteria at 2×10^5 CFU/ml and were incubated at 23°C for 10 days. **C**, Swimming motility and **D**, swarming motility assays. Bacterial cell densities were adjusted to an optical density at 660 nm of 0.3 with MMMF medium (50 mM potassium phosphate buffer, 7.6 mM $(\text{NH}_4)_2\text{SO}_4$, 1.7 mM MgCl_2 , and 1.7 mM NaCl, pH 5.7, supplemented with 10 mM each of mannitol and fructose), and 3- μ l aliquots were inoculated on the center of MMMF (0.25% agar) and SWM (0.5% peptone, 0.3% yeast extract) agar (0.5% agar) plates for swimming and swarming assays, respectively. Each experiment was repeated three times, and typical results were shown in B, C, and D. In A, values represent means and standard errors.

(Haefele and Lindow 1987; Hattermann and Ries 1989; Panopoulos and Schroth 1974). In *P. syringae* pv. *tabaci*, reductions of swimming and swarming motilities and adhesion ability were observed in both mutants D43A and D43V and were especially prominent in the D43V mutant (Fig. 6), because the D43V mutant failed to form stable and functional flagella filaments (Fig. 7). Thus, *P. syringae* pv. *tabaci* D43A and D43V failed to cause disease in host tobacco leaves although their flagellins reduced the elicitor activity that induces plant defense responses.

In animals, Toll-like receptors (TLR) have been identified as receptors for variable MAMPs (Akira et al. 2006). TLR are composed of an LRR extracellular domain, transmembrane domain, and cytoplasmic toll/interleukin-1 receptor domain. Among 13 mouse TLR, TLR5 was identified as a receptor for flagellin (Hayashi et al. 2001). TLR5 recognizes the conserved regions of both N- and C-termini in flagellin of the gram-negative bacterium *Salmonella typhimurium* and activates host inflammatory responses (Smith et al. 2003). On the other hand, the N- and C-terminal regions in flagellin were shown to be important for precise conformation of the flagellar filament and motility by point mutation analyses (Hyman and Trachtenberg 1991; Kanto et al. 1991). Interestingly, mutation in the flagellin epitope recognized by TLR5 resulted in the reduction of motility, indicating that the host immune system recognizes the region of flagellin that is essential for intermolecular interactions within flagellar protofilaments and for flagellar motility (Smith et al. 2003). Furthermore, a mutant of *Salmonella* flagellin that evades TLR5 recognition but retains motility was engineered by the introduction of an amino acid replacement at the D0 conserved region into the motility-defective mutant strain (Andersen-Nissen et al. 2005). Thus, evasion of TLR5 by flagellated bacteria might be an evolutionary strategy to overcome host immune responses. However, there is no other nucleotide substitution except D43 in the entire *fliC* gene of D43V, D43A, L36S, and K37R mutants (data not shown), indicating that compensatory mutation to restore bacterial motility did not occur outside D43. These results indicate that bacteria with D43A flagellin are still reasonably motile and their flagellin still has some elicitor activity, whereas bacteria with L36S and K37R flagellin are very motile and their flagellin has higher elicitor activity.

In addition to MAMPs, Mackey and McFall (2006) have proposed the term microbe-induced molecular patterns (MIMPs). MIMPs are categorized as modifications of host-derived molecules that are induced by the intrinsic activity of a pathogen-derived effector and are perceived by a host-defense receptor (Mackey and McFall 2006). MAMPs are molecular patterns directly perceived via direct interactions with a host defense receptor. Mackey and McFall further refined the definition of MAMPs as molecular patterns recognized independently of the microbial molecule's intrinsic activity (Mackey and McFall 2006). From the results of our study and the study of animal TLR5 and flagellins of animal pathogens, we propose that the MAMP site that is recognized may be (and, perhaps, often should be) a site that is central to the biological activity of MAMPs. Thus, the immune system has evolved an ability to precisely target a conserved site on flagellin that is essential for bacterial motility and virulence.

MATERIALS AND METHODS

Plant materials.

Arabidopsis thaliana ecotype Columbia-0 wild-type and its *fls2* mutant were used in this study. The *fls2* mutant (*fls2-4*, SALK_093905) was obtained as T-DNA insertion mutants from the Salk Institute (Alonso et al. 2003). The homozygous

mutation of *FLS2* was confirmed by PCR and sequencing of the insertion site. The insertion site of *fls2-4* was located in the coding region of the first exon. The plants were grown at 22°C with a 16-h photoperiod. Four-week-old *Arabidopsis* was used for cell death assay. Tobacco plants (*Nicotiana tabacum* L. cv. Xanthi NC) were grown at 26°C with a 16-h photoperiod. For inoculation experiments, 2- to 3-month-old plants were used.

Bacterial culture conditions.

P. syringae pv. *tabaci* 6605 wild-type and D43-substituted mutant strains were maintained in King's B (KB) medium at 27°C and *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium. To measure bacterial growth, the strains of *P. syringae* pv. *tabaci* were cultured overnight in LB with 10 mM MgCl₂, adjusted to an optical density at 660 nm (OD₆₆₀) of 0.1, and subcultured in fresh MMMF medium (50 mM potassium phosphate buffer, 7.6 mM (NH₄)₂SO₄, 1.7 mM MgCl₂, and 1.7 mM NaCl, pH 5.7, supplemented with 10 mM each of mannitol and fructose). The cell density was measured sequentially by absorbance at OD₆₆₀.

Inoculation experiments.

For the spray-inoculation experiments, overnight cultured bacterial strains in M9 medium with 0.2% glucose were harvested after centrifugation, then suspended in 10 mM MgSO₄ and 0.02% Silwet L77 (OSI Specialties, Danbury, CT, U.S.A.) at a density of 2×10^8 CFU/ml. Both leaf surfaces were inoculated and the leaves were incubated in 100% humidity for 1 day and 80% humidity for 9 to 10 days in a growth cabinet at 23°C. For inoculation by the syringe-infiltration method, overnight cultured bacterial strains were suspended in 10 mM MgSO₄ at a density of 2×10^5 CFU ml⁻¹; then, bacteria were infiltrated into the leaves using a needleless syringe. To measure bacterial populations in inoculated plant leaves, each inoculated leaf was soaked in 15% H₂O₂ for 1 min to sterilize the leaf surface; then, leaves were washed with sterile distilled water. Five leaf disks (8 mm in diameter) were punched from each leaf and ground in a mortar with a pestle. Serially diluted bacterial suspensions in sterilized water were spread on KB plates. Two days later, the numbers of colonies was counted and bacterial populations were calculated.

Generation of mutant strains.

To introduce the desired point mutations, a QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) was used. The entire *fliC* region amplified by PCR as described by Taguchi and associates (2006) was inserted into the mobilizable cloning vector pK18mobSacB (Schäfer et al. 1994) via the *Bam*HI site. This double-stranded DNA vector was used as the template. Two sets of complementary oligonucleotides containing the replacements D43V-FW (5'-caacagc gccaagTTGACgctgccggcc-3') and D43V-RV (5'-ggccggcagc GTCAACTtggcgctgttg-3') and D43A-FW (5'-caacagcgcaca AGCTgacgctgccggcc-3') and D43A-RV (5'-ggccggcagcgtc AGCTtggcgctgttg-3') were synthesized (underlines indicate the replaced nucleotide and capital letters indicate *Hinc*II or *Alu*I sites). Subsequently, PCR, *Dpn*I digestion, and transformation were performed according to the manufacturer's instructions. The desired mutations were confirmed by DNA sequence analysis; then, mutated plasmids were introduced into *E. coli* S17-1 and introduced into *P. syringae* pv. *tabaci* 6605 by conjugation. After loss of the plasmid by incubation on KB agar plates containing 10% sucrose, each mutant strain was isolated. The mutated *fliC* gene generated an additional *Hinc*II site in the corresponding positions at D43V and the *Alu*I site at D43A. Therefore, analysis of restriction fragment length polymorphism in PCR-amplified *fliC* gene revealed that

each mutant strain possesses a mutated *fliC* gene without the wild-type gene. Mutation of the *fliC* gene was also confirmed by direct sequencing of the PCR product and the mutant strains were designated D43V or D43A.

In the same way, site-directed mutagenesis for L36S and K37R was carried out using sets of primers L36S-FW (5'-tcgtctgtcttcggttcgaaatcaacagcgcc-3') and L36S-RV (5'-ggcgctgtgat ttcgaaccggaagacagacga-3') and K37R-FW (5'-tgtcttcggtctgaga atcaacagcgccaa-3') and K37R-RV (5'-ttggcgctgttgattctcagaccggaagaca-3') (underlines indicate the replaced nucleotide). All specific mutations of the *fliC* gene were confirmed by direct sequencing of PCR products from the mutants.

Purification of flagellin proteins and oligopeptides.

All *Pseudomonas* strains were inoculated in LB medium supplemented with 10 mM MgCl₂ for 24 h at 25°C. The bacterial cells were harvested by centrifugation (3,000 rpm, 10 min), suspended in one-quarter volume of MMMF medium, and incubated for 24 h at 23°C. After incubation, a 10% volume of 20% polyethylene glycol (PEG) (6000) to 1 M NaCl solution was added to the bacterial culture. The bacterial cells were harvested by centrifugation (10,000 rpm, 20 min) and suspended in 300 µl of phosphate-buffered saline buffer (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, and 1.47 mM KH₂PO₄) per 100 ml of bacterial culture. Flagella were then sheared off by vortexing. After centrifugation at 10,000 rpm for 5 min, a 10% volume of 20% PEG (6000)/1 M NaCl solution was added to the supernatant, and the supernatant was incubated on ice for 20 min. Subsequently, flagella were harvested by centrifugation at 12,000 rpm for 30 min, and the pellet was suspended in distilled water. The resultant flagella were further purified by centrifugation at 40,000 rpm for 30 min. The precipitated flagella, which consist of flagellin proteins, were dissociated in 0.1 M glycine-HCl buffer (pH 2.0) and centrifuged at 40,000 rpm for 30 min. The buffer of the flagellin solution in the supernatant was replaced with distilled H₂O by use of a spin column (VIVASPIN 20 [30,000 MWCO PES]; Vivascience, Hanover, Germany). The molecular weight, quality, and quantity of the purified flagellin were confirmed by the Bradford method and SDS-PAGE analysis. The oligopeptides flg22_{pa}, flg22_{pta}, flg22_{pta}D43A, and flg22_{pta}D43V were chemically synthesized by Hokkaido System Science (Sapporo, Japan).

Detection of dead cells.

The dead cells in the leaves were stained with lactophenol-trypan blue according to the methods described by Koch and Slusarenko (1990) and Naito and associates (2007). Whole leaves were boiled for approximately 3 min in the staining solution and decolorized in chloral hydrate solution at 2.5 g ml⁻¹ for at least 6 h.

Growth inhibition assay of *Arabidopsis* seedlings.

Growth inhibition assays of *Arabidopsis* seedlings were performed as described by Gómez-Gómez and associates (1999). Sterilized *Arabidopsis* seed were sown on 0.4% agar containing the salts of Murashige-Skoog (MS) medium (Wako, Osaka, Japan), Gamborg's vitamin solution (Sigma-Aldrich, St. Louis), 0.05% morpholineethanesulfonic acid, and 1% sucrose at pH 5.7 and incubated at 24°C with a 16-h photoperiod. Seedlings grown for 5 days on MS agar plates were transferred to liquid MS medium supplied with each flagellin or flg22 at a final concentration of 0.1 µM or water as a control (two seedlings per 400 µl of MS medium in one well of a 24-well plate). After 7 days of incubation at 24°C with a 16-h photoperiod, the seedlings were photographed and the fresh weight was measured.

Fluorometric measurement and histochemical analysis of GUS activity.

Transgenic *Arabidopsis* Col-0 possessing the *SIRKp:GUS* chimeric gene was provided by Dr. S. Robatzek (Kunze et al. 2004). Quantitative analysis of GUS enzymatic activities was carried out using the extracts from leaves of transgenic plants. Five leaf disks (5 mm in diameter) were punched from each leaf infiltrated with flagellin and flg22 oligopeptides and ground in a mortar with a pestle. The concentrations of proteins in the crude enzyme solution obtained were determined by the method of Bradford, and GUS-specific activity (nmol of 4-MU produced per minute per milligram of protein) was determined using 4-methylumbelliferyl-β-D-glucuronide as the substrate, according to the previously described method (Jefferson et al. 1987). Histochemical GUS analysis was performed with leaves. Punched leaf disks were prefixed in 1% formaldehyde in 50 mM phosphate buffer (pH 7.0) and rinsed three times in the same buffer without formaldehyde. GUS staining was performed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as the substrate as described (Jefferson et al. 1987).

Western blot analysis.

To detect flagellin in each bacterial cell, total proteins were prepared and subjected to 12% SDS-PAGE. Then, using anti-flagellin antibody, flagellin was detected as described previously (Taguchi et al. 2003b).

Motility assay and adhesion experiment.

Bacterial cells grown in LB supplemented with 10 mM MgCl₂ at 25°C were adjusted to an OD₆₆₀ of 0.3 in MMMF medium. For the swimming assay, 3 µl of bacterial suspension was inoculated in the center of the 0.25% agar MMMF plates and incubated at 23°C for 2 days. For the swarming assay, bacteria were inoculated on SWM plates (0.5% peptone, 0.3% yeast extract, 0.5% agar; Difco Laboratories, Detroit) and incubated at 27°C for 1 day (Taguchi et al. 2006). Adhesion was measured as described previously (Taguchi et al. 2006). Briefly, density-adjusted aliquots of overnight cultures of each bacterial strain were transferred to polystyrene microtiter plate wells and bacteria were incubated at 27°C for 48 h. After washing to remove loosely bound bacteria, adherent bacteria were stained with crystal violet. The amount of crystal violet eluted from stained bacteria was measured at OD₅₉₅.

Transmission electron microscopy.

The overnight culture of each bacterium in MMMF medium was observed under an electron microscope (H-7500, Hitachi, Tokyo) as described previously (Taguchi et al. 2008).

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