The Arbidopsis Receptor Kinase FLS2 Binds flg22 and Determines the Specificity of Flagellin Perception

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Flagellin, the main building block of the bacterial flagellum, acts as a pathogen-associated molecular pattern triggering the innate immune response in animals and plants. In Arabidopsis thaliana, the Leu-rich repeat transmembrane receptor kinase FLAGELLIN SENSITIVE2 (FLS2) is essential for flagellin perception. Here, we demonstrate the specific interaction of the elicitor-active epitope flg22 with the FLS2 protein by chemical cross-linking and immunoprecipitation. The functionality of this receptor was further tested by heterologous expression of the Arabidopsis FLS2 gene in tomato (Lycopersicon esculentum) cells. The perception of flg22 in tomato differs characteristically from that in Arabidopsis. Expression of Arabidopsis FLS2 conferred an additional flg22-perception system on the cells of tomato, which showed all of the properties characteristic of the perception of this elicitor in Arabidopsis. In summary, these results show that FLS2 constitutes the pattern-recognition receptor that determines the specificity of flagellin perception.

INTRODUCTION

Detection of microbial pathogens by the innate immune system of animals and plants relies on an array of pattern-recognition receptors that recognize molecular structures that are characteristic of pathogens. These pathogen-associated molecular patterns (PAMPs) play key roles as activators of the innate immune response in animals (Medzhitov and Janeway, 2002; Girardin et al., 2003; Takeda and Akira, 2003) and, analogously, as elicitors of defense responses in plants (Nürnberger and Brunner, 2002). Plants also have detection systems for molecular patterns that are specific for particular races of pathogens. Perception of these factors follows the concept of gene-for-gene interaction, with avirulence (Avr) patterns (PAMPs) play key roles as activators of the innate immune response in animals (Medzhitov and Janeway, 2002; Girardin et al., 2003; Takeda and Akira, 2003) and, analogously, as elicitors of defense responses in plants (Nürnberger and Brunner, 2002). Plants also have detection systems for molecular patterns that are specific for particular races of pathogens. Perception of these factors follows the concept of gene-for-gene interaction, with avirulence (Avr) gene products of the pathogens being sensed by cognate resistance (R) gene products of the host plants. However, as recent findings suggest, many of these R products do not interact directly with their corresponding Avr products but require other components that function as the actual pattern-recognition sites for these factors (Nimchuk et al., 2003; Jones and Takemoto, 2004). To date, few receptors for general elicitors or PAMPs have been identified in plants, each involving a different class of proteins (Urano et al., 1997; Gómez-Gómez and Boller, 2000; Mithöfer et al., 2000; Ron and Avni, 2004). In mammals, the most prominent group of pattern-recognition receptors comprises the Toll-like receptors (TLRs), a family of transmembrane proteins with leucine-rich repeat (LRR) extracellular domains. TLRs are involved in the sensing of bacteria, fungi, protozoa, and viruses (O’Neill, 2004). However, most of these TLRs are not fully characterized with respect to the exact microbial structure they recognize and whether the TLRs act as the physical receptor sites for these PAMPs. Also, it is still unknown whether all of the TLRs indeed function as PAMP receptors (O’Neill, 2004).

TLR5 is a well-studied example of a TLR that interacts directly with its single microbial ligand flagellin (Hayashi et al., 2001; Mizel et al., 2003b; Smith et al., 2003). Flagellin, the protein subunit building up the bacterial surface structure flagellum, acts as a PAMP also in plants (Felix et al., 1999; Che et al., 2000). Mammals respond to an epitope of flagellin formed by an N-terminal and a C-terminal part of the peptide chain (Smith et al., 2003). By contrast, many plants recognize a different epitope represented by flg22, a peptide spanning a single stretch of 22 amino acid residues of the most conserved part in the N terminus of flagellin (Felix et al., 1999). In previous work, a genetic approach was used to screen for mutants affected in the perception of the flg22 epitope in Arabidopsis thaliana. Several of these flos (for flagellin sensing) mutants mapped to the FLS2 gene (Gómez-Gómez and Boller, 2000). Plants carrying mutations in FLS2 lack responses to flagellin, show impaired binding of flg22, and exhibit enhanced susceptibility to bacterial infection (Bauer et al., 2001; Zipfel et al., 2004).

FLS2 carries the hallmarks of a receptor kinase: a predicted signal peptide, an extracellular LRR domain, a transmembrane domain, and an intracellular Ser/Thr protein kinase domain. Genes encoding receptor-like kinases (RLKs) form a large family in plants, with >600 members in Arabidopsis (Shiu and Bleecker, 2001). Based mainly on genetic evidence, a growing number of RLKs are implicated in the regulation of a wide range of developmental and defense-related processes (Torii, 2004). Whereas the structural features of the RLKs suggest that these proteins might act as receptors for extracellular signals, clear evidence for...
direct, physical receptor–ligand interaction has been provided for only a few of these perception systems, including those for brassinolide (Kinoshita et al., 2005), phytosulfokine (Matsubayashi et al., 2002), and the wound signal systemin (Scheer et al., 2003). Some RLKs, notably Clavata-1 (CLV1), which is involved in meristem maintenance (Jeong et al., 1999), and the S-locus receptor kinase SRK, which determines self-incompatibility in stigmas of Brassicaceae species (Stein et al., 1991), probably require additional components to form the binding sites for their corresponding signal molecules (Jeong et al., 1999; Takayama et al., 2001).

The RLK FLS2 has an extracellular domain with 28 LRRs, and this domain, like the LRR domain of TLRs in mammals (Mizel et al., 2000), might form the interaction site for flagellin. Specific, high-affinity binding sites for flg22 with the characteristics expected for a flagellin receptor have been biochemically characterized in tomato (Lycopersicon esculentum) and Arabidopsis (Meindl et al., 2000; Bauer et al., 2001). Furthermore, the Arabidopsis mutant fls2-24, carrying a point mutation in one of the LRR repeats, lacks flg22 binding activity completely. Surprisingly, though, plants carrying mutations in the kinase domain of FLS2 also show strongly reduced binding of flagellin (Gómez-Gómez et al., 2001), indicating that an intact FLS2-LRR domain alone might not suffice to form a functional binding site. Thus, the interaction of flagellin with FLS2 might be indirect, involving an additional element to form the high-affinity binding site.

In the first part of this work, we used immunoprecipitation with antibodies specific for FLS2 and chemical cross-linking with a radiolabeled derivative of flg22 to demonstrate specific, physical interaction of flg22 with the FLS2 protein in Arabidopsis. In the second part, we investigated the role of FLS2 for the specificity of flagellin perception. For this purpose, we tested the effect of heterologous expression of the Arabidopsis FLS2 gene in tomato cells. Both Arabidopsis and tomato have highly sensitive perception systems for the flg22 epitope of flagellin. However, despite the general similarity of the perception systems, tomato and Arabidopsis exhibit characteristic differences with respect to the exact structural determinants recognized (Meindl et al., 2000; Bauer et al., 2001). Here, we make use of these species-specific differences and show that tomato cells expressing FLS2 gain a perception system with the properties characteristic of that in Arabidopsis. In summary, we show that FLS2 binds flg22 and determines the specificity of this interaction. These biochemical and functional characteristics identify FLS2 as the bona fide pattern-recognition receptor for flagellin.

RESULTS

Antibodies against the C Terminus of FLS2 Specifically Detect a Polypeptide of ~175 kD in Arabidopsis

The Arabidopsis genome encodes >200 LRR RLKs that show high sequence homology for both the LRR and the kinase domains (Shiu and Bleecker, 2001). Nevertheless, the very C terminus of FLS2, represented by the sequence KANSFREDR-NEDREV, is unique to this particular RLK and also shows no obvious homology with any other protein of Arabidopsis. In extracts of Arabidopsis, polyclonal antibodies raised against this peptide specifically detected a polypeptide migrating with an apparent molecular mass of ~175 kD on protein gel blots (Figure 1). The immunoreactive 175-kD polypeptide was present in extracts of wild-type plants, expressing a functional FLS2 protein (Landsberg erecta [Ler-0] and Columbia [Col-0]), and in the mutant fls2-24, carrying a point mutation in the LRR domain of FLS2. By contrast, no signal was observed in extracts from accession Wassilewskija (Ws-0), expressing a non-functional, C-terminally truncated form of FLS2, and in the Col-0 mutant SAIL_691C4, carrying a T-DNA insertion in the promoter of FLS2 (Zipfel et al., 2004). These results clearly demonstrate the specificity of the antibodies for FLS2 with an intact C terminus.

In some extracts from cultured Arabidopsis cells but not in extracts from plant tissues, an additional immunoreactive polypeptide migrating at ~120 kD was detectable (Figure 1). FLS2 encodes a polypeptide of 126.2 kD (128.8 kD including the signal peptide), with several potential glycosylation sites in its extracellular LRR domain (Gómez-Gómez and Boller, 2000). The anti-FLS2 antibodies precipitated both the ~175-kD and the ~120-kD polypeptides from solubilized extracts of cultured cells (Figure 2A). Analysis of the tryptic digests of these polypeptides by tandem mass spectrometry confirmed the identity of the ~175-kD polypeptide with FLS2 (Mass Spectrometry Protein Sequence Database: Q9FL28_ARATH) and identified the ~120-kD polypeptide as the unrelated protein Q9FIC2_ARATH. The amino acid sequence of this protein of unknown function ends with DSEV-COOH and thus resembles the C terminus of FLS2. In the presence of an excess of the antigenic C-terminal peptide of FLS2, the 175- and 120-kD polypeptides were neither detected on protein gel blots nor observed in the immunoprecipitates (data not shown). This finding strongly suggests that the C terminus of Q9FIC2_ARATH cross-reacts with the antibodies and that this protein is expressed only in the cell cultures.

![Figure 1](image-url)
The Immunoprecipitate with Anti-FLS2 Antibodies Retains Functional Binding Sites for Flagellin

Immunoprecipitates from detergent-solubilized extracts of Arabidopsis were assayed for binding of 125I-Tyr-flg22, a radiolabeled derivative of flg22 used in binding studies (Meindl et al., 2000). Immunoprecipitates from extracts of cultured cells showed strong binding of radiolabel, and this binding was competed for by adding an excess of unlabeled flg22 (Figure 2B). Similarly, immunoprecipitates from extracts of wild-type Ler-0 but not those of the mutant fts-2-24 retained binding activity for 125I-Tyr-flg22 (data not shown). No specific binding of 125I-Tyr-flg22 was found in immunoprecipitates with control antibodies (Figure 2B). Unlabeled flg22 competed for the binding of 125I-Tyr-flg22 to immunoprecipitates with anti-FLS2 antibodies in a concentration-dependent manner, and inhibition of radioligand binding by 50% (IC50) occurred at a concentration of ~5 nM flg22 (Figure 2C). Only weak competition was observed with 100 nM flg15 or 100 nM flg22Aum (Figure 2C, inset), flg22 derivatives with weak or no affinity for the flagellin binding site in Arabidopsis (Bauer et al., 2001). Binding of 125I-Tyr-flg22 to the immunocomplex was reversible, and ~80% of radiolabel was displaced within 30 min of incubation with an excess of unlabeled flg22 (data not shown). Overall, the binding activity of the immunoprecipitates exhibited the specificity and affinity described previously for the binding of flagellin to solubilized extracts of Arabidopsis cells (Bauer et al., 2001).

Affinity Cross-Linking of 125I-Tyr-flg22 Specifically Labels a Polypeptide of ~175 kD in Cells of Arabidopsis

Covalent chemical affinity cross-linking of labeled ligands to their binding sites is a common method for the identification of receptor binding sites. For example, this method has been used successfully to characterize receptor binding sites for phytosulfokine in carrot (Daucus carota) and rice (Oryza sativa) (Matsubayashi and Sakagami, 2000; Matsubayashi et al., 2002), for the wound hormone systemin in Lycopersicon peruvianum (Scheer and Ryan, 2002), and for the self-incompatibility determinant SP11 in Brassica species (Takayama et al., 2001). In cross-linking experiments with 125I-Tyr-flg22 and leaf extracts from wild-type Ler-0 plants, we observed labeling of a band migrating at 175 kD on SDS-PAGE (Figure 3A, left). The presence of excess, unlabeled flg22 in the cross-linking assays suppressed the labeling of this band completely, and no labeling of this band was found in extracts from Ws-0 and all other FLS2 mutants affected in the binding of flg22 (data shown only for Ws-0). Cross-linking to intact cells of Arabidopsis similarly resulted in specific labeling of the ~175-kD band (Figure 3A, middle and right). The presence of excess, unlabeled flg22 in the assays suppressed the labeling of this band completely, whereas only weak or no reduction in staining was observed with 100 nM of the inactive analog flg22Aum or with 100 nM of the structurally unrelated elicitor peptide elf18 (Kunze et al., 2004). No labeling of polypeptides occurred without the addition of chemical cross-linker, but labeling of the 175-kD band occurred also in experiments with di-succinimidylsuberate or dithio-bis(succinimidylpropionate) substituting for ethylene glycol bis(succinimidylsuccinate) as the chemical cross-linker (data not shown).

In good agreement with competitive binding studies of intact cells (Bauer et al., 2001) and with studies conducted on immunoprecipitates (Figure 2C), unlabeled flg22 efficiently competed...
for labeling of the ~175-kD polypeptide with an IC50 of ~5 nM (Figure 3B). These cross-linking experiments show that the flg22 elicitor interacts specifically with a high-affinity binding site on a single ~175-kD polypeptide in Arabidopsis cells.

The anti-FLS2 antibodies were used in immunoprecipitation assays to test whether the 175-kD protein that cross-links to radiolabeled flg22 is the FLS2 protein or a different component that, by coincidence, also migrates at ~175 kD on SDS-PAGE. The anti-FLS2 antibodies efficiently and completely immunoprecipitated the radiolabeled 175-kD protein from solubilized extracts of cells cross-linked to 125I-Tyr-flg22 (Figure 3C). An identical treatment with control antibodies did not pull down any label. Immunoprecipitation of this radiolabeled polypeptide by anti-FLS2 occurred even after denaturation of the solubilized extracts by boiling in the presence of SDS and DTT. This finding demonstrates that flg22 is covalently cross-linked to FLS2 rather than to a protein that coimmunoprecipitates with FLS2.

Expression of FLS2 from Arabidopsis in Tomato Cells

To further probe the role of FLS2 as the receptor binding site for flagellin, we tested the role of the Arabidopsis FLS2 protein when heterologously expressed in cells of tomato. For this, we prepared a vector with the constitutive 35S cauliflower mosaic virus promoter driving the expression of the FLS2 coding sequence fused in frame to a triple c-myc tag. Similar to other C-terminally tagged versions of FLS2 used in earlier complementation studies (Gómez-Gómez and Boller, 2000; Zipfel et al., 2004), this construct fully restored flagellin perception when transformed into fls mutants of Arabidopsis (data not shown). Cells of the tomato line msk8 (Koornneef et al., 1987) were transformed with this construct by particle bombardment and selected for kanamycin resistance. One of the kanamycin-resistant lines that exhibited clear staining of an ~175-kD protein when analyzed on protein gel blots using anti-myc antibodies or antibodies raised against the C terminus of FLS2 was chosen for further studies.

The immunoprecipitates from detergent-solubilized extracts of tomato cells, tomato cells transformed with FLS2:3*myc, and Arabidopsis cells were examined for the presence of the FLS2 protein and flagellin binding activity (Figures 4A and 4B). The immunoprecipitate from nontransformed tomato cells neither showed a signal on a protein gel blot nor contained detectable binding sites for flagellin, indicating that the anti-FLS2 antibodies do not cross-react with the putative ortholog of FLS2 from tomato. By contrast, precipitates from Arabidopsis cells and tomato cells expressing FLS2:3*myc contained FLS2 and exhibited clear radiolabeled bands were detectable on the rest of the gel). Bottom, quantification of the label in the 175-kD band seen in the top panel. Dashed lines indicate the IC50 value for the inhibition of labeling by flg22.

**Figure 3.** 125I-Tyr-flg22 Specifically Cross-Links to a 175-kD Polypeptide That Is Immunoprecipitated by Anti-FLS2 Antibodies. (A) Leaf extracts or intact cells of the cell culture were incubated with 125I-Tyr-flg22 without competitor or with 10 μM unlabeled flg22, the biologically inactive analog flg22Atum, or the structurally unrelated peptide elf18 as indicated. After binding, cross-linking was initiated by the addition of ethylene glycol bis(succinimidylsuccinate) (EGS). Radiolabeled proteins were analyzed after separation by SDS-PAGE with a PhosphorImager. Equal loading with proteins was checked by Coomassie blue staining of the gels (data not shown). (B) Competition of label cross-linking to the 175-kD polypeptide by different concentrations of unlabeled flg22. Top, PhosphorImager picture showing the relevant part of the gel with the 175-kD band (no other
values, respectively, of two replicate measurements. 10 m activity from extracts of tomato cells expressing with anti-myc antibodies precipitated FLS2 and flagellin binding flg22 but not by 100 nM flg15 (Figure 4B). Immunoprecipitations binding of radiolabeled flg22 that was strongly competed for by 125I-Tyr-flg22 without the addition of competitor or with 100 nM flg15 or 100 nM flg22-ΔA16/17 tested (EC50 8 nM), a much higher IC50 value of 3 μM was observed for competition with flg15 (Figure 5). Tomato cells expressing the transgene FLS2 bound more radioligand than the nontransformed cells, and the competition showed characteristics observed in Arabidopsis cells, with IC50 values of 10 nM for flg22 and 8 μM for flg15 (Figure 5). Thus, tomato cells expressing FLS2 have flagellin binding sites that bind flg22 and flg15 in a manner characteristic of Arabidopsis.

The Flagellin Derivative flg22-ΔA16/17 Induces an Alkalization Response in Arabidopsis and Tomato Expressing FLS2 but Not in Tomato

These results demonstrated the expression of functional Arabidopsis-type binding sites in tomato cells transformed with FLS2. To test for the effects of these binding sites on the physiological response to flagellin, we used extracellular alkalization to compare responses in nontransformed tomato, tomato expressing FLS2, and Arabidopsis. Medium alkalization, occurring as a consequence of altered ion fluxes across the plasma membrane, is a rapid, quantitative, and highly reproducible response of cultured plant cells to treatment with various elicitors (Felix et al., 1991; Mathieu et al., 1991; Nürnberg et al., 1994) and has been used to determine the characteristics of flagellin perception in tomato and Arabidopsis (Felix et al., 1999). Tomato cells expressing FLS2 showed increased binding activity (Figure 5) but no significant increase in sensitivity toward flg22 (Figure 6). However, rather than by the number of binding sites, the sensitivity might be limited by other parameters, such as diffusion of the ligand through the cell walls or signal transmission to downstream elements. Indeed, although nontransformed Arabidopsis cells have a higher binding activity than tomato cells (Figure 5), they exhibit lower sensitivity to flg22 (Figure 6). The tomato cells expressing FLS2 showed sensitivity to flg15 that was not significantly different from that of nontransformed cells (data not shown), indicating normal functioning of the endogenous, tomato type of flagellin perception in these transformed cells.

To obtain evidence for the functionality of FLS2 in the tomato background, we screened for flg22 derivatives that would be active in Arabidopsis but not in tomato. Among the different flg22 variants synthesized to probe for structural requirements of flagellin elicitors, we found the peptide flg22-ΔA16/17, lacking one of the Ala residues at position 16 or 17, to have such characteristics. In the experiment shown in Figure 6, dose–response relationships in the different cell cultures were established by measuring the pH changes occurring within 20 min. Although clearly less active than flg22, flg22-ΔA16/17 induced medium alkalization in cells of Arabidopsis half-maximally at a concentration of 80 nM (EC50 value). By contrast, tomato cells showed no significant response even at the highest doses of 60 μM flg22-ΔA16/17 tested (EC50 > 60 μM; Figure 6). In the tomato cells expressing FLS2, however, flg22-ΔA16/17 induced alkalization in a manner similar to Arabidopsis cells, with an EC50 value of ~200 nM. The maximal response reached in these cells with saturating doses of flg22-ΔA16/17 was somewhat lower than that with flg22. This transfer of responsiveness to flg22-ΔA16/17 by expressing FLS2 provided further evidence for the functionality of FLS2 in tomato cells.
The Flagellin Derivative flg22-\(\Delta^{16/17}\) Acts as a Competitive Antagonist of Flagellin Perception in Tomato

Structural analogs of flg22 that exhibit no agonistic activity, such as flg22-\(\Delta^{16/17}\) in cells of tomato, are candidates for structures acting as competitive antagonists. Indeed, when applied together with flg22, the peptide flg22-\(\Delta^{16/17}\) behaved as a competitive antagonist of flg22 in tomato (Figure 6, left). As shown in this example, a dose of 1 \(\mu\)M flg22-\(\Delta^{16/17}\) decreased sensitivity toward flg22 by a factor of \(~10^3\). Doses of 0.1 and 10 \(\mu\)M flg22-\(\Delta^{16/17}\) caused shifts in dose dependency to flg22 of \(~10^2\)- and \(~10^4\)-fold, respectively (see Supplemental Figure 1 online). Also, antagonistic activity of flg22-\(\Delta^{16/17}\) was specific for flagellin and did not affect the responses to structurally unrelated elicitors such as chitin and xylanase (see Supplemental Figure 1 online). In different experiments with different batches of the cell cultures, the absolute pH changes varied somewhat with the density and the age of the cultures. However, the behavior described in the experiments shown in Figure 6 was highly reproducible in several independent experiments.

Antagonists as Tools to Distinguish Arabidopsis-Type and Tomato-Type Flagellin Perception

As described above for flg22-\(\Delta^{16/17}\), several other flagellin-derived peptides exhibit clear differences when assayed for responses in Arabidopsis and tomato (Felix et al., 1999; Meindl

Figure 5. Competition of \(^{125}\text{I}-\text{Tyr-flg22}\) Binding by flg22 and flg15.

Aliquots of intact tomato cells, tomato cells expressing FLS2, and Arabidopsis cells were incubated with \(^{125}\text{I}-\text{Tyr-flg22}\) and various concentrations of flg22 and flg15.

Figure 6. Activity of flg22 and flg22-\(\Delta^{16/17}\), a flg22 Derivative with an Ala Deletion at Position 16 or 17.

Alkalization response in tomato cells, tomato cells expressing FLS2, and Arabidopsis cells treated with different doses of flg22 and/or flg22-\(\Delta^{16/17}\). Values represent changes in extracellular pH occurring within 20 min of treatment. Initial pH (pHi) was 4.6 to 4.8 in the different cell cultures. Competitive antagonism of flg22-\(\Delta^{16/17}\) in nontransformed tomato cells (left, closed circles) was analyzed in cells pretreated for 6 min with 1 \(\mu\)M flg22-\(\Delta^{16/17}\), and extracellular pH was determined 20 min after the addition of the different doses of flg22.
et al., 2000; Bauer et al., 2001). The peptides used in this study and their biological activities as agonists or antagonists in tomato and Arabidopsis are summarized in Table 1. To further delineate the properties of flagellin perception attributable to the FLS2 protein, these peptides were tested for their activities in tomato cells, in tomato cells expressing FLS2, and in Arabidopsis cells (Figure 7). Confirming the findings shown above, 200 nM flg22-Δ16/17 completely inhibited the response to 1 nM flg22 in tomato cells, but this peptide was active as an agonist, either alone or in combination with 1 nM flg22, in cells of Arabidopsis as well as in tomato cells expressing FLS2 (Figure 7A).

The peptide flg15-Δ7, representing the eight amino acid residues in the middle of flg22, is inactive in Arabidopsis but acts as a competitive antagonist for flg22 in tomato (Table 1). Confirming these features, 10 μM flg15-Δ7 completely inhibited the response to 1 nM flg22 in nontransformed tomato cells but had no effect in Arabidopsis cells (Figure 7B, left and right). Importantly, 10 μM flg15-Δ7 had only a minor effect in tomato cells expressing FLS2 (Figure 7B, middle), providing further evidence for Arabidopsis-type flagellin perception in these cells.

Conversely, the peptide flg22-Δ2 could be used as a tomato-specific agonist. In Arabidopsis, this peptide has no agonist activity and exhibits an antagonistic activity only when applied in >1000-fold molar excess over flagellin agonists (Bauer et al., 2001). In accordance with these earlier results, a response to 1 nM flg22-Δ2 was observed in tomato cells but not in cells of Arabidopsis (Figure 7C). Induction of responses by flg22-Δ2 in both nontransformed and FLS2-expressing tomato cells was completely inhibited in the presence of flg15-Δ7 (Figure 7C), indicating the functioning of the tomato type of flagellin perception also in the tomato cells expressing FLS2. The presence of functional tomato-type perception was confirmed with 1 nM flg15 instead of flg22-Δ2 as the tomato-specific agonist and flg15-Δ4 instead of flg15-Δ7 as the tomato-specific antagonist (data not shown).

In summary, tomato cells expressing FLS2 respond to flagellin-derived peptides with characteristics known from both tomato and Arabidopsis. Rather than chimeric, this behavior indicates independent, parallel functioning of tomato-type and Arabidopsis-type perception in these cells. The results also demonstrate the functionality of FLS2 in the tomato background and show that this single protein determines the specificities characteristic of the perception system of Arabidopsis.

**DISCUSSION**

In the first part of this work, we used affinity cross-linking and immunoprecipitation to demonstrate that flg22 specifically interacts with FLS2 present on intact cells of Arabidopsis. In these experiments, flg22, detectable by its iodine label, got immunoprecipitated under denaturing conditions by antibodies specific for the C terminus of FLS2 and was recovered in a complex that comigrates with FLS2 on SDS-PAGE. Although this demonstrates covalent linkage between flg22 and FLS2, one can argue that flg22, rather than interacting directly with the FLS2 polypeptide, might interact with an additional, hypothetical component associated with FLS2. FLS2 does carry covalent modifications that cause the FLS2 antigen (and the cross-linked complex) to migrate at ~175 kD on SDS-PAGE rather than at the ~126 kD predicted for the polypeptide encoded by the FLS2 gene. Most, if not all, of this retardation in electrophoretic mobility appears to be attributable to protein glycosylation, because the FLS2 antigen was found to migrate with an apparent molecular mass of <130 kD after chemical deglycosylation (data not shown). Although interaction of flg22 and FLS2 most likely occurs at the polypeptide part of FLS2, the cross-linking data cannot exclude interaction occurring via the glycosyl part or an additional, as yet undetected and hypothetical component of the FLS2 receptor complex. To further test the role of FLS2 in flagellin perception, we made use of species-specific differences between tomato and Arabidopsis (Meindl et al., 2000; Bauer et al., 2001) in the second part of this work. Tomato cells expressing the Arabidopsis gene FLS2 were found to exhibit flg22 perception with Arabidopsis-type features. These features include affinity for the first seven amino acids with strong preference for flg22 over flg15 (binding assays; Figure 5), inefficiency of the middle part of flg15-Δ7 as an antagonist (Figure 7B), and importance of the amino acid residues at the C terminus for activation of the responses in flg22-Δ2 and flg22-Δ16/17 (Figures 6 and 7). Thus, the characteristics of flagellin perception that go along with the expression of AtFLS2 refer to different parts distributed over the whole flg22 epitope. It is difficult to imagine how FLS2 could cause these species-specific features of recognition without interacting directly with the flg22 epitope. In combination, the results of affinity cross-linking and heterologous expression in tomato show that FLS2 constitutes the pattern-recognition receptor that determines the specificity of flagellin perception.

FLS2 has an extracellular LRR domain, a feature it shares with many RLKs in plants but also with the Toll receptor in Drosophila and the TLRs in mammals. Generally, LRR domains are implicated in protein–protein interactions (Kobe and Kajava, 2001); therefore, we hypothesize that the LRR domain of FLS2 is responsible for interaction with flagellin. The lack of flg22 binding in the mutant fls2-24, carrying a single point mutation in one of the 28 repeats, provides a strong argument for this hypothesis (Bauer et al., 2001). It will be interesting to determine the exact site(s) on the protein at which this cross-linking occurs. However, the amount of flg22-FLS2 complex formed with our current method of cross-linking is not sufficient for analysis by tandem mass spectrometry. In mammals, flagellin has been shown to bind in a highly specific manner to the extracellular LRR domain of TLR5 (Mizel et al., 2003b; Smith et al., 2003). Thus, although the two perception systems are thought to have evolved independently by convergent evolution (Smith et al., 2003), flagellin perception by FLS2 in plants might follow the same fundamental mechanism of direct interaction with a LRR domain.

Attempts to express FLS2 in cells from other kingdoms, such as Escherichia coli, yeast, Pichia, and human embryonic kidney cells, did not lead to the accumulation of a functional flg22 binding site. Specifically, when FLS2 was expressed in human embryonic kidney cells, the FLS2 antigen migrating at ~175 kD was present but no binding activity could be detected (S. Robatzek, unpublished results). This is likely attributable to incorrect posttranslational modification or folding, to mislocalization, or to the absence of additional plant factors required for the formation of
Figure 7. Structural Analogs of flg22 as Tools to Distinguish Tomato-Type and Arabidopsis-Type Flagellin Perception.

(A) Alkalinization response in cells of tomato, tomato expressing FLS2, and Arabidopsis to treatment with flg22 and flg22-ΔA^{16/17} in the combinations indicated. Changes in extracellular pH (ΔpH) occurring within 20 min of treatment with the peptides are plotted as means (bars) and standard deviations of three replicates.

(B) Alkalinization response to treatment with flg22 and flg15-Δ7 in the combinations indicated.

(C) Alkalinization response to treatment with flg22-Δ2 and flg15-Δ7 in the combinations indicated.
functional binding sites in the heterologous expression system. In previous work, we found binding activity of the flagellin receptor to depend on an intact kinase domain of FLS2 (Bauer et al., 2001; Gómez-Gómez et al., 2001), indicating that the LRR domain of FLS2 alone might not be sufficient to form a stable, functional binding site. This is in accordance with previous reports on two other RLKs of Arabidopsis, CLV1 and SRK, for which intrinsic kinase activity seems to be essential for the assembly of the receptor complex or for the formation of ligand binding sites (Trotchaud et al., 1999; Takayama et al., 2001). Demonstration of functional binding sites for plant receptors expressed in cells of other kingdoms has been reported only for the tomato xylanase receptor expressed in mammalian COS-7 cells (Ron and Avni, 2004) and the brassinolide binding epitope of the tomato xylanase receptor expressed in tobacco cells (Scheer et al., 2003), the systemin receptor expressed in tobacco (Scheer et al., 2003), the tomato xylanase receptor expressed in mammalian COS-7 cells (Ron and Avni, 2004) and the brassinolide binding epitope of BRI1 in E. coli (Kinosita et al., 2005). Similarly, expression of functional plant receptors in heterologous plant systems has been described for only a few examples, including the tomato systemin receptor expressed in tobacco (Scheer et al., 2003), the tomato xylanase receptor expressed in tobacco cells (Ron and Avni, 2004), and the flagellar receptor of Arabidopsis expressed in tomato described in this report.

Because FLS2 expressed in tomato cells forms a functional binding site and also induces physiological responses, it seems to properly interact with the downstream signaling components of tomato. This indicates conservation of these elements in both species and conservation of the part of the flagellar receptor that interacts with them. The anti-FLS2 antibodies do not cross-react with the tomato ortholog, indicating that the C terminus is not conserved. Interestingly, in none of the experiments with tomato cells expressing FLS2 did we obtain evidence for a hybrid or chimeric functioning of Arabidopsis and tomato receptors. A hybrid-type functioning could arise for features of perception that depend on oligomerization, either of the FLS2 protein with its tomato ortholog or with other, hypothetical components that determine tomato-type perception. Rather, the transformed tomato cells also show tomato-type perception, indicating independent, parallel functioning of tomato and Arabidopsis receptors in these cells. Compared with Arabidopsis, tomato appears to have a perception system that is more efficient and that responds to a smaller part of the flg22 epitope. Assuming that the flagellar receptor in tomato is a receptor kinase closely related to FLS2, it will be interesting to identify this tomato ortholog and test its function in Arabidopsis. Beyond that, hybrid or chimeric forms of LeFLS2 and AtFLS2 should allow identification of the protein domain(s) responsible for the species-specific features of flagellin perception and receptor activation.

Although the results of this work give FLS2 a primary role as the receptor site determining the specificity of flagellin perception, they do not exclude the possibility that additional protein components are required for the formation of a functional receptor complex. In particular, such elements might be involved in the transmission of the extracellular flg22 signal across the plasma membrane and the activation of the signal output in the cytoplasm. In previous work, we described the binding and activation of the flagellar receptor as a two-step process that follows the address-message concept first put forward for the activation of receptors for neuropeptides in animals (Schwizer, 1980). According to this concept, the N-terminal part of flg22 functions as the address that first binds to the receptor, and the C-terminal part of flg22 then acts as the message activating the receptor (Meindl et al., 2000; Bauer et al., 2001). At least theoretically, such a two-step recognition mechanism could involve two different proteins. However, because both the address and the message part are perceived in an Arabidopsis-type manner in tomato cells expressing FLS2, this strongly indicates that both steps occur on the FLS2 protein alone. Based on models of receptor activation for single transmembrane-spanning receptors in animals, one can envisage the second step to coincide with a conformational change in the extracellular LRR domain. This switch would then allow a change in the homodimerization or heterodimerization of receptor components, leading to concomitant rearrangements of receptor components in the cytoplasm. Assembly of a multireceptor complex involving oligomerization in both the homo and hetero manner has been described for TLR in animals (Underhill, 2003). In particular, some of the responses induced by flagellin in animal cells have been shown to involve signaling via TLR5/TLR4 complexes (Mizel et al., 2003a). In plants, receptor homodimerization has been proposed to occur for SERK1, which is involved in somatic embryogenesis development (Shah et al., 2001). Receptor activation involving homodimerization has been proposed for CLV1 with CLV2 (Jeong et al., 1999) and BRI1 with BAK1 (Li et al., 2002; Nam and Li, 2002; Russinova et al., 2004). In many plant receptor-like proteins, several Cys residues are conserved in the regions of the protein that flank the LRR domain. These residues, which also occur in FLS2, have been proposed to be involved in forming intermolecular disulfide bridges, allowing stable homodimer formation or coupling to signaling partners in the receptor complex (Trotchaud et al., 2001).

Table 1. Sequences of Peptides and Their Biological Activities in Cells of Tomato and Arabidopsis

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Tomato</th>
<th>Sequence</th>
<th>Arabidopsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>flg22</td>
<td>Agonist</td>
<td>QRLSTGSRINSARDDDAAGLQIA</td>
<td>Agonist</td>
</tr>
<tr>
<td>flg15</td>
<td>Agonist</td>
<td>RINSARDDDAAGLQIA</td>
<td>Weak agonist</td>
</tr>
<tr>
<td>flg22-32</td>
<td>Agonist</td>
<td>QRLSTGSRINSARDDAAAGLQ</td>
<td>Weak antagonist</td>
</tr>
<tr>
<td>flg22-3A16/17</td>
<td>Antagonist</td>
<td>QRLSTGSRINSARDD-A-GLQIA</td>
<td>Weak agonist</td>
</tr>
<tr>
<td>flg15-37</td>
<td>Weak antagonist</td>
<td>RINSARDD</td>
<td>Inactive</td>
</tr>
</tbody>
</table>

Categorization of peptides as agonists or antagonists is based on results obtained in this report and in work published previously (Felix et al., 1999; Bauer et al., 2001).
et al., 1987) was maintained and subcultured as described (Felix et al.,
1993). The tomato (Lycopersicon esculentum), were grown as described (May and Leaver,
1999; Van der Hoorn et al., 2005). Building on the methods of immunoprecipitation and chemical cross-linking established in this work, we intend to address the molecular composition of the receptor complex and to study processes of homodimerization and heterodimerization important for the activation of the flagellin receptor.

Plant genomes encode large numbers of receptor kinases that are structurally related to FLS2. Most of these RLKs are orphan with respect to their functions or ligands. Ligand–receptor interactions and transmembrane signaling are poorly understood processes in plants, and one can anticipate that many of these receptors share mechanisms of receptor activation. The well-characterized interaction of flg22 with FLS2 should serve as an excellent model system to study the molecular composition of this activation process.

METHODS

Peptides and Radioiodination

Flg22 and other flagellin-derived peptides were synthesized by F. Fischer (Friedrich Miescher Institute). The elicitor-active peptide elf18, representing the N-acetylated first 18 amino acid residues of bacterial EF-Tu, was synthesized as described previously (Kunze et al., 2004). Peptides were dissolved in water (stock solutions of 1 to 10 mM) and diluted in a solution containing 0.1% BSA and 0.1 M NaCl. Tyr-flg22 was labeled with 125I dissolved in water (stock solutions of 1 to 10 mM) and diluted in a solution containing 0.1% BSA and 0.1 M NaCl. Tyr-flg22 was labeled with 125I by Anawa Trading to yield 125I-Tyr-flg22 with a specific radioactivity of >2000 Ci/mmol.

Immunological Techniques

Polyclonal rabbit antibodies were produced against the C-terminal peptide KANSFREDRNEDREV of FLS2 (Eurogentec). Antibodies were affinity-purified on Affigel-15 beads (Bio-Rad) coupled to the antigenic peptide. These antibodies were used on protein gel blots using goat anti-rabbit IgG coupled to alkaline phosphatase (Sigma-Aldrich) to detect and stain for immunoreactive proteins. For immunoprecipitation, affinity-purified anti-FLS2 antibodies were incubated with solubilized proteins at 4°C on a rotary shaker. After 2 h of incubation, an excess of protein A–Sepharose (Amersham Biosciences) was added for 1 h, and the pellet was washed twice with detergent buffer and twice with binding buffer. The samples were further analyzed either by boiling for 5 min in Laemmli buffer and protein gel blot analysis or by measurement of flg22 binding activity. Anti-myc antibodies recognizing the 10-amino acid epitope N-acetylated first 18 amino acid residues of bacterial EF-Tu, was verified by sequencing.

Cell Expression Cultures of Arabidopsis, Tomato, and Tomato Expressing FLS2

Cell cultures of Arabidopsis thaliana, originally derived from plant tissue of accession Landsberg erecta, were grown as described (May and Leaver, 1993). The tomato (Lycopersicon esculentum) cell line msk8 (Koornneef et al., 1987) was maintained and subcultured as described (Felix et al., 1999).

For expression of FLS2 in tomato cells, the genomic sequence encoding FLS2 was amplified by PCR from the pBBFLS2 vector (Gómez-Gómez and Boller, 2000) and fused in frame to a PKS vector encoding a triple c-myc peptide (three repeats of EKGISEEDL) (Evans and Hancock, 1985) in a pSK vector. This fusion was then cloned into the pCAMBIA 2300 vector containing a cauliflower mosaic virus 35S promoter and the nopaline synthase terminator (www.cambia.org.au). The final construct, termed P35S:FLS2:3’myc, was verified by sequencing.

For transformation, tomato cells were collected on sterile filter paper and incubated for 4 h on agarose-solidified 0.7% (w/v) cell suspension medium supplemented with 0.5 M mannose. Cells were then bombarded at 650 p.s.i. with gold particles coated with 10 µg of plasmid P35S:FLS2:3’myc (Biolistic particle-delivery system, model PDS-1000/HE; BioRad). The filters with the bombarded cells were further incubated for 1 d on medium containing mannose in darkness, for 1 d on medium without mannose in normal light conditions, and then on plates containing medium with 50 µg/mL kanamycin. Growing calli of resistant cells were further propagated under kanamycin selection (50 µg/mL), first on agarose-solidified medium and then in liquid medium.

Protein Extraction

Plants and cells were homogenized in extraction buffer (25 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 10 mM MgCl2) supplemented with the protease inhibitors (cocktail P9599 from Sigma-Aldrich). After centrifugation at 10,000g for 30 min, the pellet (P1) was resuspended in binding buffer. For immunoprecipitation assays, proteins were solubilized from P1 with a buffer containing 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (w/v) octylphenoxypolyethoxethanol (Nonidet P-40), 0.1% (w/v) SDS, and 0.5% (w/v) sodium deoxycholate as detergents. After overnight incubation with slight shaking, the solution was centrifuged and supernatant containing solubilized proteins was used for immunoprecipitation. For immunoprecipitation of extracts from cross-linked cells, the same procedure was used except that 1 mM DTT was added as a reducing agent and samples were boiled for 5 min before the addition of antibodies. The BenchMark precasted protein ladder (Invitrogen) was used as a molecular mass standard for proteins.

Measurement of the Alkalinization Response

To measure the alkalinization response, aliquots of the cell suspensions, 5 to 8 d after subculture, were incubated in open flasks on a rotary shaker at 150 cycles/min and extracellular pH was measured with small combined
glass pH electrodes. Values of pH were either recorded continuously using a pen recorder or measured 15 or 20 min after the start of the experimental treatment.

Accession Number

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AI5g46330.

Supplemental Data

The following material is available in the online version of this article.

Supplemental Figure 1. The Flagellin Derivative flg22-A16/17 Acts as a Competitive Antagonist of Flagellin Perception in Tomato.

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The Arabidopsis Receptor Kinase FLS2 Binds flg22 and Determines the Specificity of Flagellin Perception

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