Bacterial disease resistance in *Arabidopsis* through flagellin perception

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Plants and animals recognize microbial invaders by detecting pathogen-associated molecular patterns (PAMPs)[1–5] such as flagellin[6–10]. However, the importance of flagellin perception for disease resistance has, until now, not been demonstrated[7–11]. Here we show that treatment of plants with flg22, a peptide representing the elicitor-active epitope of flagellin[6], induces the expression of numerous defence-related genes and triggers resistance to pathogenic bacteria in wild-type plants, but not in plants carrying mutations in the flagellin receptor gene *FLS2*. This induced resistance seems to be independent of salicylic acid, jasmonic acid and ethylene signalling. Wild-type and *fls2* mutants both display enhanced resistance when treated with crude bacterial extracts, even devoid of elicitor-active flagellin, indicating the existence of functional perception systems for PAMPs other than flagellin. Although *fls2* mutant plants are as susceptible as the wild type when bacteria are infiltrated into leaves, they are more susceptible to the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 when it is sprayed on the leaf surface. Thus, flagellin perception restricts bacterial invasion, probably at an early step, and contributes to the plant's disease resistance.

In plants, disease resistance has been studied most thoroughly in cases that depend on the presence of specific resistance genes (*R* genes) conferring immunity to particular races of pathogens. The proteins encoded by *R* genes were shown to mediate specific recognition of factors specified by particular avirulence genes (*Avr* genes) in the pathogens[12,13]. In addition to *R*-gene-related mechanisms, plants have broader, more basal perception systems for patterns characteristic for entire groups or classes of microorganisms, so-called general elicitors[14], which are conceptually equivalent to PAMPs[1–5]. In contrast to *R*-gene-dependent defence, the responses to general elicitors do not always result in the cell death associated with the hypersensitive response, and the exact role of general elicitors in plant disease resistance is still unclear. PAMPs that act as general elicitors in plants include chitin[15] and ergosterol[16] from fungi, and flagellin[6] and lipopolysaccharides[17] from bacteria. Flagellin, the subunit building the filament of the bacterial flagellum, is also recognized as a PAMP in mammals, by way of the Toll-like receptor TLR5 (refs 9, 10). In *Arabidopsis*, perception of flagellin occurs by recognition of the most conserved domain in its amino terminus, represented by the peptide flg22 (ref. 6). Perception of this elicitor-active domain depends on the LRR-type receptor kinase FLS2 (flagellin sensing 2)[8] and activates a downstream mitogen-activated protein kinase pathway, composed of *At*MEKK1, *At*MKK4/*At*MKK5 and *At*MPK3/*At*MPK6 (ref. 7; the prefix *At* indicates *Arabidopsis thaliana*). flg22 induces numerous defence-related genes in *A. thaliana*, and the responses triggered by flg22 show great similarity to *R*-gene-mediated responses[18].

Here we studied the role of flagellin perception in bacterial disease resistance. In a first step we extended a previous transcriptional analysis[18] by comparing flg22-induced changes in intact wild-type and *fls2* mutant seedlings, using the full-genome Gene-Chip ATH1 (Affymetrix) of *A. thaliana* (about 23,000 genes). After a 30-min treatment of wild-type seedlings with flg22, 966 genes were categorized as upregulated, 625 of them more than 2.5-fold; and 202 were categorized as downregulated, 35 of them more than 2.5-fold (Supplementary Fig. 1 and Supplementary Table 1). In seedlings of the flagellin-insensitive mutant *fls2-17*, carrying a point mutation in the kinase domain of FLS2 (G1064R)[8], treatment with flg22 showed minor changes in six genes only (less than twofold changes). These genes do not belong to those regulated by flg22 in wild-type seedlings (Supplementary Table 1), indicating random fluctuations. This result demonstrates the validity of the criteria used to classify changes in the wild type as significant even below the 2.5-fold threshold, and it clearly shows that flagellin perception and signalling depend absolutely on the presence of a functional FLS2 receptor.

As well as a large group of genes with unknown functions (328 genes), a considerable number of the upregulated genes can be classified as being involved in signal perception (155 genes encoding receptor-like kinases (RLK) and *R* genes), signal transduction (145 genes), transcriptional regulation (87 genes), and potential antimicrobial action (29 genes) (Supplementary Table 2). Among the genes that are rapidly induced at the transcriptional level are the following genes (see MIPS database, http://mips.gsf.de/prog/thal/db/index.html): *FLS2* (At5g46330), *MEKK1* (At4g08500), *MKK4* (At1g51660), *MPK3* (At3g45640) and *WRKY22* (At4g01250). These genes encode elements that have previously been shown to be involved in the perception and transmission of the flg22 signal[7,8]. A similar positive feedback regulation with transcriptional activation of the components involved in the perception and signalling has been reported for the innate immune response in *Drosophila*[19,20]. Interestingly, our previous analysis of promoter sequences from flg22-induced genes revealed an over-representation of W-boxes[18], that is, *cis*-elements, which confer the specific binding of WRKY

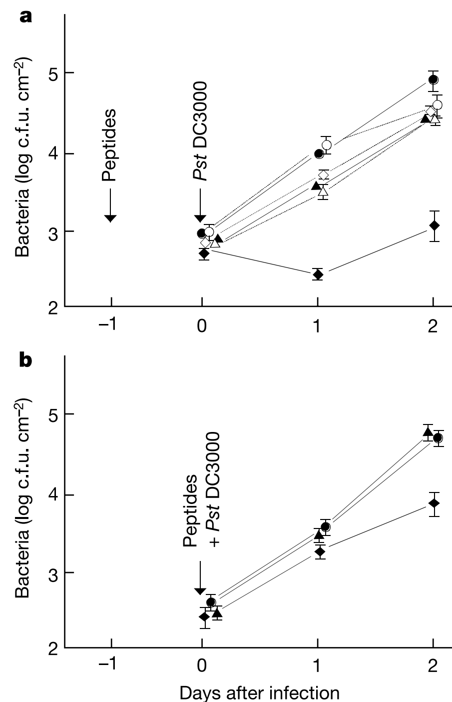

Figure 1 Treatment with flagellin limits *Pst* DC3000 growth. **a**, *Arabidopsis* wild-type L*er*-0 (filled symbols) and *fls2-17* (open symbols) plants were pretreated for 24 h by leaf infiltration with 1 μM flg22 (diamonds) or flg22$^{A.tum}$ (triangles). Subsequently, leaves were infected with 10^5 c.f.u. ml^{-1} *Pst* DC3000, and bacterial growth was assessed 1 and 2 days after infection. Control L*er*-0 and *fls2-17* plants (circles) were not pretreated before bacterial infection. **b**, L*er*-0 plants were infiltrated simultaneously with 1 μM flg22 or flg22$^{A.tum}$ and 10^5 c.f.u. ml^{-1} *Pst* DC3000. Controls were treated with bacteria only. Results shown are means ± s.e.m. ($n = 8$).

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transcription factors[21]. A similar over-representation of W-boxes was found when promoter sequences from all flg22-induced *RLK* genes were analysed (Supplementary Table 4). Because several WRKY factors are among the strongly induced genes after 30 min, it will be interesting to test whether a self-amplification system leads to even more pronounced induction of these genes after prolonged treatment with flg22. The induced RLKs and *R* genes constitute one-sixth of all upregulated genes (Supplementary Tables 2 and 3). With regard to the numbers of genes comprising these families in the *Arabidopsis* genome, this indicates an over-representation of 4.3-fold for RLKs and 3.8-fold for *R* genes, respectively. In summary, as well as the induction of numerous elements of the defence response, flagellin treatment seems to induce factors with an important function in the amplification of the signal and factors leading to an enhanced sensitivity of the plant to further stimuli sensing the presence of invading microorganisms. In particular, one could speculate that some of the induced RLKs and *R* genes might be involved in the recognition of other, as yet unidentified, PAMPs or *Avr* signals.

Transient overexpression of constitutively active MEKK1, MKK4, or wild-type WRKY29 resulted in reduced disease symptoms after treatment with *Pseudomonas syringae* pv. *maculicola* ES4326 or *Botrytis cinerea*[7]. To test whether direct induction of this signalling chain by flagellin leads to increased plant resistance, growth of pathogenic bacteria *in planta* was addressed after pretreatment of leaves with the flg22 elicitor. Wild-type and *fls2-17* mutant *Arabidopsis* plants were pretreated either with flg22 or the inactive analogue[22] flg22$^{A.tum}$ by leaf infiltration 1 day before challenge with pathogenic *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) bacteria (Fig. 1a). In wild-type plants that received either no pretreatment (controls) or pretreatment with flg22$^{A.tum}$, bacteria multiplied at the same rate. However, bacterial growth was strongly decreased in plants pretreated with flg22 (about 100-fold difference at 2 days after infection). In *fls2-17* plants, pretreatment with flg22 did not lead to a decreased growth of bacteria (Fig. 1a). This result shows that the induction of resistance depends on a functional FLS2, and also that flg22 has no antimicrobial activity itself. Decreased bacterial growth was also observed, but less so, when flg22 peptide was applied concomitantly with the bacterial inoculum (Fig. 1b), indicating that exposure of elicitor-active flagellin present in the injected *Pst* DC3000 bacteria might be a limiting factor for efficient induction of the basal resistance, at least in the absence of *R*-gene-dependent detection of *Avr* factors.

Analysis of mutants and transgenic plants have revealed the importance of the salicylic acid, jasmonic acid and ethylene pathways in *Arabidopsis* resistance against pathogens[23]. We tested the requirements of flg22-induced resistance for these previously identified defence signalling elements in plants mutated in *NPR1*, *EDS1*, *SGT1*, *RAR1*, *ETR1*, *EIN2*, *JAR1*, *PAD2* or *PAD4*, or in plants overexpressing *NahG*. Whereas bacterial growth showed some accession-dependent and mutation-dependent variation, all mutants still exhibited a significant flg22-induced reduction in bacterial growth (Table 1). Thus, the genes tested are not required for flg22-induced resistance. It is surprising that *NPR1*, *EDS1* and *PAD4*, which are essential for salicylic-acid-mediated resistance[23] and are transcriptionally induced as rapidly as 30 min after treatment of seedlings with flg22 (Supplementary Table 2), are not involved in the observed flg22-induced resistance. Furthermore, *PR1*, whose induced expression through NPR1 is a marker for salicylic-acid-mediated resistance[23], is activated 24 h after treatment with flg22 (ref. 24). However, our findings are consistent with the fact that flg22- and chitin-induced phosphorylation of the ankyrin-repeat protein *At*Phos43 was independent of NPR1 and salicylic acid[25]. flg22 activates the production of ethylene and triggers a rapid oxidative burst[6]. We therefore propose that flg22 induces the activation of the salicylic acid, jasmonic acid and ethylene pathways in parallel and that knocking out a single pathway alone does not abolish the induction of resistance. Alternatively, signalling elements that are as yet unknown, or the induction of antimicrobials, reactive oxygen species or cell wall reinforcement, might be responsible for inhibiting bacterial growth.

To test the involvement of PAMP perception systems other than flg22–FLS2 in resistance, we pretreated wild-type and mutant *fls2-17* plants with crude extracts obtained from *Pseudomonas syringae* pv. *syringae* (*Pss*), *Pst* DC3000 and *Agrobacterium tumefaciens*. All bacterial extracts—even that from *A. tumefaciens*, a species with an inactive flg22 peptide sequence[22]—induced medium alkalinization of *Arabidopsis* cell cultures (data not shown) and therefore showed clear elicitor activity. Pretreatment of plants with all the bacterial extracts resulted in a decreased growth of the pathogenic bacteria *Pst* DC3000 in comparison with that in control wild-type plants (Fig. 2). The same effect was also observed in *fls2-17* plants, but to a smaller extent in the case of a pretreatment with *Pst* DC3000 extracts. Thus, extracts from the tested bacteria contain at least one elicitor of resistance distinct from flagellin, and we propose that *Arabidopsis* has additional detection systems for these, as yet undefined, PAMPs.

Because flg22 perception induced disease resistance in plants, we tested whether plants lacking flagellin perception are more susceptible to pathogenic bacteria carrying elicitor-active flagellin. Inter-

Table 1 **flg22-induced resistance in plants affected in salicylic acid, jasmonic acid and ethylene signalling**

	Bacterial count (log c.f.u. cm^{-2})	
Line	flg22$^{A.tum}$	flg22
Col-0	4.6 ± 0.2	3.4 ± 0.2
NahG	5.9 ± 0.01	4.7 ± 0.1
etr1-3	4.4 ± 0.3	2.7 ± 0.2
ein2-1	3.5 ± 0.25	2.7 ± 0.3
jar1-1	4.6 ± 0.15	3.5 ± 0.2
pad2-1	5.0 ± 0.1	3.9 ± 0.3
pad4-1	5.5 ± 0.2	3.6 ± 0.2
L*er*-0	5.1 ± 0.1	3.5 ± 0.1
fls2-17	4.8 ± 0.25	4.9 ± 0.1
eds1-2	5.6 ± 0.05	3.7 ± 0.2
sgt1b-3	5.0 ± 0.2	3.6 ± 0.05
rar1-13	5.2 ± 0.2	3.5 ± 0.2
No-0	5.0 ± 0.2	2.6 ± 0.25
npr1-5	4.5 ± 0.15	2.2 ± 0.05

The following were pretreated for 24 h with 1 μM flg22 or flg22$^{A.tum}$: *Arabidopsis* transgenic *NahG* and mutants *etr1-3*, *ein2-1*, *jar1-1*, *pad2-1* and *pad4-1* in a Col-0 background; mutants *fls2-17*, *eds1-2*, *sgt1b-3* and *rar1-13* in a L*er*-0 background; and mutant *npr1-5* in a No-0 background. Subsequent leaf infection with 10^5 c.f.u. ml^{-1} *Pst* DC3000 was performed, and bacteria were counted 2 days after infection as described in Methods.

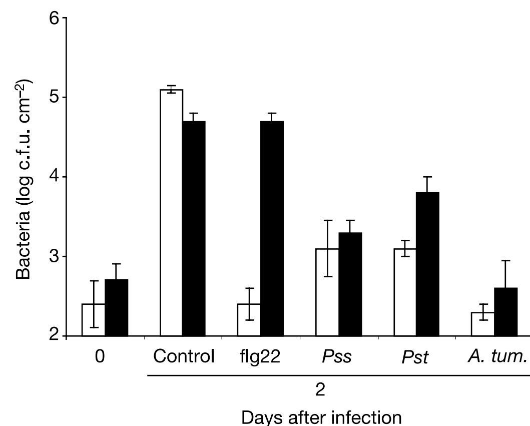

Figure 2 Treatment with different bacterial extracts limits subsequent growth of *Pst* DC3000 in L*er*-0 and *fls2-17* plants. L*er*-0 (open bars) and *fls2-17* (filled bars) plants were either left untreated, pretreated for 24 h with 1 μM flg22 or pretreated with one of the following bacterial extracts: *Pseudomonas syringae* pv. *syringae* (*Pss*) (3 mg ml^{-1}), *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (3 mg ml^{-1}) or *Agrobacterium tumefaciens* (*A. tum.*) (10 mg ml^{-1}). Subsequent leaf infection with 10^5 c.f.u. ml^{-1} *Pst* DC3000 was performed, and bacterial growth was assessed 2 days after infection. Results shown are means ± s.e.m. ($n = 8$).

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estingly, *Pst* DC3000 bacteria infiltrated directly into the intercellular leaf space grew at the same rate in *fls2-17* as in wild-type plants (Fig. 1a) and caused the same visible disease symptoms within the first week after infection (data not shown). In addition, various non-pathogenic or avirulent strains of *Pseudomonas* and *Xanthomonas* grew at the same, restricted, rate in both the *fls2-17* and wild-type plants (data not shown). Under natural conditions, *Pst* DC3000 enters host plants, usually the leaves, through wounds or natural openings such as stomata, and then spreads and multiplies to high population densities in intercellular spaces[26]. Thus, the infiltration of bacteria with a syringe might bypass the first steps of the natural infection process, notably the steps of invasion and spreading that probably rely on flagella-based motility. Bacterial motility might not be important within the intercellular spaces, because non-motile mutants of *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *malvacearum* were similar to the parental strains in their ability to grow after vacuum infiltration into bean and cotton leaves, respectively[26]. We therefore infected *A. thaliana* plants by spraying *Pst* DC3000 bacteria onto leaf surfaces. Under these conditions, *fls2-17* plants showed a faster and more severe development of disease symptoms than wild-type plants (Fig. 3a). These stronger symptoms correlated with higher numbers of bacteria in *fls2-17* leaves (Fig. 3b), a difference that was particularly pronounced in younger leaves. Higher sensitivity of *fls2-17* mutants, compared to wild-type plants, was found in all of three independent experiments. Because *fls2-17* mutants originate from a population mutagenized with ethyl methane sulphonate, to exclude the possibility that the enhanced sensitivity of *fls2-17* was due to a genetic difference other than the one in the *FLS2* gene, we tested a second, independent, mutant affected in the *FLS2* gene in a Col-0 background. This mutant, carrying a T-DNA insertion in the promoter region abolishing expression of the *FLS2* gene (checked by reverse transcriptase polymerase chain reaction; data not shown), also showed enhanced sensitivity to *Pst DC3000* in comparison with its wild-type Col-0 background (Supplementary Fig. 2). In addition, the ecotype Ws-0 presents a flagellin-insensitive phenotype[22,24], formerly attributed to a mutation in a hypothetical *FLS1* gene[24] but recently shown to be a natural *fls2* mutant carrying a point mutation that resulted in a stop codon in the kinase domain of *FLS*2 (S.R., unpublished observations). In comparison with the accession Col-0, Ws-0 plants exhibited faster and more severe development of disease symptoms after being sprayed with *Pst* (data not shown). However, Ws-0 plants transformed with a functional *FLS2* gene, under the control of its native promoter sequence, acquired responsiveness to flg22 (Supplementary Fig. 3) and became less susceptible to *Pst* DC3000 (Fig. 3c), indicating that the natural deficiency in flagellin perception in the ecotype Ws-0 can be complemented with the wild-type *FLS2* gene.

Enhanced disease susceptibility to airborne infection with *Mycobacterium tuberculosis* or *M. avium* has been observed in knockout mice lacking a single Toll-like receptor (TLR2). However, a more drastic effect on susceptibility was observed in mice lacking MyD88, a signal adaptor protein thought to be required for transfer of signals coming from all TLRs[27]. This indicates redundancy of the recognition process and the involvement of several TLRs in the innate immune system of animals. Interestingly, a common dominant TLR5 stop codon polymorphism abolishes flagellin signalling and is associated with susceptibility to Legionnaires' disease in humans[28]. Similarly, the results presented above provide a first example that perception of a single general elicitor or PAMP makes a difference for plant defence. Although the sensing of flagellin by FLS2 is an important initial checkpoint for controlling or restricting bacterial invasion in *Arabidopsis* leaves, it is not the only checkpoint; detection systems for additional bacterial PAMPs can be expected to have similar and complementary functions in controlling pathogen invasion at different steps of the infection process. The identification of these additional PAMP(s) and of the corresponding receptor(s) represents an exciting goal for the future. □

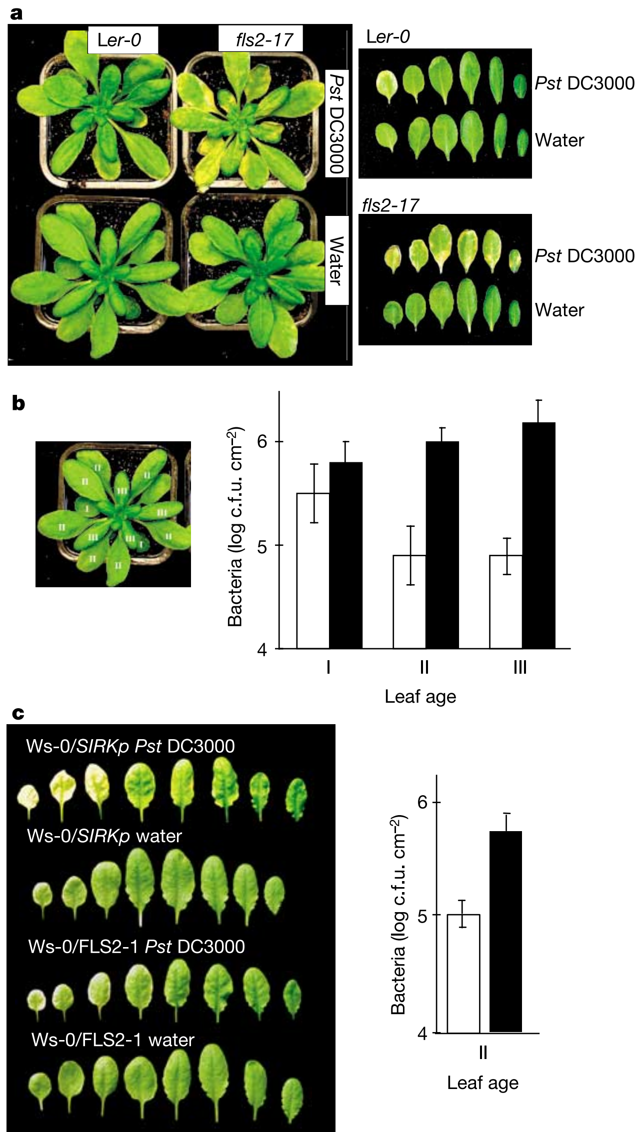

Figure 3 Bacterial disease resistance is determined by flagellin perception. **a**, A *FLS2* loss-of-function mutation, *fls2-17*, leads to enhanced disease susceptibility. Left: wild-type and *fls2-17* mutant plants were sprayed with *Pst* DC3000 bacteria or with water and photographed 4 days later. Right: symptoms after 4 days in a series of leaves of decreasing age. **b**, Number of *Pst* DC3000 bacteria extracted from wild-type (open bars) and *fls2-17* mutant plants (filled bars) 4 days after infection. Leaves were grouped by age as depicted on the left. **c**, A gain-of-function transgene of *FLS2* leads to decreased susceptibility in the accession Wassilewskaya (Ws-0), which lacks a functional *FLS2* gene. Ws-0 was stably transformed with *FLS2p::FLS2-3xmyc* (line FLS2-1; open bars), or *SIRKp::GUS* (line SIRKp; filled bars) as a control. Plants were sprayed with 5×10^8 c.f.u. ml^{-1} *Pst* DC3000, or water. Pictures were taken for a series of leaves of decreasing age (left), and bacteria were extracted and counted from leaves of age class II (right), 4 days after infection. Results are means ± s.e.m. ($n = 8$).

Methods

Plant material

All plants were grown at 20–21 °C with 65% humidity under light (about 100 μmol m^{-2} s^{-1}) in an 8 h light/16 h dark cycle in environment-controlled chambers. Plants aged 5–6 weeks were used for the infection experiments.

Flagellin treatment

Treatments with flg22 or flg22$^{A.tum}$ were performed by pressure infiltration (needle-less syringes) of 1 μM peptide solution into the leaves. For each treatment, four to eight plant replicates were used, and each experiment was repeated at least twice.

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Bacterial growth assays

Bacterial strains used in this study were *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), *Pst* DC3000 *AvrRpm1*, *Pst* DC3000 *AvrRps4*, *Pst* DC3000 *AvrRpt2*, *Pseudomonas syringae* pv. *tomato* DC3000 *HrpS*$^-$, *Pseudomonas syringae* pv. *syringae* (*Pss*), *Pseudomonas syringae* pv. *tabaci*, *Pseudomonas syringae* pv. *glycinae*, *Pseudomonas syringae* pv. *phaseolicola*, *Pseudomonas fluorescens*, *Pseudomonas brassicacearum*, *Xanthomonas axonopodis* pv. *citri* and *Agrobacterium tumefaciens*. All strains were grown at 28 °C on King's B medium (40 g l^{-1} proteose, 20 g l^{-1} glycerol, 15 g l^{-1} agar) containing the appropriate antibiotics for selection. Syringe and spray inoculations, and bacterial growth *in planta*, were performed as described[29]. In brief, for syringe inoculation, the bacteria were scraped off a fresh plate, resuspended in sterile water to 10^5 colony-forming units (c.f.u.) ml^{-1}, and pressure-infiltrated into leaves with a needleless syringe. For spray inoculation, overnight *Pst* DC3000 cultures were collected, washed once and resuspended in sterile water. Plants were sprayed with a bacterial suspension containing 5×10^8 c.f.u. ml^{-1} bacteria with 0.04% Silwet L-77 (Lehle Seeds). Leaves were harvested and surface sterilized (30 s in 70% ethanol, followed by 30 s in sterile distilled water) for the spray inoculation method. Leaf discs from two different leaves were ground in 10 mM $MgCl_2$ with a Microfuge tube glass pestle. After grinding of the tissue, the samples were thoroughly vortex-mixed and diluted 1:10 serially. Samples were finally plated on NYGA solid medium (5 g l^{-1} bactopeptone, 3 g l^{-1} yeast extract, 20 ml l^{-1} glycerol, 15 g l^{-1} agar) supplemented with the appropriated antibiotic. Plates were placed at 28 °C for 2 days, after which the colony-forming units were counted.

Treatment with bacterial extracts

Extracts from *Pss* and *Pst* DC3000 were prepared as described[6], freeze-dried and dissolved in water (3 mg ml^{-1}). *A. tumefaciens* bacteria were harvested by centrifugation, washed once with water and lysed by incubation in lysozyme solution (0.2 mg ml^{-1}) for 30 min at 37 °C and homogenization with a Polytron. The soluble supernatant was freeze-dried and redissolved in water (10 mg ml^{-1}).

Generation of transgenic plants

Arabidopsis thaliana Ws-0 plants were transformed with a *FLS2p::FLS2-3xmyc* construct. The *FLS2* promoter up to −988 base pairs was amplified by polymerase chain reaction (PCR) and introduced into the *Eco*RI and *Hin*dIII sites of p*CAMBIA 2300* (www.cambia.org.au), additionally adding *Bam*HI and *Kpn*I restriction sites upstream of the *Hin*dIII site. The *FLS2* gene triple Myc-tag fusion was amplified by PCR and cloned into the *Bam*HI and *Kpn*I sites of p*CAMBIA*, and the construct was verified by sequencing. Stable transgenic lines were generated with the *A. tumefaciens*-mediated gene transfer procedure. Independent transformed plant pools were kept separate for the selection of independent transgenic lines based on their kanamycin resistance. Functional complementation of Ws-0 by *FLS2p::FLS2-3xmyc* was assayed with standard procedures[24]. Expression of FLS2-3xmyc protein was confirmed by western blot analysis with anti-Myc antibodies (Supplementary Fig. 3). For control transformation, a *SIRKp::GUS* construct[30] was used. Plants of the T_2 generation were chosen for the bacterial spraying experiments.

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Cdc42 and mDia3 regulate microtubule attachment to kinetochores

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During mitosis, the mitotic spindle, a bipolar structure composed of microtubules (MTs) and associated motor proteins[1,2], segregates sister chromatids to daughter cells. Initially some MTs emanating from one centrosome attach to the kinetochore at the centromere of one of the duplicated chromosomes. This attachment allows rapid poleward movement of the bound chromosome. Subsequent attachment of the sister kinetochore to MTs

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