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Allelic variation in two distinct *Pseudomonas syringae* flagellin epitopes modulates the strength of plant immune responses but not bacterial motility

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Summary

- The bacterial flagellin (FliC) epitopes flg22 and flgII-28 are microbe-associated molecular patterns (MAMPs). While flg22 is recognized by many plant species via the

pattern recognition receptor FLS2, neither the flgII-28 receptor nor the extent of flgII-28 recognition by different plant families is known.

- Here we tested the significance of flgII-28 as a MAMP and the importance of allelic diversity in flg22 and flgII-28 in plant–pathogen interactions using purified peptides and a *Pseudomonas syringae fliC* mutant complemented with different *fliC* alleles.

- Plant genotype and allelic diversity in flg22 and flgII-28 were found to significantly affect the plant immune response but not bacterial motility. Recognition of flgII-28 is restricted to a number of Solanaceous species. While the flgII-28 peptide does not trigger any immune response in Arabidopsis, mutations in both flg22 and flgII-28 have *FLS2*-dependent effects on virulence. However, expression of a tomato allele of *FLS2* does not confer to *Nicotiana benthamiana* the ability to detect flgII-28 and tomato plants silenced for *FLS2* are not altered in flgII-28 recognition.

- Therefore, MAMP diversification is an effective pathogen virulence strategy and flgII-28 appears to be perceived by a yet unidentified receptor in the Solanaceae although it has an *FLS2*-dependent virulence effect in Arabidopsis.

Keywords

Introduction

Perception of conserved molecular patterns of microbes, called PAMPs (pathogen-associated molecular patterns) or MAMPs (microbe-associated molecular patterns), is an important first line of plant defense known as pattern-triggered immunity (PTI) (Jones & Dangl, 2006; Segonzac & Zipfel, 2011). Perception of the 22-amino acid flagellin epitope flg22 is one of the most studied examples of PTI. The flg22 epitope directly binds to the pattern recognition receptor (PRR) FLS2 (Chinchilla *et al.*, 2006), after which FLS2 interacts with the adaptor protein BAK1 (Chinchilla *et al.*, 2007b; Heese *et al.*, 2007) and several other receptor-like kinases (RLKs) (Roux & Zipfel, 2012). These interactions lead to the rapid efflux of Ca^{2+} and activation of calcium-dependent protein kinases (CDPKs) (Boudsocq *et al.*, 2010), generation of reactive oxygen species (ROS) (Felix *et al.*, 1999) and activation of MAP kinase cascades (Rasmussen *et al.*, 2012) triggering a complex defense response which includes *FLS2*-dependent stomatal closure to interfere with pathogen invasion (Melotto *et al.*, 2006; Zeng & He, 2010), callose deposition to strengthen plant cell walls (Gomez-Gomez & Boller, 2000), and induction of pathogenesis-related genes to restrict pathogen growth (Gómez-Gómez *et al.*, 1999; Chinchilla *et al.*, 2007a). Pre-treatment of Arabidopsis plants with flg22 epitope before pathogen inoculation decreases pathogen growth while *fls2* mutant plants are more susceptible to *Pseudomonas syringae* pv. tomato (*Pto*) infection following spray inoculation (Zipfel *et al.*, 2004) demonstrating that flg22 recognition by FLS2 has biological relevance. Interestingly, flagellin recognition evolved in parallel in plants and animals (Ausubel, 2005; Zipfel & Felix, 2005). In mammals, the PRR TLR5 recognizes extracellular flagellin (Hayashi *et al.*, 2001) and the intracellular receptor NLRC4 recognizes flagellin inside macrophages (Kofoed & Vance, 2011; Zhao *et al.*, 2011).

An important strategy of plant pathogens to avoid PTI is injection of immunity-suppressing effector proteins directly into host cells (Chisholm *et al.*, 2006; Jones & Dangl, 2006; Cunnac *et al.*, 2011). This strategy is best exemplified by the molecular mechanisms of the type III-secreted effector proteins AvrPto and AvrPtoB of *Pto*, both of which suppress

FLS2-mediated immunity (Shan *et al.*, 2008; Xiang *et al.*, 2008; Cheng *et al.*, 2011; Martin, 2012). Since MAMP-containing proteins and other molecules are by definition essential for a pathogen's life cycle and/or pathogenicity (Jones & Dangl, 2006; Segonzac & Zipfel, 2011), pathogens cannot avoid PTI by losing essential proteins containing MAMPs and allelic variation of MAMPs is expected to be limited by evolutionary constraints on their structure (Bittel & Robatzek, 2007; Boller & Felix, 2009; McCann *et al.*, 2012). Nonetheless, a few studies suggest that some pathogens are able to alter MAMPs to avoid PTI. For example, a single amino acid change in flg22 of the plant pathogen *Xanthomonas campestris* pv. *campestris* severely attenuated or eliminated perception of flg22 by FLS2 (Sun *et al.*, 2006); however, the mutation had no effect on pathogen growth during infection. Furthermore, no known alleles of flagellin from *Ralstonia solanacearum* elicit PTI (Pfund *et al.*, 2004), but the effect of the evasion of flagellin recognition on pathogen growth during infection is not known. Also, post-translational modifications of flagellin, including glycosylation, can have a major impact on the elicitation activity of flagellin (Taguchi *et al.*, 2003; Takeuchi *et al.*, 2003). Flagellin proteins from the human pathogens *Bartonella bacilliformi, Campylobacter jejuni*, and *Helicobacter pylori* escape detection by TLR5 through mutations of amino acids in the known interaction surface (Andersen-Nissen *et al.*, 2005). Diversity of flagellin perception due to allelic variation in *FLS2* has also been demonstrated in that the flg15 epitope of *Escherichia coli* flagellin is recognized by tomato (*Solanum lycopersicum*) but not by Arabidopsis or *Nicotiana benthamiana* (Bauer *et al.*, 2001; Meindl *et al.*, 2000).

We previously determined that a second epitope of flagellin, termed flgII-28, is sufficient to trigger immunity in tomato (Cai *et al.*, 2011). The flgII-28 epitope was identified based on two non-synonymous mutations in almost identical strains of the bacterial speck disease pathogen *Pto*, which suggested selection for evasion of flgII-28 perception by tomato. In fact, the two derived alleles of flgII-28 (flgII-28$_{K40}$ and flgII-28$_{Col338}$), which are present in *Pto* strains typical of recent bacterial speck disease outbreaks, trigger a weaker immune response in tomato cultivar cv 'Chico III' than the ancestral flgII-28$_{T1}$ allele, which was the predominant allele of *Pto* strains that caused disease outbreaks before 1980 (Cai *et al.*, 2011). Some *Pto* strains also have a mutation in flg22 but this mutation does not significantly affect the strength of the tomato immune response (Cai *et al.*, 2011).

Here we find that there is significant variation both in the strength of PTI elicited by different flagellin proteins in the same plant and in perception of the same flagellin among different plants. Importantly, we show a significant effect of allelic variation in flg22 and flgII-28 on the outcome of plant–pathogen interaction in the absence of any effects on bacterial motility, thus revealing that allelic variation in MAMPs is an important PTI-avoidance mechanism in the evolutionary arms race between *P. syringae* pathogens and plants. Intriguingly, the effect of allelic variation in flgII-28 on bacterial growth on Arabidopsis is *FLS2*-dependent although recognition of the flgII-28 peptide appears to be *FLS2*-independent based on multiple lines of evidence. This suggests that flgII-28 may indirectly modulate flg22 perception by FLS2 and that Solanaceous plants are likely equipped with a second flagellin receptor that recognizes flgII-28.

Materials and Methods

Peptide synthesis and storage

Peptides were synthesized to 70–90% purity by EZ Biolab (Carmel, IN, USA) with the exception of the flg22 consensus peptide (QRLSTGSRINSAKDDAAGLQIA) used in Figs 4(d) and Supporting Information S6 which was synthesized by Biomatik (Cambridge, Ontario, Canada). Water was added to the peptides to bring them to a concentration of 5 μM except for the flgII-28 peptides which were insoluble in water (for some alleles) and were

instead initially dissolved in a solution of 50% DMSO and diluted further in water. Dissolution in DMSO is unlikely to affect the ROS elicitation potential of the peptides because flgII-28$_{T1}$ is an equivalent elicitor on *S. lycopersicum* whether dissolved in water or 50% DMSO (not shown). To maintain activity, peptides were stored in small aliquots to avoid multiple freezing and thawing. All dilutions to obtain working concentrations were done in ultrapure H$_2$O. Peptide solutions were stored at −20°C for short-term usage or −80°C for long-term storage.

Measurement of ROS generation

Previously described protocols were used to quantify production of ROS generation following elicitation with peptides (Chakravarthy *et al.*, 2010). Briefly, leaf disks of 4-wk-old plants (except eggplant and bean for which 6-wk-old and 3-wk-old plants, respectively, were used) were punched out with a #1 cork borer and floated adaxial side up overnight at room temperature in 200μl ddH$_2$O in individual wells of a clear-bottom 96 well microassay plate (Greiner Bio-one, Germany). 16 h later, the water was replaced with a solution containing 1μM peptide (unless concentration otherwise noted in figure), 34 μg ml^{-1} luminol, and 20μg ml^{-1} horseradish peroxidase (Sigma, St Louis, MO, USA) all in ddH$_2$O. Luminescence of each well was then immediately measured using a Synergy HT plate reader (Biotek, Winooski, VT, USA) using the bottom camera at optical sensitivity of 225 every 3 min for 60 min. For comparison among different peptides, multiple leaf punches were taken from the same leaf to allow for the peptides to act on the same leaf (though different sections) to control for variability among leaves. 6 to 8 leaves were used for each experiment.

The following plants were screened via the ROS assay in this work: *Arabidopsis thaliana* ecos. 'Columbia-0', 'Cvi-0', and 'WS' (TAIR, Stanford, CA, USA); Radish cv 'Champion' (Ferry Morse, Futon, KY, USA); Turnip cv 'Purple Top White Globe' (Ferry Morse, Futon, KY); Cauliflower cv 'Toscana 2 (Ferry Morse, Futon, KY)'; Snapdragon cv 'Cook's Tall' (Cooks Garden, Warminster, PA, USA); Morning glory (Ferry Morse, Futon, KY, USA); Celery cv 'S. V. Pascal' (Seeds of Italy, Harrow, UK); Bean cv 'Red Mexican' (Vermont Bean Seed Company, Randolph, WI, USA); Tomato cvs. 'Rio Grande', 'Chico III', 'Sunpride' (Bavicchi, Ponte San Giovani, Italy) and 'Roter Gnom' (Canadian plant genetic resource center, Ottowa, Canada); Pepper cvs. 'California Wonder' (Wyatt-Quarles Seed, Garner, NC, USA) and 'Jalapeno Early' (Burpee, Warminster, PA, USA); Eggplant cv 'MM643' (INRA, Avignon, France); *Nicotiana benthamiana, N. tabacum* cv 'Burly' (personal lab stocks), and Potato cv 'Red Maria' (Maine Potato Lady, Guilford, ME, USA).

Measurement of ethylene production following peptide treatment

Fully expanded leaves of 4–6-wk-old *Solanum lycopersicum* cv 'MoneyMaker' (Thompson & Morgan, Ipswich, UK) and *N. benthamiana*, both grown in long day conditions (14 h photoperiod, 25°C), were cut into 2 mm slices and floated on water overnight. Leaf slices were transferred to 6ml glass tubes containing 0.5ml of an aqueous solution of the peptide being tested. Vials were closed with rubber septa and ethylene accumulating in the free air space was measured by gas chromatography after 3.5–4 h incubation.

Bayesian analysis of ROS assays

For analysis of ROS production, we modeled the data for each plant–peptide combination using a shifted Gompertz-decay curve (Eqn 4 in Methods S1). Estimation of the unknown modeling parameters was performed using a Markov Chain Monte Carlo algorithm. To initially address whether ROS is elicited by a plant-peptide combination, we calculated the posterior probability of the null hypothesis (no ROS response) given the data. That is, we fit the data to the Gompertz-decay with the parameters $\beta_1 = \beta_2 = 0$ – a function of a flat line with

transformed into *E. coli* S17-1. The *fliC* deletion mutant was obtained by conjugation and homologous recombination. Specific deletions were confirmed by PCR using primers PC1 and PC4.

Cloning of *fliC* and complementation of *Pto* DC3000Δ*fliC*

Genomic DNA was extracted from the *Pto* strains DC3000, K40, T1, and ES4326 and used as a template for PCR amplification of *fliC*. Primers (Table S1) were designed to amplify 326bp upstream (except for ES4326 with 327bp upstream) and 52bp downstream of the *fliC* ORF with *Sac1* and *Xho1* restriction enzyme sites on the forward and reverse primers respectively. This region contains both the *fliC* ORF and nearly the entire flanking regions up to the flanking genes in the flagellum gene cluster. PCR products were cleaned using AccuPrep PCR purification kit (Bioneer, Alameda, CA, USA) and digested by *SacI* and *XhoI* (NEB, Ipswich, MA, USA) at 37°C for 3 h. The broad expression vector pME6010 was similarly digested with *SacI* and *XhoI*. All digested products were separated using gel electrophoresis and cleaned using *AccuPrep* gel extraction kit (Bioneer) and each PCR product was individually ligated into the digested pME6010 vector using DNA ligase (Takara Bio, Shiga, Japan). Because of the orientation of restriction enzyme sites in the multiple cloning site of pME6010, each PCR product was ligated 3′ to 5′ with respect to the *Pk* promoter of 6010; therefore, *fliC* is under control of its native promoter in these vectors. The resulting vectors were transformed via conjugation into *Pto* DC3000 *fliC*.

Plant protection assay and plant infection assays

Plant protection assays were performed similar to previously described protocols (Zipfel *et al.*, 2004; Cai *et al.*, 2011). 16 h before infection, 1μM of each peptide was infiltrated into the adaxial side of at least 5 attached leaves on 2–4 different 4- or 5-wk-old plants via blunt end syringe and marked to indicate sites of infiltration. Plants were placed in high humidity for 16 h before spray inoculation of 10ml of bacterial suspension per pot at an OD_{600} of 0.01 using a Preval® sprayer. Silwet L-77 was not added to the bacterial suspension to avoid creating artificial conditions where flagella are likely less essential. The plants were then covered with clear plastic flat covers to maintain high humidity for 16 h following inoculation. After the number of days indicated in each figure legend, regions of leaves that had been previously infiltrated by peptide were removed using a $0.52mm^2$ cork borer and placed in a tube containing 200μL of 10mM $MgSO_4$ solution and three 2mm glass beads. The tube was placed in a mini bead beater (Biospec Products, Inc., Bartlesvill, OK, USA) and shaken for 90 s to grind the leaf and release endophytic bacteria. This suspension was diluted in 10mM $MgSO_4$ and plated on a King's B media plate with appropriate antibiotic selection. After 2 d, colony-forming units were counted. Similar procedures were used to quantify growth of the *Pto* DC3000 *fliC* strains on *A. thaliana* and *S. lycopersicum. A. thaliana fls2* mutant plants (Xiang *et al.*, 2008) were kindly provided by Jian-min Zhou.

For inoculation by infiltration, plants were infiltrated via a blunt-end syringe at an OD_{600} of either 1×10^{-3} (*Arabidopsis*) or 1×10^{-4} (tomato). Population assays were performed as described above.

Results

Diversity exists in flg22 and flgII-28 in the *P. syringae* species complex

Since we had found unexpected diversity among *Pto* strains in both the flg22 and flgII-28 epitopes (Cai *et al.*, 2011), we assembled the flg22 and flgII-28 alleles from all available genomes of the *P. syringae* species complex. The positions within flg22 and flgII-28 that are mutated in *Pto* strains are conserved in *P. syringae* strains belonging to other pathovars (Table S2); however, some strains show overall sequence variation in both epitopes,

particularly *P. syringae* pv. *maculicola* ES4326, a strain recently reassigned to *P. cannabina* pv. *alisalensis* (*Pcal*) (Bull *et al.*, 2010). The subset of flg22 and flgII-28 alleles tested in this paper are shown in Fig. 1.

Mapping the locations of flg22 and flgII-28 to the known flagellin structure (Yonekura *et al.*, 2003) revealed that flg22 and flgII-28 are structurally similar regions of flagellin: both are found within loop regions located between alpha helices (data not shown). Loops define the angle between flanking alpha helixes and are thus important determinants of protein structure, which may in fact explain the relative sequence conservation of both flg22 and flgII-28.

flgII-28 recognition is limited to a subset of the *Solanaceae* family

We have previously shown that the flgII-28 alleles of *Pto* strains K40 (same genotype as LNPV17.41) and Col338 trigger less ROS production and less stomatal closure than the flgII-28 allele of *Pto* T1 on tomato cv 'Chico III'(Cai *et al.*, 2011). To test whether plants other than tomato can recognize flgII-28, ROS production was measured in 18 different plant genotypes across six different families, with multiple *Brassicaceae* and *Solanaceae* species tested. The posterior probability ($P(H_0|Data)$) that the observed data for each plant-peptide combination can be fit to a function with no slope (i.e. no ROS production; see methods for more details) was then calculated. While $flg22_{T1}$ triggered ROS generation in 15 of the tested plants, $flgII-28_{T1}$ triggered ROS production only in 6 tested plants – all of which were in the *Solanaceae* family (Table 1). Two of the plants that did not respond to $flg22_{T1}$ were ecotypes of *Arabidopsis* known to be defective in the *FLS2* locus: Ws-0 (Gómez-Gómez *et al.*, 1999) and Cvi-0 (Dunning *et al.*, 2007); the third was celery (*Apium graveolens*). The $flgII-28_{T1}$ peptide, on the other hand, only elicited ROS in the three tested tomato cultivars, the two tested cultivars of pepper (*Capsicum annuum*), and the one tested cultivar of potato (*Solanum tuberosum*) (Table 1). Graphical representations of select ROS curves are depicted in Fig. S2.

The relative strength of the immune response triggered by flgII-28 and flg22 alleles is dependent on plant genotype

To test whether the derived flgII-28 alleles ($flgII-28_{K40}$ and $flgII-28_{Col338}$) are generally weaker elicitors of PTI compared to the ancestral $flgII-28_{T1}$ allele, we performed ROS analyses for both the ancestral and derived flgII-28 peptides on all *Solanaceae* tested above. We indeed observed that the derived alleles of flgII-28 triggered significantly less ROS than $flgII-28_{T1}$ on both tested cultivars of tomato. By contrast, the derived alleles of flgII-28 triggered only slightly less ROS on pepper (Fig. 2).

To determine if differences in ROS elicitation were statistically relevant, we modeled the ROS data for each plant-peptide combination using a shifted Gompertz-decay curve and determined differences in the kinetics of the ROS response between different peptides on the same plants (See the Materials and Methods section, Methods S1, and Fig. S1 for more information). Using this approach to compare the ROS curves depicted in Fig. 2, $flgII-28_{K40}$ and $flgII-28_{Col338}$ were found to give rise to ROS elicitation with significantly lower peak intensity and longer offset than $flgII-28_{T1}$ in both tomato cv 'Rio Grande' and tomato cv 'Sunpride'. Contrastingly, no significant differences among peptides were detected in ROS elicitation in pepper cv 'Jalapeno Early' (Tables S3, S4). Extending this statistical analysis to all other plants capable of detecting $flgII-28_{T1}$ (Table 1), $flgII-28_{K40}$ and $flgII-28_{Col338}$ were found to elicit significantly less ROS than $flgII-28_{T1}$ on potato cv 'Red Maria' and tomato cv 'Chico III'. However, as for pepper cv 'Jalapeno Early', the derived flgII-28 peptides do not trigger significantly less ROS on pepper cv 'CA Wonder' (Tables S5, S6).

A single nonsynonymous SNP distinguishes the flg22 allele of the *Pto* strain Col338 from the flg22 allele present in all other *Pto* strains including *Pto* DC3000 and T1 (Fig. 1). Peptides of this flg22 variant triggered stronger responses in some *Solanaceae* but weaker responses in others when compared to flg22$_{DC3000}$ (Fig. 2, Tables S7 and S8).

Ethylene production is another quantifiable PTI response (Boller and Felix, 2009). Fig. 3 shows that flgII-28$_{K40}$ and flgII-28$_{Col338}$ elicit significantly less ethylene biosynthesis in tomato cv 'Money Maker' than flgII-28$_{T1}$ further confirming that flgII-28$_{K40}$ and flgII-28$_{Col338}$ trigger weaker immune responses in tomato compared to flgII-28$_{T1}$. Moreover, flgII-28$_{T1}$ is at least as active in eliciting ethylene biosynthesis in tomato as flg22.

Overall, these data indicate that allelic variability in both epitopes of flagellin and in the plant PRRs that recognize them determine the intensity of PTI suggesting co-evolution between MAMPs and PRRs. We conclude that the derived lineages of *Pto* have evolved to avoid flagellin recognition by *S. lycopersicum* and/or *S. tuberosum* (and possibly other related *Solanaceae* not tested here) but these mutations in flagellin do not make it universally stealthier in all plant interactions.

Arabidopsis cannot perceive flgII-28 peptide

We further tested the ability of *A. thaliana* to detect flgII-28 in four independent PTI assays: the plant protection assay (Zipfel *et al.*, 2004), callose deposition (Adam & Somerville, 1996), ethylene production (Boller & Felix, 2009), and cell culture alkalinization (Meindl *et al.*, 1998). All four assays confirmed that Arabidopsis does not respond to flgII-28 peptide (Fig. S3A,B,E,F). We also observed that the flgII-28 allele of the Arabidopsis and tomato pathogen *Pto* DC3000, which differs by two amino acid residues from flgII-28$_{T1}$ (Fig. 1), does not elicit ROS in *A. thaliana* either (Fig. S3C), nor do two other alleles of *Pto* flgII-28 (Fig. S3D). Overall, detection of flgII-28 seems limited to a subset of species in the *Solanaceae* family, contrasting with flg22, which is broadly recognized across the plant kingdom (Albert *et al.*, 2010).

FLS2 is not sufficient for recognition of flgII-28

To test whether flgII-28 is recognized by FLS2, we transiently expressed *FLS2* from either tomato cv 'Roter Gnom' (Robatzek *et al.*, 2007), which is capable of detecting both flgII-28 and flg22 (Fig. S4), or *A. thaliana*, which is non-responsive to flgII-28 (Table 1), in non-responsive *N. benthamiana* plants. Plants were treated with flg22, flgII-28, or flg15$_{E. coli}$ – a truncation variant of flg22 that is recognized by tomato but not by *A. thaliana* or *N. benthamiana* (Bauer *et al.*, 2001; Robatzek *et al.*, 2007). Both FLS2 proteins accumulated in *N. benthamiana* (Fig. S5), and expression of *SlFLS2* conferred to *N. benthamiana* responsiveness to flg15$_{E. coli}$ (Fig. 4a), confirming functionality of the receptor. However, transient expression of either allele of *FLS2* did not confer responsiveness to flgII-28 (Fig. 4a).

Addition of flgII-28 peptide to a *S. peruvianum* cell culture saturated for the flg22 response triggers a further increase in extracellular alkalinization, and, vice versa, addition of flg22 peptide to *S. peruvianum* cell culture saturated for the flgII-28 peptide triggers further increase in extracellular alkalinization (Fig. 4b). Taken together, these results suggest that flgII-28 is recognized by a PRR distinct from FLS2.

Moreover, virus-induced gene silencing (VIGS) of *FLS2* in tomato had no effect on flgII-28 recognition but significantly attenuated ROS production elicited by flg22 treatment (Fig. 4c). Recent sequencing of the tomato genome (Consortium, 2012) revealed a close paralog located adjacent to the original *FLS2* on chromosome 2, which we designated as *FLS2.2*.

The DNA fragment in our FLS2-VIGS construct, cloned using *FLS2.1* (Solyc02g070890) has 8 stretches of at least 21 nucleotides with perfect identity to *FLS2.2*, (Solyc02g070910) and expression of both genes was decreased by *c.* 40% (Fig. S6). These data suggest that neither copy of *SlFLS2* contributes to recognition of flgII-28.

However, pretreatment of tomato leaf disks with flg22 significantly reduced ROS elicitation by subsequent addition of flg22 or flgII-28 relative to mock pretreatment (Fig. 4d) suggesting that flg22 and flgII-28 recognition share at least one common downstream signaling component. Because flgII-28 typically elicits significantly more ROS than flg22 in tomato, this result is not simply due to exhaustion of reagents used in the assay or exhaustion of ROS substrates within cells following flg22 pretreatment.

Alleles of *Pseudomonas syringae* flg22 are not universally recognized in either *Brassicaceae* or *Solanaceae*

The recently sequenced *Pcal* ES4326 strain (Baltrus *et al.*, 2011) causes disease in *Arabidopsis* (Dong *et al.*, 1991) and is related to strains that cause disease on various other *Brassicaceae* (Bull *et al.*, 2010, 2011). Because flg22$_{ES4326}$ is the most divergent flg22 allele compared to all other known *P. syringae* flg22 epitopes (only 12 identities between flg22$_{ES4326}$ and flg22$_{DC3000}$; Fig. 1; Table S2), we hypothesized that ES4326 and ES4326-like strains evolved a flg22 sequence adapted to avoid recognition. Indeed, flg22$_{ES4326}$ failed to elicit PTI based on ROS assays and did not promote resistance to *P. syringae* in either Arabidopsis (Fig. 5a,b; Table S9) or tomato (Fig. 5c,d; Table S9).

Therefore, diversity within *P. syringae* flg22 does not only affect the strength of the PTI response but can even lead to complete avoidance of PTI

When performing plant protection assays in tomato, pretreatment with flg22$_{ES4326}$ consistently led to more pathogen growth compared to the mock-treated plants (Fig. 5d), a result confirmed on a second tomato cultivar (Fig. S7). We hypothesized that flg22$_{ES4326}$ may interact with FLS2 in an antagonistic manner, reducing the detection of native flagellin from the pathogen during infection. Such characteristics have been described for truncated versions of flg22 (Felix *et al.*, 1999) and flg22$_{Rsol}$, the flg22 variant of *Ralstonia solanacearum* (Mueller *et al.*, 2012). To test this hypothesis we used cultured cells of wild tomato *S. peruvianum*, which respond to flg22$_{DC3000}$ and flgII-28$_{T1}$ with a rapid extracellular alkalinization response (Fig. 5e). As hypothesized, treating the cells with flg22$_{ES4326}$ did not lead to extracellular alkalinization but inhibited a subsequent response to flg22 (Fig. 5e) confirming that flg22$_{ES4326}$ acts as an antagonist for flg22 possibly by engaging the FLS2 receptor without triggering an immune response. Notably, pretreatment with flg22$_{ES4326}$ did not reduce the alkalinization response to flgII-28$_{T1}$ (Fig. 5f) further corroborating that flgII-28 is not an active ligand for FLS2.

Allelic diversity in FliC does not impact motility in a *Pto* DC3000δ*fliC* background

MAMPs are considered to be favorite targets for recognition by the plant immune system because they represent conserved structures with essential roles in invariant microbial processes. Thus, we tested whether the observed mutations in flagellin affect the swimming motility of *P. syringae*, which is the primary function of the flagellum. To quantify swimming motility, soft-agar swim plates were used in which flagellar-motile bacteria will spread from an initial point of inoculation (Hazelbauer *et al.*, 1969). In this assay, wild-type *Pto* strain K40 and *Pcal* strain ES4326 both were significantly less motile than the other tested strains (Fig. S8). However, besides the allelic differences in *fliC* there are many more genetic differences between the analyzed strains. Therefore, to specifically quantify the effect of the *fliC* SNPs in an isogenic background, we deleted the endogenous *fliC* gene in *Pto* DC3000 and complemented it with four different *fliC* alleles under the control of their

native promoters. As expected, *Pto* DC3000 *fliC* was found to be significantly impaired in motility. By contrast, strains complemented with the four *fliC* alleles were indistinguishable from each other and swam out from the initial point of inoculation at a level just below that of wild-type *Pto* DC3000 (Fig. 6). We conclude that the mutations in flagellin do not substantially affect motility.

Allelic diversity in *fliC* affects disease development but not growth of *P. syringae* in tomato

To test whether the *fliC* mutations affect bacterial growth *in planta*, tomato plants were infiltrated with *Pto* DC3000 wild-type, *Pto* DC3000 *fliC*, and the four complemented *Pto* DC3000 *fliC* strains described above. We hypothesized that the alleles of *fliC* containing the stealthier versions of flgII-28 would allow for higher growth compared to *Pto* DC3000. However, all strains grew to equally high population densities both 3 and 4 d following inoculation by syringe infiltration (Figs S9, 7a, respectively) and spray inoculation (Fig. S10A). It is surprising that even the uncomplemented *Pto* DC3000 *fliC* strain was able to reach a population density as high as that of the other strains even following spray inoculation in the absence of a surfactant. We hypothesize that *Pto* DC3000 either uses forms of motility other than swimming during infection of tomato or that the cost of being non-motile is counterbalanced by lower PTI elicitation due to the absence of flg22 and flgII-28 in this mutant.

Intriguingly, while the various *fliC* alleles did not affect bacterial growth in the *Pto* DC3000 *fliC* background, they did lead to significant differences in the development of leaf necrosis (Fig. 7b,c) suggesting that *in planta* expression of FliC protein can interfere with symptom development, which was recently demonstrated (Wei *et al.*, 2012), in an allele-dependent manner.

Allelic diversity in *fliC* affects virulence of *P. syringae* in *Arabidopsis*

To test the effects of the identified SNPs in a second pathogen - host interaction, *Arabidopsis* was inoculated with the complemented *Pto* DC3000 *fliC* strains. Interestingly, *Pto* DC3000 *fliC* complemented with *fliC*$_{K40}$ grew to significantly lower population densities than the other strains following inoculation by infiltration (Fig. 8a). Because flagella are known to play only a minor role in apoplastic growth (Schreiber & Desveaux, 2011), we also queried for differences in fitness among the strains following spray infection and confirmed the fitness defect of *Pto* DC3000 *fliC* complemented with *fliC*$_{K40}$ (Fig. S10B). Importantly, no surfactants were used in the spray inoculations to avoid creating an artificial condition in which flagella are less important. This strain also triggered less severe disease symptoms (Figs S10D, S11). The difference between *Pto* DC3000 *fliC* complemented with either *fliC*$_{K40}$ or *fliC*$_{T1}$ is particularly intriguing considering that the only amino acid difference between these two strains is a single S F mutation in the flgII-28 region of flagellin – a region that does not, as an isolated peptide, elicit PTI in *Arabidopsis.* To determine if the observed differences between the two strains were *FLS2*-dependent, leaves of an *fls2 Arabidopsis* mutant line (Xiang *et al.*, 2008) were inoculated by either spraying or infiltration. Interestingly, the differences between the two strains were abolished (Figs 8b, S10C). The differences were also abolished in *Arabidopsis* ecotypes that have a defect in *FLS2* (Fig. 8c). We conclude that diversity in flagellin beyond flg22 can significantly affect *P. syringae* virulence in an *FLS2*-dependent manner.

To rule out the possibility that the observed differences between complemented *Pto* DC3000 *fliC* strains were due to mutations accumulated during the transformation of the *Pto* DC3000 *fliC* strain with the different *fliC* alleles and not due to the different *fliC* alleles themselves, the results for growth and necrosis development in tomato and growth in

Arabidopsis were confirmed with a second set of independently transformed isolates of all strains (Fig. S12).

Discussion

Since flg22 was found to be sufficient to elicit PTI in multiple clades of seed plants belonging to angiosperms and even gymnosperms (Felix *et al.*, 1999; Albert *et al.*, 2010), studies of flagellin-triggered plant immunity have mainly focused on the flg22 epitope, though a flg22-independent flagellin perception system has been proposed for rice (Che *et al.*, 2000). However, in performing a population genomics analysis of a large collection of *Pto* strains (Cai *et al.*, 2011) we identified a second PTI-eliciting flagellin epitope, flgII-28. Studying pathogen diversity can thus lead to new insights into plant – microbe interactions (Cai *et al.*, 2011) and provides an efficient strategy for the identification of new MAMPs, as recently confirmed by others (McCann *et al.*, 2012).

Continuing characterization of flagellin diversity, here we have shown that: flgII-28 elicits immunity in several *Solanaceae* species but not in any members of other plant families tested so far; significant and biologically relevant allelic diversity exists at both MAMP loci of flagellin (flg22 and flgII-28), demonstrating the ability of adapted pathogens to overcome PTI through MAMP diversification; allele-dependent differences in PTI are also dependent on plant genotype, implying natural variability in the plant receptor(s) for these epitopes; the PTI-altering mutations in flagellin do not come at the cost of microbial fitness in the tested conditions, suggesting that MAMPs are under weaker purifying selection than previously thought; FLS2 is likely not the flgII-28 receptor although allelic diversity in flgII-28 affects plant virulence in an *FLS2*-dependent manner in *Arabidopsis*.

flgII-28 elicits PTI responses in several Solanaceae species

Based on the flgII-28 alleles and plant species analyzed here, recognition of flgII-28 is limited to a subset of *Solanaceae* species. The most obvious explanation for this result is that flgII-28 recognition evolved in a relatively recent ancestor of some *Solanaceae*. However, at this point we cannot exclude the possibility that flgII-28 recognition is widespread in the plant kingdom and that the plants that were non-responsive to flgII-28 in this study recognize not yet tested alleles of flgII-28.

Significant diversity in MAMPs and MAMP perception reveals an evolutionary PTI arms race

Because of the relative differences in the strength of PTI triggered in closely related *Solanaceae* by the different alleles of the flgII-28 peptide, we infer the importance of allelic variability in both MAMPS and corresponding plant PRRs. Also, Vetter *et. al.* recently described extensive variation in recognition of flg22 across *Arabidopsis* ecotypes due to allelic differences in *FLS2* sequences, differences in FLS2 protein abundance, and differences in downstream signaling components (Vetter *et al.*, 2012). However, the observed variability in flgII-28 recognition among *Solanaceae* appears to be primarily linked to sequence diversity of a putative flgII-28 receptor. In fact, differences in abundance of a receptor or in elements in downstream signaling between plant species would equally affect recognition of all flgII-28 alleles and not lead to relative differences in recognition of flgII-28 alleles as we observed.

Since flgII-28$_{K40}$ and flgII-28$_{Col338}$ elicit less PTI in tomato and almost entirely replaced the ancestral flgII-28$_{T1}$ alleles in *Pto* populations in Europe and North America over the last 30 yr, we had hypothesized that these alleles recently evolved under selection pressure in tomato agricultural settings (Cai *et al.*, 2011). However, we had also noticed that the

flgII-28$_{K40}$ allele appeared in two separate genetic lineages of *Pto* (Cai *et al.*, 2011) suggesting that these alleles might have been acquired through horizontal gene transfer. Since we now found that the same alleles also trigger less PTI in other plants, like potato, it is plausible that these alleles pre-existed in the *P. syringae* population, had evolved under selection pressure for PTI-avoidance on hosts other than tomato, and were only later acquired by *Pto* through horizontal gene transfer.

On the other hand, the significant reduction in bacterial growth and disease development in Arabidopsis during *Pto* DC3000 infection due to the flagellin allele of *Pto* K40 compared to *Pto* DC3000 shows that adaptation for PTI avoidance on some plant species (here tomato) may have a collateral effect of increasing PTI on other plants (here Arabidopsis). This may explain why the mutations that gave rise to the flgII-28 alleles of *Pto* K40 and Col338 are not present in any other sequenced *P. syringae* strains. For the first time, effects of flg22 and flgII-28 on bacterial growth were observed by expressing different *fliC* alleles under their native promoters in the same genetic pathogen background during infection. These results thus reveal the biological significance of allelic diversity in MAMPs.

The complete absence of PTI elicited by the flg22 allele of P*cal* ES4326 in *Arabidopsis* and tomato is also remarkable. Interestingly, P*cal* ES4326 does not have the effector *avrPto*, which interferes with FLS2 kinase activity (Baltrus *et al.*, 2011) and the PcalES4326 homolog of the effector *avrPtoB* is missing the ubiquitin-ligase domain which has been reported to ubiquitinate FLS2 leading to its degradation (Guttman *et al.*, 2002). This suggests that evasion of PTI through allelic diversification at MAMP loci is similarly efficient in overcoming PTI as delivery of PTI-suppressing effector proteins.

Diversity in flg22 and flgII-28 among closely related pathogens is particularly striking because MAMPs are considered to be essential for important functions (Jones & Dangl, 2006; Bittel & Robatzek, 2007; Lacombe *et al.*, 2010), such as motility in the case of flagellin, and thus under purifying selection. However, to our surprise, none of the differences in flg22 or flgII-28 led to deficiencies in motility *in vitro* when placed in the same genetic background (*Pto* DC3000 *fliC*) demonstrating that there is significant room for divergence at both of these epitopes without cost to motility, thus unsettling the theory that PTI is more evolutionarily stable than its effector-recognizing corollary –Effector Triggered Immunity. Importantly, the differences in flgII-28 and flg22 within the *Pto* lineage are the only polymorphisms in FliC; therefore, the success of these mutations cannot be explained by intragenic compensatory changes, which have previously been implicated as essential for bacteria to mutate MAMPs to avoid recognition but maintain function (Andersen-Nissen *et al.*, 2005).

What is the flgII-28 receptor?

In bacterial flagellin, flg22 and flgII-28 are physically linked by a stretch of only 33aa residues, hinting at the possibility that both epitopes might act via the same receptor, FLS2. However, when applied as separate synthetic peptides both epitopes act as distinct and independent MAMPs on tomato cells. For example, cells pretreated with highly saturating concentrations of one of the peptides still respond to the other peptide with further response (Fig. 4b). Therefore, the hypothesis of FLS2 as receptor for both epitopes would imply that: flgII-28 and flg22 have distinct binding sites within FLS2 because the two peptides do not compete for binding (Fig. 4b); and that binding of flgII-28 is a peculiar feature of some of the Solanaceous FLS2 orthologs. As an argument against this hypothesis, we observed that gene silencing of *FLS2* in tomato attenuated the response to flg22 but not flgII-28 in the ROS assays. Importantly also, heterologous expression of the tomato ortholog *Sl FLS2* is not sufficient to confer recognition of flgII-28 to *N. benthamiana*. This strongly suggests that tomato has at least one additional factor that specifies perception of flgII-28. We tentatively

termed this yet-to-be-identified factor FLS3 (Flagellin sensing3). At present, we cannot exclude a role of FLS2 in perception of flgII-28. For example, the postulated FLS3 might be a co-receptor or signaling component that is specifically required for FLS2-dependent detection of flgII-28 and only present in Solanaceae species that respond to flgII-28. However, based on the cumulative evidence from our experiments, we rather conclude that it is more likely that the postulated FLS3 acts as the genuine receptor for flgII-28. Candidates for such a FLS3 receptor are among the hundreds of orphan RLKs and receptor-like proteins in higher plants, many of which show species specific variation that could fit to the occurrence of flgII-28 perception in plants.

However, if FLS2 is not the flgII-28 receptor, how can we explain the *FLS2*-dependent virulence effect of mutations in flgII-28 during infection of Arabidopsis? Multiple assays showed that flgII-28 peptide itself is not an elicitor in Arabidopsis (Fig. S2) and flgII-28 variants only had an *FLS2*-dependent virulence effect when in the context of the entire *FliC* protein. Therefore, Arabidopsis might have an FLS3 allele that cannot bind flgII-28 peptide by itself but requires a larger region of FliC for interacting with flgII-28. FLS2 may act as a necessary co-receptor for FLS3 and FLS2-flg22 binding may even be necessary to place the flgII-28 region of FliC in proximity of the flgII-28 binding site of FLS3. This is reminiscent of the mammalian macrophage flagellin receptor NLCR4 which acts as a co-receptor hub for multiple MAMPs – both flagellin and Type III secretion system components – after the elicitors bind to the NOD-like receptors NAIP5 and NAIP2 respectively (Kofoed & Vance, 2011; Zhao *et al.*, 2011). An equally likely alternative hypothesis is that mutations in flgII-28 lead to *FLS2*-dependent PTI modulation through an indirect mechanism. For example, mutations in flgII-28 may alter the release of flagellin monomers during flagella assembly or they may cause conformational changes in the FliC structure altering the exposure of flg22 to FLS2.

An emerging parallel in plant and animal innate immunity

Interestingly, the proposed TLR5 recognition site of flagellin in animals (Yoon *et al.*, 2012) partially overlaps with flgII-28 while flg22 seems not to play any role in TLR5 –flagellin binding. Therefore, future studies of FLS3-mediated recognition of flagellin in plants, in particular, identification of FLS3 and elucidation of flgII-28-FLS3 interaction will be highly informative in unraveling a new, intriguing example of convergent evolution in plants and animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Position:1 30                                          51 84                                                 111 282
   DC3000:···· TRLSSGLKINSAKDDAAGLQ I A ···· ESTNILQRMRELAVQSRNDSNSATDREA ····
       T1:···· TRLSSGLKINSAKDDAAGLQ I A ···· ESTNILQRMRELAVQSRNDSNSSTDRDA ····
      K40:···· TRLSSGLKINSAKDDAAGLQ I A ···· ESTNILQRMRELAVQFRNDSNSSTDRDA ····
Colombia338:···· TRLSSGLKI I SAKDDAAGLQ I A ···· ESTNILQRMRELVVQSRNDSNSSTDRDA ····
    ES4326:···· ERLSTGKKINTASDDAGGSVTQ ···· ESVSILQRMRELAVQSRNDSNSSEGRDA ····
              flg22                                         flgII-28
```

Fig. 1.
Alignment of flg22 and flgII-28 regions of FliC of the six *Pto* strains (listed on left) used in
this study. Numbers on top row indicate the amino acid positions within the flagellin
protein. Bold/underlined letters indicate single nucleotide polymorphisms (SNPs).
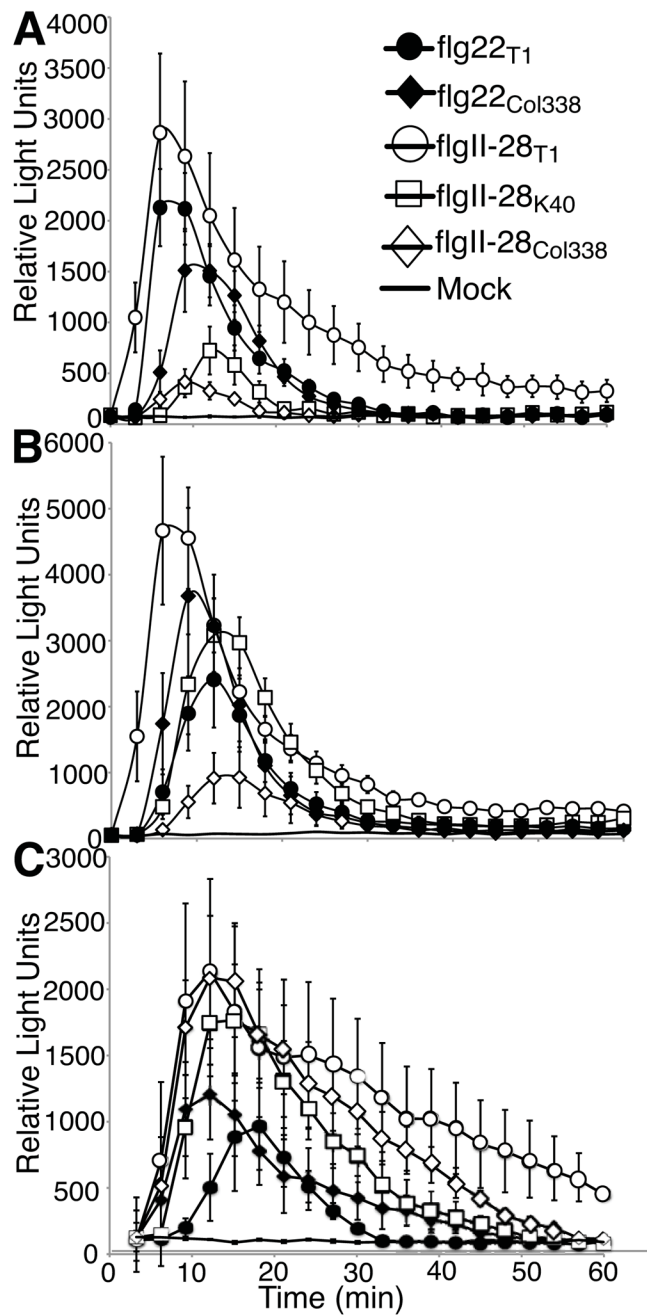
Fig. 2.
Pepper and tomato plants differ in their relative sensitivity to the flgII-28 alleles from *Pto* strains T1, Col338, and K40. Leaf punches of either *Solanum lycopersicum* cv 'Rio Grande' (a), *Solanum lycopersicum* cv 'Sunpride' (b), or *Capsicum annuum* cv 'Jalapeno Early' (c) were treated with 1μM (a, b) or 100nM (c) of indicated peptides and reactive oxygen species (ROS) was measured for 1 h immediately following treatment. Data shown are the average of 6 replicate leaves and error bars represent ± SE. Similar results were obtained in at least 4 independent experiments.
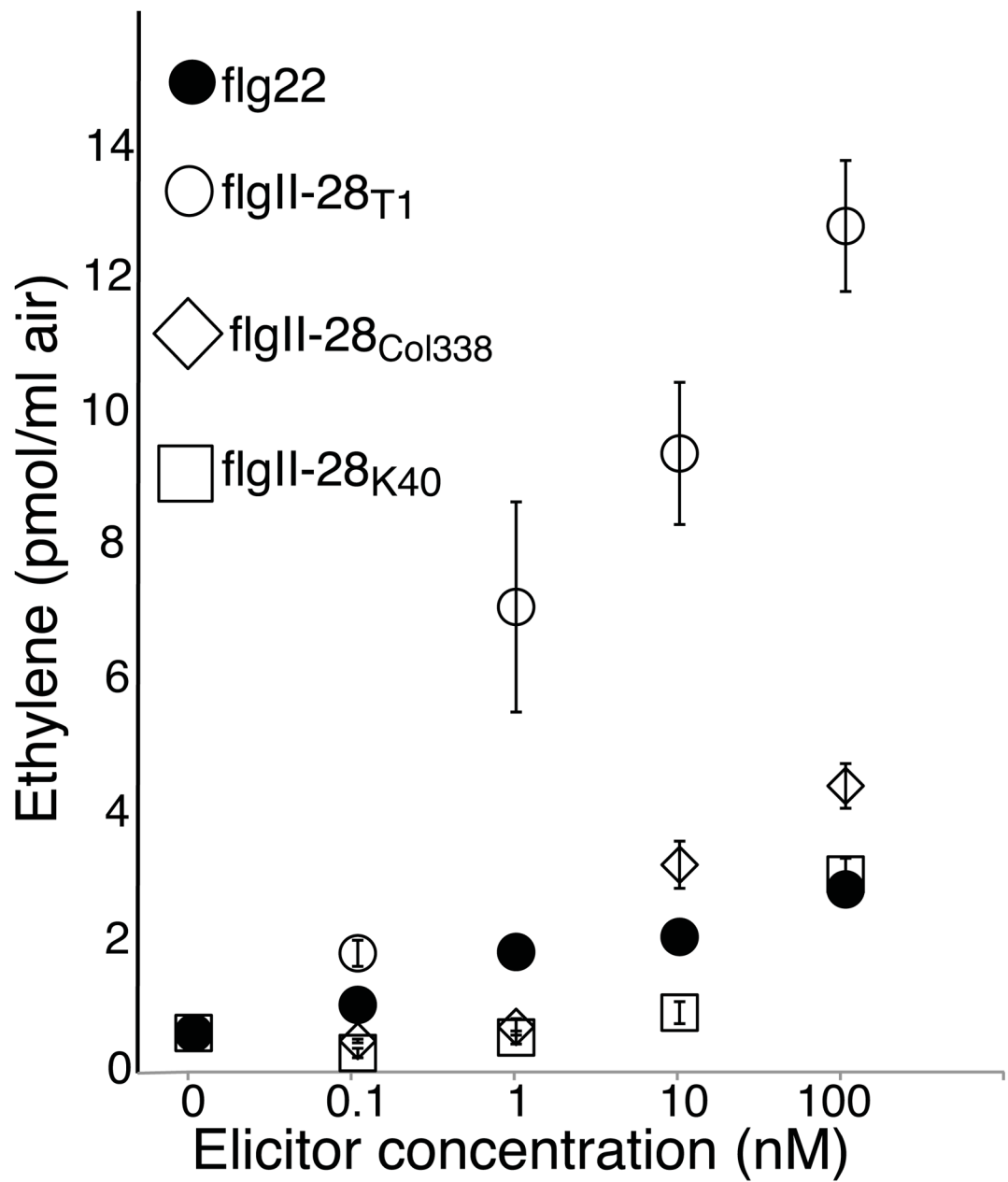
Fig. 3.
Pattern-triggered immunity (PTI)-responses of tomato to flgII-28 alleles from *Pto* strains T1, Col338, and K40. Leaf strips of 4-wk-old *Solanum lycopersicum* cv 'Money Maker' were treated with the indicated peptides at concentrations ranging from 0.1 to 100nM and ethylene accumulating during 4 h of treatment was measured. Data shown are the average of 4 replicates and error bars represent ± SE. Similar results were obtained in 2 independent experiments.
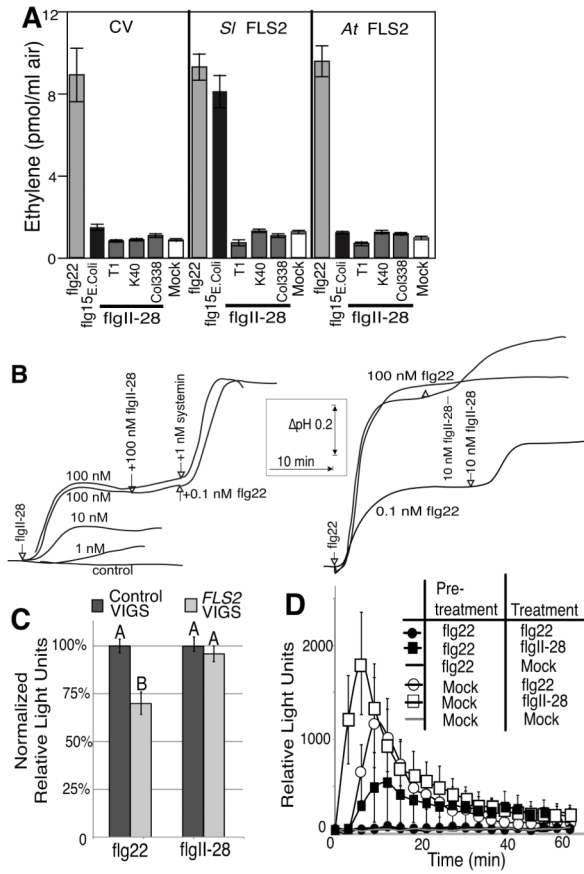
Fig. 4.

Tomato FLS2 is not sufficient for perception of flgII-28$_{T1}$. (a)Transient expression of FLS2 in *Nicotiana benthamiana* does not lead to the detection of flgII-28$_{T1}$. Leaf punches from leaves agrotransformed with control vector (CV), tomato FLS2 (*Sl FLS2*) and Arabidopsis FLS2 (*At FLS2*) were treated with indicated peptides at 1μM concentration and total ethylene production was measured 3.5 h after treatment. Data shown are the average of 6 replicates; error bars represent ± SE. (b) Alkalinization of extracellular pH in cell cultures derived from the wild tomato species *peruvianum* to treatment with the peptides flgII-28$_{T1}$, flg22, and systemin. (c) The oxidative burst triggered by 10nM flg22 (grey bars) or 10nM flgII-28$_{T1}$ (white bars) was quantified in *S. lycopersicum* cv 'Rio Grande' silenced for the flagellin receptor *FLS2* (*n*=48; 6 experiments) or the flagellin co-receptor *Bak1* (*n*=18; 3 experiments) compared to non-silenced control plants (*n*=48; 6 experiments). Total reactive oxygen species (ROS) production was measured over a 45 min time period, and the average of 3–4 leaf disks per plant was calculated. Bar values represent the normalized mean and the error bars ± SE. The statistical significance between silenced and control plants for each peptide treatment was assessed using Student's *t*-test with the alpha level 0.005. (d) Leaf punches of 4-wk-old tomato cv 'Rio Grande' were treated with either 1μM of flg22 or H$_2$O (mock) as indicated and then 50 min later treated with indicated peptides and ROS was measured for 1 h immediately following treatment. Similar results were obtained in 4 independent experiments. Data shown are the average of 6 replicate leaves.
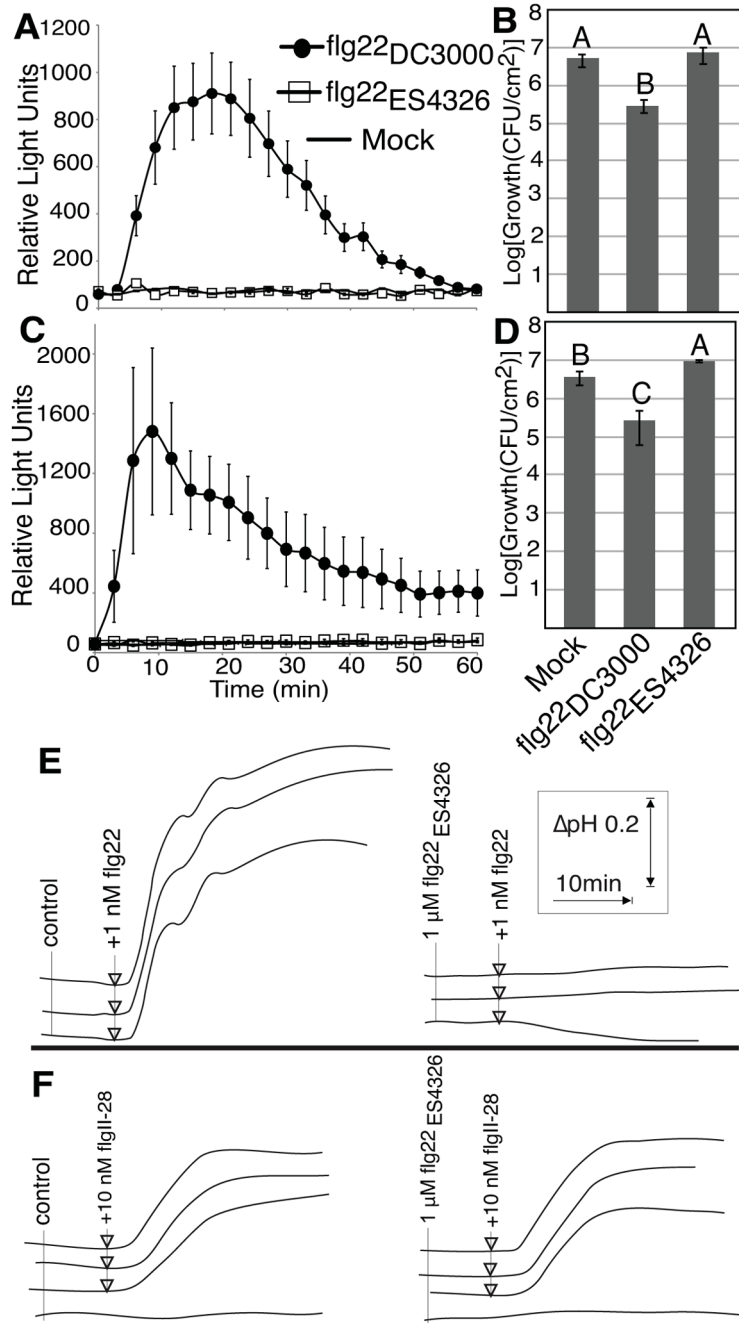
Fig. 5.
The flg22 allele of *Pcal* ES4326 is inactive as a microbe-associated molecular patterns (MAMP) but acts as an antagonist for flg22. Reactive oxygen species (ROS) production in leaf punches of either *A. thaliana* ecotype Col-0 (a) or tomato cv 'Chico III' (c) with 1μM of indicated peptides. flg22$_{DC3000}$, circles; flg22$_{ES4326}$, squares; mock, lines. Data shown are the average of 6 replicate leaves and error bars represent standard error. Similar results were obtained in at least 3 independent experiments. Plant protection in leaves of *A. thaliana* ecotype Col-0 (b) or tomato cv 'Chico III' (d) after infiltration with 1μM flg22 or 1 μM flg22$_{ES4326}$. Leaves were inoculated with either strain *Pto* DC3000 (b) or strain *Pto* DC3000 *avrpto1 avrptoB* (d) by spray infection 16 h later and total bacterial populations

were quantified at 4 d. Different letters indicate significant differences at the 0.05 alpha level in an unpaired Student's *t*-test. Data represent the average of 4 replicate leaves and error bars represent standard error. Similar results were obtained in at least 3 independent experiments. (e, f): Extracellular pH in *S. peruvianum* (wild tomato) cell cultures treated with 1μM flg22$_{ES4326}$ and 1nM flg22 (e) or 10nM flgII-28 (f) as indicated by the arrows. Each line represents the pH readout from a single well of cell culture.
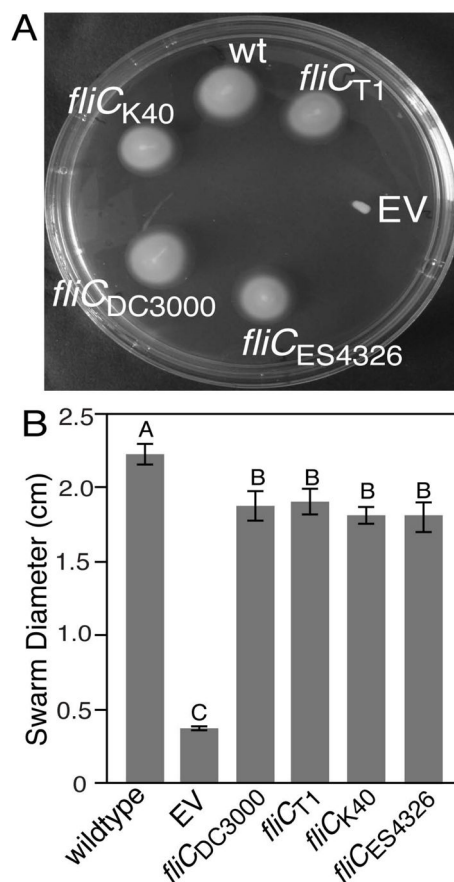
Fig. 6.
The alleles of *fliC* from *Pto* strains T1, DC3000, K40 and *Pcal* ES4326 reconstitute motility in *Pto* DC3000 *fliC*. (a) Swim size on a KB swim plate (0.3% agar) 2 d following toothpick inoculation with wild-type *Pto* DC3000 (wt), *Pto* DC3000 *fliC* with an empty vector (EV) or *Pto* DC3000 *fliC* complemented with the indicated alleles of *fliC*. (b) Quantification of swim size from 8 replicate plate. Error bars represent ± SE. Letters indicate significant differences at the 0.05 alpha level in an unpaired Student's *t*-test. Similar results were obtained in 3 independent experiments.
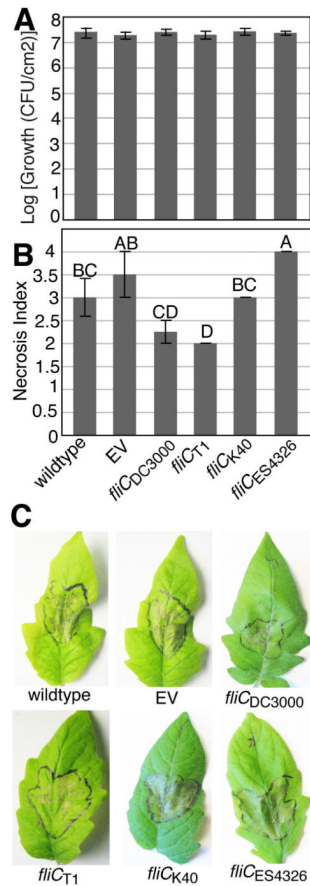
Fig. 7.

The different alleles of *fliC* have no effect on bacterial growth in tomato leaves but contribute differentially to induction of necrosis. (a) 4-wk-old tomato cv 'Rio Grande' plants were infiltrated with either wild-type *Pto* DC3000 (wt), *Pto* DC3000 *fliC* with an empty vector (EV) or *Pto* DC3000 *fliC* complemented with the indicated alleles of *fliC* at 1×10^{-4} O.D. Growth was quantified 4 d following infection. (b) The same plants used to quantify growth were scored for necrosis severity on the scale: 1, no necrosis; 2, moderate necrosis; 3, heavy necrosis; 4, total necrosis. (c) Representative pictures of necrosis induced by the different *Pto* DC3000 *fliC* complemented strains. Similar results were obtained in 4 independent experiments. Data shown are the average of 4 replicate leaves and error bars represent ± SE.

Fig. 8.
The different of alleles of *fliC* affect virulence on *Arabidopsis* in an *FLS2*-dependent manner. 4-wk-old Col-0 wild-type plants (a) or *fls2* mutant plants (b) were syringe infiltrated with either wild-type *Pto* DC3000 (wt), *Pto* DC3000 *fliC* with an empty vector (EV) or *Pto* DC3000 *fliC* complemented with the indicated alleles of *fliC* at 1×10^{-3} O.D. Bacteria were quantified at 0 and 4 d following infection. (c) The indicated strains were syringe infiltrated into either Col-0 or *fls2* null ecotypes Ws-0 or Cvi-0 at 1×10^{-3} O.D. Growth was quantified 4 d following infection. Data shown are the average of 8 replicate leaves. Error bars represent ± SE. Similar results for all sections were obtained in at least 3 independent experiments.

Table 1

Production of reactive oxygen species (ROS) in response to either flg22$_{T1}$ or flgII-28$_{T1}$ across the plant kingdom

		Elicitor:	
		flg22	flgII-28
Plant	cv/eco.	P(H0|Data)[a]	P(H0|Data)[a]
Brassicaceae			
Arabidopsis	Ws-0	\ (0.98)	\ (0.99)
Arabidopsis	Cvi-0	\ (1.00)	\ (0.99)
Arabidopsis	Col-0	+ (0.00)	\ (0.98)
Cauliflower	Toscana 2	+ (0.00)	\ (0.96)
Turnip	P. T. W. Globe	+ (0.00)	\ (0.99)
Radish	Champion	+ (0.00)	\ (0.99)
Fabaceae			
Bean	Red Mex	+ (0.00)	\ (0.20)
Convolvulaceae			
Morning Glory*	-	+ (0.03)	\ (0.70)
Apiaceae			
Celery	S. V. Pascal	\ (0.96)	\ (0.76)
Plantaginaceae			
Snapdragon	Cook's Tall	+\ (0.11)	\ (0.79)
Solanaceae			
Tomato	ChicoIII	+ (0.00)	+ (0.00)
Tomato	Sunpride	+ (0.00)	+ (0.00)
Tomato	Rio Grande	+ (0.00)	+ (0.00)
Potato	Red Maria	+\ (0.15)	+ (0.00)
Bell Pepper	CA Wonder	+ (0.00)	+ (0.00)
Jalap. Pepper	Early	+ (0.00)	+ (0.00)
Eggplant[b]	MM643	+ (0.00)	\ (1.00)
Tobacco	Burly	+ (0.00)	\ (0.99)
N. benthamiana	-	+ (0.00)	\ (0.99)

ROS response of selected species of the *Brassicaceae*, *Solanaceae* and other plant families to flg22 and flgII-28 peptides. +, strong response; \, no response; +\, weak to no response. Data represent the analysis from one experiment of 6 leaf punches. Similar results were obtained in 2–12 independent experiments (plants tested in only 2 experiments labeled with *).

[a] Posterior probability of the data being fit to the null model (no response – the function being fit is a flat line with no slope) explaining the observed data (*i.e.* the probability that there is no measurable production of ROS). See the Materials and Methods section for more details.

[b] In ~25% of experimental repeats flgII-28 did trigger significant amounts of ROS production cautioning the variability among plants in this assay.