Sensitivity of Different Ecotypes and Mutants of Arabidopsis thaliana toward the Bacterial Elicitor Flagellin Correlates with the Presence of Receptor-binding Sites*

Zsuzsa Bauer, Lourdes Gómez-Gómez, Thomas Boller, and Georg Felix†

From the Friedrich Miescher-Institute, CH-4002 Basel, Switzerland

Flagellin, the main building block of the bacterial flagellum, acts as potent elicitor of defense responses in different plant species. Genetic analysis in Arabidopsis thaliana identified two distinct loci, termed FLS1 and FLS2, that are essential for perception of flagellin-derived elicitors. FLS2 was found to encode a leucine-rich repeat transmembrane receptor-like kinase with similarities to Toll-like receptors involved in the innate immune system of mammals and insects. Here we used a radiolabeled derivative of flg22, a synthetic peptide representing the elicitor-active domain of flagellin, to probe the interaction of flagellin with its receptor in A. thaliana. The high affinity binding site detected in intact cells and membrane preparations exhibited specificity for flagellin-derived peptides with biological activity as agonists or antagonists of the elicitor responses. Specific binding activity was measurable in all ecotypes of A. thaliana that show sensitivity to flagellin but was barely detectable in the flagellin-insensitive ecotype Ws-0 affected in FLS1. A strongly impaired binding of flagellin was observed also in several independent flagellin-insensitive mutants isolated from the flagellin-sensitive ecotype La-er. In particular, no binding was found in plants carrying a mutation in the LRR domain of FLS2. These data indicate that the formation of functional receptor-binding sites depends on genes encoded by both loci, FLS1 and FLS2. The tight correlation between the presence of the binding site and elicitor response provides strong evidence that this binding site acts as the physiological receptor of flagellin.

Induction of active defense responses by plants depends on the detection of the invading pathogen or detection of the stress condition. A variety of chemically different substances, either originating from microorganisms or released from the plant cells in the course of injury, have been shown to act as potent elicitors of active defense responses in plants. Perception of these elicitors is thought to occur via specific receptors present in the plant hosts (1). Microbial elicitors have been classified into two groups: The first group, the “general elicitors,” are characteristic for whole groups or classes of microorganisms. Perception for these general elicitors is thought to occur via specific receptors and to signal the presence of “nonself” in general, i.e. the mere presence of fungi or bacteria (1, 2). The second group comprises the race-specific elicitors encoded by Avr (avirulence) genes present in particular races of pathogens that elicit defense responses in plant hosts carrying the corresponding resistance genes. The interaction of these specific elicitors and the gene products underlies the classic gene-for-gene interaction (3, 4), and it has been postulated that the products of the resistance genes function as receptors for the race-specific elicitors (5).

Flagellin, the subunit building up the filament of bacterial flagella, has been identified as a potent general elicitor, active in Arabidopsis thaliana, tomato, and other plant species (6). Elicitor activity could be attributed to the most conserved domain within the N-terminal part of flagellin. flg22, a synthetic peptide comprising the core 22 amino acids, exhibited full elicitor activity and induced responses in tomato and A. thaliana at subnanomolar concentrations. Interestingly, flagellins of the plant-associated species Agrobacterium and Rhizobium exhibit exceptional divergence of this domain, and synthetic peptides representing these divergent sequences did not induce responses in tomato and A. thaliana (6, 7).

In A. thaliana seedlings, flg22 elicits rapid general defense responses like ethylene production and oxidative burst but leads also to a striking growth retardation on prolonged treatment (7). Among different ecotypes tested, only ecotype Ws-0 proved insensitive to flagellin. Crosses of Ws-0 with the sensitive ecotypes Col-0 and La-er demonstrated that a single dominant locus on chromosome 5, termed FLS1, determines sensitivity to flagellin (7). In addition, several mutants that are not affected in growth by the flg22 peptide were isolated by screening a mutagenized population of the sensitive ecotype La-er. At least two independent point mutations mapped to a single gene encoding a putative membrane receptor-kinase with an extraplasmatic leucine-rich-repeat (LRR) domain (8). This gene mapped to a locus genetically closely linked but different from FLS1 and was consequently termed FLS2. Complementation of these two mutants with the FLS2 gene of the wild type fully restored responsiveness to flagellin (8). The product of FLS2 encodes a receptor kinase with high homology to the resistance gene Xa21 from rice responsible for resistance to Xanthomonas oryzae (9), and its LRR domain is similar to that of the Cf gene family from tomato, providing resistance to various strains of Cladosporium fulvum (5, 10).

The present work aimed at defining the physiological role of FLS1 and FLS2 for flagellin perception in A. thaliana. Similar to the situation with most resistance genes, FLS1 and FLS2 are decisive for the formation of a functional perception system, but it remains to be demonstrated whether one or both encode the receptor site that physically interacts with the elicitor.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: Friedrich Miescher-Institute, P.O. Box 2543, CH-4002 Basel, Switzerland. Tel.: 41-61-6975240; Fax: 41-61-6974527; E-mail: Felix@fmi.ch.

1 The abbreviations used are: LRR, leucine-rich repeat; MES, 4-morpholinethanesulfonic acid.
ligand. To study elicitor binding, we adapted the binding assay established previously for detection and characterization of flagellin receptor-binding sites in tomato (11) to *A. thaliana*. Although similar in many aspects to tomato, perception of flagellin by *A. thaliana* showed characteristic differences with respect to the structural determinants of peptides recognized as agonists or antagonists. Applying the modified binding assay, we studied the presence of the flagellin receptor-binding site in *A. thaliana* differing in their sensitivity to flagellin because of changes in *FLS1* and *FLS2*. The presence of binding sites and sensitivity to flagellin were closely linked in all plants tested, demonstrating that both *FLS1* and *FLS2* are important for the formation of functional binding sites acting as receptors for the flagellin elicitor.

**EXPERIMENTAL PROCEDURES**

*Flagellin-derived Peptides and Radiiodination*—The flagellin-derived peptides were synthesized according to the consensus sequence for the most highly conserved region in the N terminus of eubacterial flagellin (6). flg22, Tyr-flg22, flg15, flg13, flg22-2, flg152, flg152, and flg222 were synthesized and purified on reversed phase high pressure liquid chromatography by F. Fischer (Friedrich Miescher Institute). Peptides were dissolved in H2O (stock solutions of 1–10 mM) and diluted in a solution containing 0.1% bovine serum albumin and 0.1 M NaCl. Tyr-flg22 was iodinated using chloramine T to I-Tyr-flg22 (11) or labeled with [125I]iodine to yield 3-[125I]iodotyrosine-flg22 (I-Tyr-flg22) with a specific radioactivity of >2000 Ci/mmol by Anawara Trading SA (Wangen, Switzerland). Flagellin protein was purified as described before (6).

*Plants and Cell Suspension Cultures of *A. thaliana*—*A. thaliana* seedlings of ecotype La-er, Zurich and Ws-0 were obtained from J. Paszkowski (Friedrich Miescher Institute). Seeds of ecotypes Col-0, Mühlen, Estland, Cvi, AUA-Rhon, Ns-0, Col-PRL, and Kandavilli were obtained from Lele Seeds (Round Rock, TX). Mutants insensitive to treatment with flag22 were selected from ethyl methanesulfonate-mutagenized La-er seedlings (Lele Seeds) as described before (8). The seeds were grown in soil in growth chambers programmed for cycles of 12 h of light at 20°C and 12 h of dark at 16°C with 70% relative humidity.

Cell cultures of *A. thaliana*, originally derived from plant tissue of ecotype Landsberg erecta, were grown as described (12). The cells were subcultured at weekly intervals and used for assays 6–8 days after subculture, containing ~80 mg cells/ml (fresh weight).

*Measurement of Alkalization Response*—Aliquots of the *A. thaliana* cell suspension were incubated in open flasks on a rotary shaker at 150 cycles/min (7). Extracellular pH was measured with a small combined glass pH electrode (Metrohm, Herisau, Switzerland) and either recorded continuously using a pen recorder or measured 20 min after start of the experimental treatment.

*Preparation of Microsomal Membranes from *A. thaliana* Cells*—For preparation of microsomal membranes, 100 g of cells were transferred to 200 ml of binding buffer (25 mM MES/KOH, pH 6.0, 3 mM MgCl2, 10 mM NaCl) supplemented with 4 mM dithiothreitol and broken in a Parr cell disruption bomb (Parr Instrument Co., Moline, IL) as described before for tomato cells (11). The homogenate was sequentially centrifuged at 10,000 × g for 20 min to yield pellet 1 (P1) and at 100,000 × g for 45 min to yield pellet 2 (P2) containing microsomal membranes. The pellets were resuspended in binding buffer, and protein concentrations were determined by the Micro BCA protein assay kit from Pierce.

*Preparation of Plant Homogenates*—Individual *A. thaliana* plants, weighing 0.1–0.5 g fresh weight, were homogenized in 1–5 ml ice-cold binding buffer (10 ml of buffer is tissue) with a Polytron mixer (Kienematica AG, Littau-Luzern, Switzerland). Big fragments of tissues were removed by passing the homogenate through one layer of Miracloth (Calbiochem).

*Binding Assays with Intact Cells, Microsomal Membrane Preparations, and Plant Homogenates*—Aliquots of cells, microsomes, or plant homogenates were incubated in binding buffer in a total volume of 100 μl with [125I]Tyr-flg22 (60 fmol in standard assays; >2000 Ci/mmol) for 25 min either alone (total binding) or with 10 μM of competing flag22 (non-specific binding). Cells, microsomes, or crude extracts were collected by vacuum filtration on glass fiber filters (Macherey-Nagel MN GF-2, 2.5-mm diameter, preincubated with 1% bovine serum albumin, 1% bacto-trypton, and 1% bactopepton in binding buffer) and washed for 10 s with 15 ml of ice-cold binding buffer. The radioactivity retained on the filters was determined by γ-counting. Specific binding was calculated by subtracting nonspecific binding from total binding.

For equilibrium binding assays, aliquots of plant homogenates containing 500 μg of protein were incubated with radioligand and competing unlabeled flag22 as described above. After incubation for 20 min on ice, the free label was separated from label bound to P1 by centrifugation (10,000 × g for 5 min).

**RESULTS**

*Flagellin-derived Peptides Exhibit Agonistic and Antagonistic Activity in Cells of *A. thaliana*—*Flagellin and synthetic peptides corresponding to the highly conserved N-terminal domain spanned by flag22 act as potent elicitors in tomato and *A. thaliana* (6). Suspension-cultured plant cells react to elicitors within minutes and have thus been widely used to study elicitor perception and elicitor responses (1). Changes in protein phosphorylation, activation of MAP kinases, and altered ion fluxes across the plasma membrane, including increased influx of K+ and Cl− and increased influx of H+ and Ca2+ are among the earliest responses observed (13–16). These alterations in ion fluxes precede the actual defense responses and are believed to be involved in elicitor signaling. We used medium alkalization, an easily measurable consequence of the changed ion fluxes, as a rapid, sensitive, and quantitative bioassay to assess structural requirements important for elicitor activity of flagellin-derived elicitors. flg15, a peptide lacking the 7 amino acid residues at the N terminus of flag22, was nearly as active as flag22 in tomato but showed ~100-fold lower activity than flag22 in *A. thaliana* (6). Peptides shortened at the C-terminal end exhibited an even more drastic difference in their activity in cells of tomato and *A. thaliana*. flag22-Δ2, a peptide shortened by 2 amino acid residues at the C terminus, triggers medium alkalization in tomato cells nearly as efficiently as flag22 (EC50 of ~0.03 mM; data not shown) but failed to induce significant medium alkalization in cells of *A. thaliana* when added in concentration up to 30 μM (Fig. 1A). When added concomitantly with 3 μg/ml, a excess of 30 μg/ml flag22-Δ2 nearly completely suppressed alkalization induced by 3 μg/ml flag22 alone (Fig. 1A). The suppressive effect of flag22-Δ2 was competitive and could be overcome by increasing concentrations of flag22. In the presence of 10 μg/ml flag22-Δ2 the dose of flag22 required to induce a half-maximal response (EC50) increased from ~0.3 to ~10 nM (Fig. 1B). In further experiments with different concentrations of flag22-Δ2 and flag22, a KI of ~100 nM was estimated for flag22-Δ2 (data not shown).

The antagonistic activity depended on the intact peptide flag22-Δ2 and was rapidly degraded by pretreatment of the peptide with trypsin. Similarly, the shorter peptide flag15-ΔΔ exhibited no significant activity as antagonist of flag22 when tested in concentrations up to 30 μg/ml (data not shown). In contrast, a small, transient acidification of the extracellular medium similar to the one observed after treatment with flag22-Δ2 (Fig. 1A) was also found with flag15-ΔΔ and with flag22-Δ2 after treatment with trypsin (data not shown), indicating that the slight acidification was due to smaller peptide fragments, free to enter as yet unidentified contaminants of the peptide preparations.

In summary, flagellin perception by *A. thaliana* and tomato exhibit characteristic differences, although both species recognize essentially the same conserved domain in the flagellin protein. In particular, both the N-terminal as well as the C-terminal parts of the domain spanned by flag22 appear to be of
greater importance for biological activity in *A. thaliana* than in tomato cells.

**Binding of 125I-Tyr-flg22 to Intact Cells and Microsomal Membrane Preparations of *A. thaliana*—In previous experiments we used 125I-Tyr-flg22, a radioactive derivative of flg22, to establish a binding assay for flagellin elicitors in tomato (11). When we attempted to apply this assay to *A. thaliana*, we initially failed to detect specific binding sites in cells and membrane preparations. Variation of the experimental parameters showed that binding in *A. thaliana* had a pH optimum between pH 5 and 6, and buffering to pH 7, as used for assays with tomato, reduced binding by >90% (data not shown). Similarly, binding was sensitive to concentrations of 100 mM NaCl, KCl, or MgCl₂ (data not shown). Under appropriately modified and optimized assay conditions, buffering at pH 6.0 and lowering the concentration of NaCl to 10 mM, specific binding of 125I-Tyr-flg22 to *A. thaliana* cells and membrane preparations could readily be detected (Fig. 2).

In accordance with the rapid onset of physiological responses like medium alkalinization, intact cells showed rapid binding of the radioligand (Fig. 2A). Even at 4°C, maximal binding was reached within 20 min, and label associated with cells remained essentially stable for at least 90 min. Nonspecific binding, assayed in the presence of an excess of 10 μM unlabeled Tyr-flg22, remained low throughout the experiment (Fig. 2A).

Nonreversibility of binding was peculiar for binding of flagellin both to intact cells and membrane preparations of tomato (11). In contrast, binding of 125I-Tyr-flg22 to microsomal membranes of *A. thaliana* proved to be reversible, and 60 ± 10% of label was replaced within 20 min in six independent membrane preparations. One example is shown in Fig. 2B.
membranes (15 μg of microsomal protein) at 4 °C for 25 min in the absence (total binding, open squares) or presence (nonspecific binding, open circles) of 10 μM flg22. To determine the specific binding (filled diamonds), nonspecific binding was subtracted from total binding. Kd and Bmax were determined by curve fitting to rectangular hyperbola (y = Bmax * x/Kd + x), where y = bound ligand and x = free ligand.

binding site, we incubated increasing concentrations of 125I-Tyr-flg22 with intact cells and microsomal membranes (Fig. 3). Various concentrations of 125I-Tyr-flg22, diluted with nonlabeled Tyr-flg22 to a specific radioactivity of 710 Ci/mmol, were incubated with aliquots of intact cells (A) (9 mg fresh weight) or microsomal membranes (B) (15 μg of microsomal protein) at 4 °C for 25 min in the absence (total binding, open squares) or presence (nonspecific binding, open circles) of 10 μM flg22. To determine the specific binding (filled diamonds), nonspecific binding was subtracted from total binding. Kd and Bmax were determined by curve fitting to rectangular hyperbola (y = Bmax * x/Kd + x), where y = bound ligand and x = free ligand.

FIG. 3. Saturation of binding to intact cells and microsomal membranes. Various concentrations of 125I-Tyr-flg22, diluted with nonlabeled Tyr-flg22 to a specific radioactivity of 710 Ci/mmol, were incubated with aliquots of intact cells (A) (9 mg fresh weight) or microsomal membranes (B) (15 μg of microsomal protein) at 4 °C for 25 min in the absence (total binding, open squares) or presence (nonspecific binding, open circles) of 10 μM flg22. To determine the specific binding (filled diamonds), nonspecific binding was subtracted from total binding. Kd and Bmax were determined by curve fitting to rectangular hyperbola (y = Bmax * x/Kd + x), where y = bound ligand and x = free ligand.

FIG. 4. Competition of I-Tyr-flg22 binding by flagellin and different peptides. Binding assays with 125I-Tyr-flg22 (0.6 nM) and various concentrations of the flagellin derived peptides Tyr-flg22, flg22, flg22Δ2, flg15, flg22A tum, and flg13. The results were obtained with different batches of microsomal membrane preparations using 60 μg of protein/assay and are presented as the percentages of total binding. Total binding (100%) ranged between 14000 and 18000 cpm, and nonspecific binding (5–10%) ranged between 1000 and 2000 cpm, respectively.

Apart from the apparent change in reversibility, the binding characteristics in cells, crude extracts, P1, and microsomal membrane fraction P2 were indistinguishable with respect to affinity, measured with saturation kinetics, and specificity, tested in competition assays with different flagellin-derived peptides (data not shown). This indicates that binding activity detected in these different fractions all represent the same binding site.

**Specificity of the Binding Site for Flagellin and Biologically Active Flagellin-derived Peptides**—The specificity of binding was tested in competitive binding assays with increasing concentrations of flagellin protein or various flagellin-derived peptides as competitors of 125I-Tyr-flg22. Examples for competition by Tyr-flg22, flg22, flg15, flg22Δ2, flg22A tum, and flg13 in binding assays with microsomal preparations are shown in Fig. 4. In binding competition assays with intact cells, the peptides tested exhibited the same relative order with similar IC50 values (data not shown). In Fig. 5, the IC50 values for flagellin protein and various peptides, deduced from dose-competition curves such as the ones shown in Fig. 4, were plotted against their respective activity for induction of a half-maximal alkalization response (EC50 values). The most efficient competition and the highest biological activity were observed for Tyr-flg22 and its iodinated form I-Tyr-flg22 (values for IC50 of 4 nM and for EC50 of 0.2 nM, respectively). flg22 was ~3–5-fold less efficient in both assays, whereas intact flagellin protein was ~20-fold less active. Peptides shortened at the N terminus, flg15 and flg13, showed decreasing binding affinity in parallel to dropping elicitor activity. flg22Δ2, acting as antagonist for biological activity (Fig. 1), strongly competed for binding with an IC50 only 10-fold lower than that of flg22. Weak competition of binding was observed for flg15–32 when added in millimolar concentrations. Peptides corresponding to the homologues of flg15 from Agrobacterium tumefaciens and Rhizobium meliloti
ing buffer and assayed the crude homogenates for binding of radioligand increased linearly with the amount of protein, corresponding to −10 mg of plant tissue, were sufficient to detect significant binding. Thus, the assay was sensitive enough to measure binding activity in homogenates of individual plants.

In contrast to ecotype La-er, seedlings of ecotype Ws-0 exhibit no sensitivity to treatment with flagellin. This insensitivity was previously attributed to a single locus termed FLS1 (7). When assayed for flagellin binding, homogenates of Ws-0 showed greatly reduced specific binding compared with the flagellin-sensitive ecotype La-er (Fig. 6A). Although the difference between total and nonspecific binding was close to the detection limit in Ws-0 (Fig. 6A), a very low specific binding activity was detectable in most repetitions with independent homogenates of Ws-0 (n > 6; data not shown). Mixtures of homogenates from La-er and Ws-0 plants exhibited binding corresponding to the arithmetic of the mixtures, indicating that no soluble factors inhibit or enhance binding in the two homogenates (data not shown).

Reduced binding, as observed in homogenates of Ws-0, could indicate a reduced number of binding sites, or it could reflect a reduced affinity of these sites. A reduced affinity could lead to the loss of bound radioligand during the washing step used to remove unbound ligand in the binding assays. To test this possibility we performed equilibrium binding assays with separation of bound and unbound ligand by centrifugation (Fig. 6B). Although background in assays with excess unlabeled flagellin was higher than in standard assays with washing on filters, it clearly demonstrated significant specific binding in La-er and a strongly reduced number of binding sites in ecotype Ws-0.

Several additional ecotypes of A. thaliana were assayed for the presence of flagellin-binding sites and their response to treatment with flagellin. In Fig. 7, binding activity in homogenates was compared with the flagellin-dependent induction of ethylene biosynthesis in leaf tissues of these ecotypes. With the exception of Ws-0, all ecotypes showed clear induction of ethylene biosynthesis and significant specific binding of to flagellin (8). The two mutants fls2-24 and fls2-17 carry two different point mutations in the FLS2 gene encoding
open bars) of 125I-Tyr-flg22 were measured in homogenates of different ecotypes. Bars and error bars represent the means and S.D. values of three replicates with three replicates. B, total binding (shaded bars) and nonspecific binding (open bars) of 125I-Tyr-flg22 were measured in homogenates of different ecotypes. Bars and error bars represent the means and S.D. values of three homogenates obtained from three individual plants of every ecotype.

a putative receptor kinase (8). Confirming previous results (17), these mutants exhibited strongly reduced flagellar binding that was fully restored in plants complemented with the wt-FLS2 gene (Fig. 8). The gene(s) affected in the two additional mutant lines fls1-2 and fls1-19 has not yet been identified, but the mutants are nonallelic with FLS2, carry no mutation in the sequence encoding FLS2, and also show wild type expression of the FLS2 gene. These mutants also exhibited strongly reduced total binding and little changes in nonspecific binding such that the specific binding was close to the detection limit (Fig. 8). Mutant plants fls2-24, carrying a point mutation in one of the leucine rich repeats of FLS2 (8), exhibited no specific binding in all experiments. In contrast, as observed above for Ws-0, the mutants fls1-2, fls1-19, and fls2-17 appeared to have a little specific binding (Fig. 8). From the data of several independent repetitions (n = 3) with different sets of plants, we estimated that these mutants contained significant but ~6.5-10-fold lower binding than La-er wild type plants. In summary, because mutants fls1-2 and fls1-19 as well as plants of ecotype Ws-0 are affected in a locus different from FLS2, these data indicate that gene products of at least two loci are required to form functional receptor binding sites.

**DISCUSSION**

**The High Affinity Binding Site in A. thaliana Exhibits Characteristics Expected for a Functional Flagellin Receptor**—The binding site for flagellin studied in this report shows characteristics expected for a receptor with respect to affinity and specificity for flagellin-derived ligands with activity as agonist or antagonist of elicitor responses. For all flagellin-derived peptides tested, the apparent affinity for the binding site in competition assays correlated with their relative ability to induce or inhibit flagellin-dependent responses in A. thaliana cells. Binding was saturable and exhibited high affinity with an apparent \( K_d \) of ~1.3 nM. As previously observed in tomato (11), occupancy of the binding site and the strength of the alkalization response do not show a linear, one-to-one, relationship. At least for the alkalization response, used to quantify elicitor activity, a full response is triggered when only a small percentage of binding sites are occupied. The presence of “spare receptors” is characteristic for many receptor-mediated signaling processes in animals and has been observed before for perceiving a putative receptor kinase (8). The gene(s) affected in the two additional mutant lines fls1-2 and fls1-19 has not yet been identified, but the mutants are nonallelic with FLS2, carry no mutation in the sequence encoding FLS2, and also show wild type expression of the FLS2 gene. These mutants also exhibited strongly reduced total binding and little changes in nonspecific binding such that the specific binding was close to the detection limit (Fig. 8). Mutant plants fls1-2, carrying a point mutation in one of the leucine rich repeats of FLS2 (8), exhibited no specific binding in all experiments. In contrast, as observed above for Ws-0, the mutants fls1-2, fls1-19, and fls2-17 appeared to have a little specific binding (Fig. 8). From the data of several independent repetitions (n = 3) with different sets of plants, we estimated that these mutants contained significant but ~6.5-10-fold lower binding than La-er wild type plants. In summary, because mutants fls1-2 and fls1-19 as well as plants of ecotype Ws-0 are affected in a locus different from FLS2, these data indicate that gene products of at least two loci are required to form functional receptor binding sites.

**DISCUSSION**

**The High Affinity Binding Site in A. thaliana Exhibits Characteristics Expected for a Functional Flagellin Receptor**—The binding site for flagellin studied in this report shows characteristics expected for a receptor with respect to affinity and specificity for flagellin-derived ligands with activity as agonist or antagonist of elicitor responses. For all flagellin-derived peptides tested, the apparent affinity for the binding site in competition assays correlated with their relative ability to induce or inhibit flagellin-dependent responses in A. thaliana cells. Binding was saturable and exhibited high affinity with an apparent \( K_d \) of ~1.3 nM. As previously observed in tomato (11), occupancy of the binding site and the strength of the alkalization response do not show a linear, one-to-one, relationship. At least for the alkalization response, used to quantify elicitor activity, a full response is triggered when only a small percentage of binding sites are occupied. The presence of “spare receptors” is characteristic for many receptor-mediated signaling processes in animals and has been observed before for perceiving a putative receptor kinase (8). The gene(s) affected in the two additional mutant lines fls1-2 and fls1-19 has not yet been identified, but the mutants are nonallelic with FLS2, carry no mutation in the sequence encoding FLS2, and also show wild type expression of the FLS2 gene. These mutants also exhibited strongly reduced total binding and little changes in nonspecific binding such that the specific binding was close to the detection limit (Fig. 8). Mutant plants fls1-2, carrying a point mutation in one of the leucine rich repeats of FLS2 (8), exhibited no specific binding in all experiments. In contrast, as observed above for Ws-0, the mutants fls1-2, fls1-19, and fls2-17 appeared to have a little specific binding (Fig. 8). From the data of several independent repetitions (n = 3) with different sets of plants, we estimated that these mutants contained significant but ~6.5-10-fold lower binding than La-er wild type plants. In summary, because mutants fls1-2 and fls1-19 as well as plants of ecotype Ws-0 are affected in a locus different from FLS2, these data indicate that gene products of at least two loci are required to form functional receptor binding sites.

**DISCUSSION**

**The High Affinity Binding Site in A. thaliana Exhibits Characteristics Expected for a Functional Flagellin Receptor**—The binding site for flagellin studied in this report shows characteristics expected for a receptor with respect to affinity and specificity for flagellin-derived ligands with activity as agonist or antagonist of elicitor responses. For all flagellin-derived peptides tested, the apparent affinity for the binding site in competition assays correlated with their relative ability to induce or inhibit flagellin-dependent responses in A. thaliana cells. Binding was saturable and exhibited high affinity with an apparent \( K_d \) of ~1.3 nM. As previously observed in tomato (11), occupancy of the binding site and the strength of the alkalization response do not show a linear, one-to-one, relationship. At least for the alkalization response, used to quantify elicitor activity, a full response is triggered when only a small percentage of binding sites are occupied. The presence of “spare receptors” is characteristic for many receptor-mediated signaling processes in animals and has been observed before for perceiving a putative receptor kinase (8). The gene(s) affected in the two additional mutant lines fls1-2 and fls1-19 has not yet been identified, but the mutants are nonallelic with FLS2, carry no mutation in the sequence encoding FLS2, and also show wild type expression of the FLS2 gene. These mutants also exhibited strongly reduced total binding and little changes in nonspecific binding such that the specific binding was close to the detection limit (Fig. 8). Mutant plants fls1-2, carrying a point mutation in one of the leucine rich repeats of FLS2 (8), exhibited no specific binding in all experiments. In contrast, as observed above for Ws-0, the mutants fls1-2, fls1-19, and fls2-17 appeared to have a little specific binding (Fig. 8). From the data of several independent repetitions (n = 3) with different sets of plants, we estimated that these mutants contained significant but ~6.5-10-fold lower binding than La-er wild type plants. In summary, because mutants fls1-2 and fls1-19 as well as plants of ecotype Ws-0 are affected in a locus different from FLS2, these data indicate that gene products of at least two loci are required to form functional receptor binding sites.
remains to be tested. Nevertheless, a 3-5-fold enrichment of binding activity/mg of protein was observed for the microosomal fraction compared with P1, indicating membrane localization. Binding activity could be solubilized from the microsomal preparations by detergents (data not shown), thus further supporting membrane association of this binding site.

**Comparison of Flagellin Perception in Tomato and A. thaliana**—Perception of flagellin in tomato and in *A. thaliana* shows clear overall similarity but exhibits characteristic differences in detail. Perception in both species occurs with specificity for the same conserved domain of the flagellin molecule. However, *A. thaliana* exhibited preference for peptides spanning a somewhat larger domain than tomato. For the N-terminal side this is exemplified by the larger differences in activities for flg22 and flg15 in *A. thaliana* compared with tomato. For the C-terminal side, this is most striking for peptides that lack the 2 amino acid residues present at the C-terminal end (flg15Δ2 and flg22Δ2). Although these peptides retained nearly full elicitor activity in tomato, they proved completely inactive as elicitors in *A. thaliana*. In tomato, abrupt loss of elicitor activity occurred for peptides lacking four amino acid residues (flg15Δ4) (6). Interestingly, in both species these C-terminally truncated peptides that lack agonist activity were found to act as competitive inhibitors or antagonists of flagellin responses, suggesting a common mechanism of signal perception. Thus, as proposed for tomato (11), activation of flagellin receptor in *A. thaliana* appears to occur as a two-step process according to the address-message concept with the N-terminal part required for binding (address) and the C-terminal part for activation (message).

Cultured cells of tomato and *A. thaliana* showed similar numbers of binding sites (1–3 pmol/g fresh weight) and affinity for flg22 (*K*<sub>d</sub> values or half-saturation at 1–3 nM). Binding to intact cells of both species appeared nonreversible. In tomato, nonreversibility of interaction persisted in membrane preparations and even solubilized membrane preparation (11), excluding internalization as explanation for this phenomenon. Rather, we hypothesized that the two-step mechanism discussed above involves binding as a first step (reversible) and a process of intra- or inter-molecular isomerization leading to “locking” of the ligand as a second step. In *A. thaliana*, interaction of radioligand with binding sites in membrane preparations and the fraction P1 was essentially reversible. With respect to the two-step mechanism proposed for receptor activation and the multi-component character of the functional receptor discussed below, one can speculate that this change in reversibility of binding could be due to disassembly of the receptor complex during cell disruption. Indeed, in several assays with cells killed by freezing and thawing or in cell homogenates prepared by disruption with a polytron blender, reversibility of binding characteristics varied between non-reversible as in the assays with intact cells and reversible as in the assays with microsomal membranes. Clearly, further experiments aimed at the purification and identification of the elements involved in flagellin perception are required to clear this point.

**Correlation between the Presence of Binding Sites and Sensitivity to Flagellin in A. thaliana Plants**—The quantitative binding assay established for cells and cell extracts from tissue culture could be applied to study presence of flagellin-binding sites in homogenates obtained from individual *A. thaliana* plants. All ecotypes and plants that exhibited physiological responses to treatment with flg22 also showed clear and significant binding activity. In contrast, both, the flagellin-insensitive ecotype Ws-0 and all *fls* mutants tested were impaired in the binding of flagellin. This correlation provides strong evidence for a functional role of this binding site as the physiological receptor for flagellin. Surprisingly, none of the flagellin-insensitive plants appeared to be affected solely in an element of signal transmission downstream of the initial binding step.

**Several Components Are Involved in the Formation of a Functional Receptor Complex—FLS2**, a gene affected in several independent mutants exhibiting flagellin insensitivity, encodes a receptor kinase protein with a predicted extraplasmatic LRR domain, a transmembrane domain, and a cytoplasmic serine/threonine kinase domain (8). These structural elements are typical for a class of plant resistance genes (9, 10) and also for perception of endogenous regulators in plant morphogenesis (29, 30). LRR domains are involved in protein-protein interactions and consist of a conserved part, postulated to play a structural role, and a variable part, thought to be important for specificity of protein interaction and binding (31). Thus, LRR domains in receptor proteins are primary candidates for signal binding sites. Most elegantly, this was recently demonstrated by fusing the LRR and transmembrane domain of the Arabidopsis receptor kinase BR11, which is implicated in brassinosteroid signaling, to the serine/threonine kinase domain of *SA21*, the rice disease resistance receptor (32). This chimeric receptor expressed in rice cells was found to mediate plant defense responses upon treatment with brassinosteroids. The resistance gene Cf-9 of tomato, essential for perception of the corresponding fungal elicitor AVR9, belongs to a class of resistance genes consisting of an extraplasmatic LRR domain and a transmembrane domain but lacking a protein kinase domain (33). However, high affinity binding sites specific for AVR9 were not restricted to tomato plants carrying the Cf-9 gene, indicating the involvement of further components in the perception of this elicitor (20).

Evidence for more than one component involved in elicitor perception were also obtained for syringolides, water-soluble, low molecular weight bacterial elicitors that trigger defense responses in soybean cultivars carrying the Rpg4 resistance gene (34). Resistance is attributed to the gene product of the Rpg4 gene, but syringolides were found to specifically and strongly interact with a cytoplasmic protein present also in all cultivars not carrying the Rpg4 gene (35). Similarly, the high affinity glucon-binding protein identified as receptor for the hepta-β-glucoside elicitor bears no recognizable domains that could explain its function in transmembrane signaling (27), and the functional receptor might involve additional components as well (28).

In the present work we compared the presence of flagellin-binding sites in different mutants affected in response to this elicitor. Complete absence of specific binding was observed for *fls-2*, a mutant changed in a single amino acid in one of the LRR in FLS2. Complementation of this mutant restored responses to flagellin (8) as well as binding activity (Ref. 17 and Fig. 8). Clearly, these data are in good accordance with the hypothesis that the LRR domain of FLS2 is the binding site that physically interacts with flagellin. However, at present, direct evidence for this interaction is lacking. Heterologous expression of FLS2 in *E. coli* and rice cells did not result in functional flagellin binding and ectopic over-expression of FLS2 in *A. thaliana* under the 35 S *CaMV* promoter did not measurably change the level of binding activity (data not shown). Most surprisingly, flagellin-insensitive *A. thaliana* plants *fls-2-17* carrying a mutation in the putative cytoplasmic kinase domain of FLS2 (8) also exhibited strongly reduced binding of the flagellin elicitor. These findings served as a basis for a more detailed study that confirmed the role of the kinase domain and the phosphorylation state for the formation of functional flagellin-binding sites and flagellin perception (17).

Whereas the results discussed above do not exclude a direct
role of the LRR domain in flagellin binding, they indicate that FLS2 alone does not account for the flagellin receptor. Evidence for a second component with FLS2. Ws-0 bears no apparent changes in the FLS2 gene, and FLS2 transcripts were detected in Ws-0 (8). Consequently, FLS2 and FLS1 are both necessary for the formation of functional binding sites for flagellin. Crossings of La-er FLS2 mutants with Ws-0 (FLS1) showed co-dominance and partial sensitivity to flagellin in F2 plants, suggesting an interaction between the two gene products for formation or stability of the binding site (8).

In conclusion, the data presented demonstrate that FLS2 together with additional components such as FLS1 are essential elements of the flagellin receptor complex. Further identification of the binding site by biochemical purification will help to elucidate the composition and functioning of the flagellin receptor.

Acknowledgments—We thank Thomas Meindl for help in establishing binding studies for A. thaliana, Franz Fischer for the synthesis of various peptides, and Thomas Sebastian Nühse for maintaining the cell cultures.

REFERENCES
1. Boller, T. (1995) Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 189–214
2. Heath, M. C. (2000) Curr. Opin. Plant Biol. 3, 315–319
3. Flor, H. H. (1971) Annu. Rev. Phytopathol. 9, 275–296
4. Keen, N. T. (1992) Plant Mol. Biol. 19, 109–122
5. Hammond-Kosack, K. E., and Jones, J. D. G. (1997) Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 575–607
6. Felix, G., Duran, J. D., Volko, S., and Boller, T. (1999) Plant J. 18, 265–276
7. Gómez-Gómez, L., Felix, G., and Boller, T. (1999) Plant J. 18, 227–284
8. Gómez-Gómez, L., and Boller, T. (2000) Mol. Cell 5, 1003–1011
9. Song, W.-Y., Pi, L.-Y., Wang, G.-L., Gardner, J., Holsten, T., and Ronald, P. C. (1997) Plant Cell 9, 1279–1287
10. Parmiske, M., Hammond-Kosack, K. E., Golstein, C., Thomas, C. M., Jones, D. A., Harrison, K., Wolff, B. B. H., and Jones, J. D. G. (1997) Cell 91, 821–832
11. Meindl, T., Boller, T., and Felix, G. (2000) Plant Cell 12, 1785–1794
12. May, M. J., and Leaver, C. J. (1993) Plant Physiol. 103, 621–627
13. Felix, G., Grasskopf, D. G., Regenass, M., and Boller, T. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8831–8834
14. Mathieu, Y., Kurkdjian, A., Xia, H., Guenn, J., Keller, A., Sprio, M. D., O'Neill, M., Albersheim, P., and Darvill, A. (1991) Plant J. 1, 335–343
15. Nürnberger, T., Nennstiel, D., Jabs, T., Sacks, W. R., Halfbroad, K., and Scheel, D. (1994) Cell 78, 449–460
16. Suzuki, K., and Shishini, H. (1995) Plant Cell 7, 639–647
17. Gómez-Gómez, L., Bauer, Z., and Boller, T. (2001) Plant Cell 13, 1155–1163
18. Baureithel, K., Felix, G., and Boller, T. (1994) J. Biol. Chem. 269, 17931–17938
19. Cheong, J. J., Alba, R., Cote, F., Enkerli, J., and Hahn, M. G. (1995) Plant Physiol 103, 1173–1182
20. Koooman-Gersmann, M., Honée, G., Bonnema, G., and De Wit, P. J. G. M. (1996) Plant Cell 8, 929–938
21. Shiroya, N., Ehsiu, N., Kamada, Y., Kaku, H., Cohn, J., and Its, Y. (1996) Plant Cell Physiol. 37, 894–898
22. Bourque, S., Binet, M. N., Ponchet, M., Pugin, A., and Lebrun-Garcia, A. (1999) J. Biol. Chem. 274, 34699–34705
23. Alfano, J. R., and Collmer, A. (1996) Science 274, 2860–2863
24. Ji, C., Boyd, C., Slaymaker, D., Okinaka, Y., Takeuchi, Y., Takeuchi, M., Yamaoka, N., and Keen, N. T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3306–3311
25. Shimpe, C., Boyer, C., Slaymaker, D., Okinaka, Y., Takeuchi, M., Takeuchi, Y., and Keen, N. T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3306–3311
26. Umemoto, N., Hakuni, M., Iwamatsu, A., Yoshikawa, M., Yamaoka, N., and Ishida, I. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1029–1034
27. Almers, F., Fleigmann, J., Neuhaus-Url, G., Schwarz, H., and Ebel, J. (2000) Biol. Chem. 381, 705–713
28. Clark, S. E., Williams, R. W., and Meyerowitz, E. M. (1997) Cell 89, 575–585
29. Trott, P. D., and Deisenhofer, J. (1995) Nature 374, 183–186
30. He, Z., Wang, Z. Y., Li, J., Zhu, Q., Lamb, C., Ronad, P., and Chory, J. (2000) Science 288, 2360–2363
31. Jones, D. A., Thomas, C. M., Hammond-Kosack, K. E., Balint-Kurti, P. J., and Jones, D. J. G. (1994) Science 266, 789–793
32. Atkinson, M. M., Midland, S. L., Slaymaker, D., Yoshikawa, M., Yamaoka, N., and Keen, N. T. (1997) Plant Cell 9, 1425–1433
Sensitivity of Different Ecotypes and Mutants of *Arabidopsis thaliana* toward the Bacterial Elicitor Flagellin Correlates with the Presence of Receptor-binding Sites

Zsuzsa Bauer, Lourdes Gómez-Gómez, Thomas Boller and Georg Felix

*J. Biol. Chem.* 2001, 276:45669-45676.
doi: 10.1074/jbc.M102390200 originally published online September 19, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102390200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 19 of which can be accessed free at http://www.jbc.org/content/276/49/45669.full.html#ref-list-1