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### Permalink

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### Journal

Proceedings of the National Academy of Sciences of the United States of America, 108(22)

### ISSN

1091-6490

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### Publication Date

2011-05-31

Peer reviewed

# The *Arabidopsis* flagellin receptor FLS2 mediates the perception of *Xanthomonas* Ax21 secreted peptides

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Detection of microbes by plants relies in part on an array of pattern-recognition receptors that recognize conserved microbial signatures, so-called "microbe-associated molecular patterns." The *Arabidopsis thaliana* receptor-like kinase FLS2 is the pattern-recognition receptor for bacterial flagellin. Similarly to FLS2, the rice transmembrane protein XA21 is the receptor for the sulfated form of the *Xanthomonas oryzae* pv. *oryzae* secreted protein Ax21. Here we show that Ax21-derived peptides activate *Arabidopsis* immunity, triggering responses similar to those elicited by flagellin, including an oxidative burst, induction of defense-response genes, and enhanced resistance to bacterial pathogens. To identify *Arabidopsis* Xa21 functional homologs, we used a reverse genetics approach to screen T-DNA insertion mutants corresponding to all 47 of the *Arabidopsis* genes encoding non-RD kinases belonging to the interleukin-1 receptor-associated kinase (IRAK) family. Surprisingly, among all of these mutant lines, only *fls2* mutants exhibited a significant loss of response to Ax21-derived peptides. Ax21 peptides also failed to activate defense-related responses in a *fls2-24* mutant that does not bind Flg22. Moreover, a Flg22Δ2 variant of Flg22 that binds to FLS2 but does not activate FLS2-mediated signaling suppressed Ax21-derived peptide signaling, indicating mutually exclusive perception of Flg22 or Ax21 peptides by FLS2. The data indicate that FLS2 functions beyond flagellin perception to detect other microbe-associated molecular patterns.

innate immunity | broad spectrum MAMP recognition | non-RD kinases

Pattern-recognition receptors (PRRs) that recognize conserved microbial signatures, which are referred to as microbe-associated molecular patterns (MAMPs), are a key mechanism by which plants and other organisms detect microbes (1). Among several MAMPs detected by *Arabidopsis thaliana*, flagellin is the best studied. In *Arabidopsis*, the leucine-rich repeat (LRR) transmembrane receptor kinase FLAGELLIN SENSITIVE 2 (FLS2) is essential for flagellin perception (2). A 22-aa synthetic peptide (Flg22) corresponding to the recognized domain of flagellin activates FLS2-dependent signaling, triggering the same responses as the native flagellin protein from *Pseudomonas syringae* pv. *tabaci* (3). Flg22-triggered responses include activation of MAPK cascades, up-regulation of defense genes, transient production of an H<sub>2</sub>O<sub>2</sub> oxidative burst, deposition of callose, and enhanced resistance against pathogens (2, 4, 5).

The *Arabidopsis* FLS2 receptor belongs to the IRAK family of receptor like kinases (RLKs), which includes two other well characterized MAMP receptors, *Arabidopsis* EFR (TU-elongation factor-receptor 1) and rice XA21 (*Xanthomonas* resistance protein 21) (6). These RLKs carry the non-RD domain, a motif that is found in many IRAK kinases that function in immune signaling pathways (6). The *Arabidopsis* genome encodes 47 non-RD IRAK kinases, of which 35 are RLKs and 12 are predicted to be cytoplasmic (6, 7).

XA21 recognizes the conserved *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) Ax21 secreted protein (8). Rice plants carrying the *Xa21* gene are fully resistant to *Xoo* carrying Ax21. A synthetic sulfated 17-aa peptide (axY<sup>s</sup>22) derived from Ax21 (residues 17–33

of the Ax21 protein) binds XA21 and triggers enhanced resistance against *Xoo*. Replacing sulfated Tyr (at amino acid position 22 of Ax21) by Ala abolishes Ax21 perception, indicating that the sulfated Tyr residue is required for activity (9).

Here we present data showing that Ax21-derived peptides are also recognized by *Arabidopsis*, and surprisingly, that this recognition is mediated by FLS2, which was previously thought to be highly specific for flagellin. If individual MAMP receptors are capable of recognizing a variety of MAMPs, it increases the spectrum of microbe-derived molecules that can activate an immune response using a relatively limited number of PRRs.

## Results

**Ax21-Derived Peptides Are Perceived by *Arabidopsis*.** Because Ax21 is conserved among *Xanthomonas* species, including *Xanthomonas campestris campestris* (*Xcc*), which is an *Arabidopsis* pathogen, we hypothesized that *Arabidopsis* may be able to respond to Ax21 similarly to other well-characterized MAMPs. Although supernatant extracts from *Xcc* do not normally elicit rice XA21-mediated immunity, they do when a plasmid containing the putative sulfotransferase *RaxST* from *Xoo* is introduced into the otherwise unrecognized *Xcc* bacterial strain (10), suggesting that the examined *Xcc* strains have the capability of normally secreting nonsulfated Ax21. Bacterial extracts from an isogenic *Xcc* Δ*flhC* mutant expressing a variant of flagellin that is not perceived by *Arabidopsis*, still triggered MAMP responses similar to wild-type *Xcc*, indicating that other MAMPs from *Xcc* besides flagellin are recognized by *Arabidopsis* (11), one of them potentially being Ax21.

To test whether Ax21-derived peptides are perceived by *Arabidopsis*, we infiltrated axY22, the nonsulfated version of the sulfated 17-aa peptide recognized by rice, into wild-type Col-0 plants carrying either *WRKY11p::GUS* or *MYB51p::GUS* reporter constructs. These two promoter-reporter constructs were previously shown to be activated by a variety of MAMPs, including Flg22 and Elf26 (a synthetic 26-aa peptide corresponding to elongation factor EF-Tu) (12). Indeed, infiltration of 1 μM axY22 triggered activation of *WRKY11p::GUS* and *MYB51p::GUS* similar to 1 μM Flg22 (Fig. 1). Surprisingly, the sulfated version of axY22 (axY<sup>s</sup>22), as well as a derivative of axY<sup>s</sup>22 that is unable to trigger XA21-mediated immunity, axY22A, which contains alanine instead of tyrosine at position 22 (9), also activated the *Arabidopsis* MAMP reporters (Fig. 1).

Treatment of plants with MAMPs has been shown to trigger a complex and multilayered defense response, including elicitation of a transient H<sub>2</sub>O<sub>2</sub> burst (2). To facilitate the study of Ax21 perception in *Arabidopsis*, we developed a miniaturized and

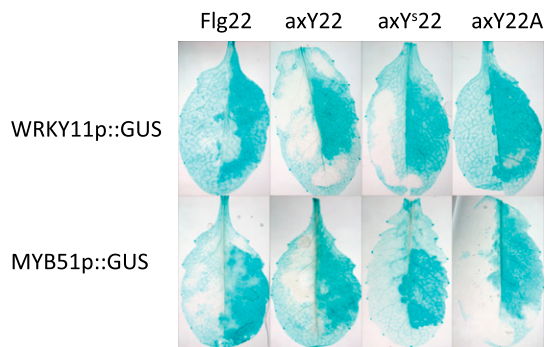
Author contributions: C.H.D., S.-W.H., P.C.R., and F.M.A. designed research; C.H.D., Y.A.M., and T.K. performed research; C.H.D., Y.A.M., T.K., S.-W.H., A.F.B., P.C.R., and F.M.A. analyzed data; and C.H.D. and F.M.A. wrote the paper.

The authors declare no conflict of interest.

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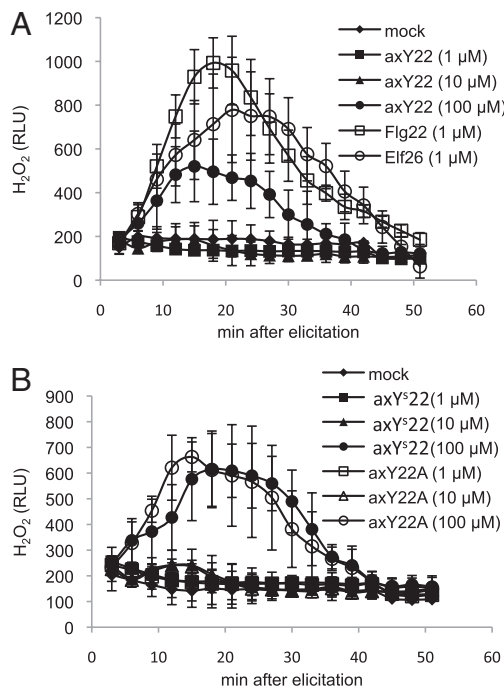
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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1106366108/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1106366108/-DCSupplemental).



**Fig. 1.** Ax21-derived peptides trigger MAMP-responsive gene expression in *Arabidopsis*. Leaves from 6-wk-old Col-0 transgenic plants carrying *WRKY11::GUS* or *MYB51::GUS* constructs were infiltrated with 1  $\mu$ M peptides (Right) or water (Left) and stained with GUS, as described in *SI Methods*.

relatively high throughput assay to measure  $H_2O_2$  bursts in 10-d-old seedlings germinated in liquid MS medium in 96-well assay plates. In agreement with the results shown in Fig. 1, peptides previously shown to be either active (axY<sup>S22</sup>) or inactive (axY22, axY22A) in rice elicited an  $H_2O_2$  burst in *Arabidopsis* (Fig. 2). However, in contrast to adult *Arabidopsis* plants, where relatively low concentrations of Ax21 peptides (1  $\mu$ M) were sufficient to trigger reporter gene expression (Fig. 1), significantly higher concentrations (~100  $\mu$ M) of the Ax21 peptides were necessary to elicit  $H_2O_2$  production in seedlings. The effective concentration Ax21 peptides that arise at sites of bacterial infection are not known, but concentrations of exogenously applied axY<sup>S22</sup> peptide

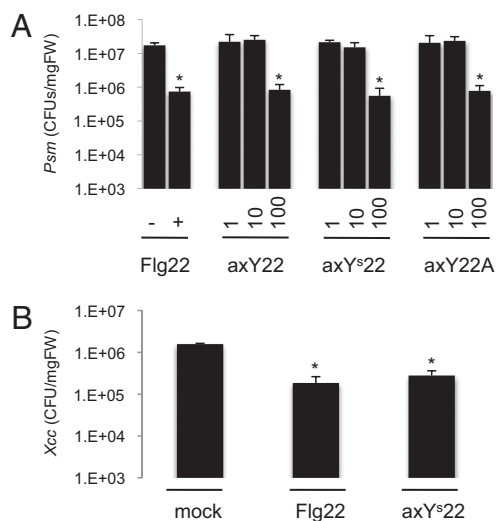


**Fig. 2.** Ax21-derived peptides trigger a hydrogen peroxide burst. Ten-day-old Col-0 wild-type seedlings were grown in 96-well plates and mock-treated or elicited with axY22, Flg22, or Elf26 (A) or with axY<sup>S22</sup> or axY22A (B) at the indicated concentrations: axY<sup>S22</sup> is the sulfated 17-mer that is active in rice; axY22 is the nonsulfated version of axY<sup>S22</sup>; axY22A contains a Tyr→Ala substitution. Neither axY22 nor axY22A can trigger rice XA21-mediated immunity. Each point represents the mean of six seedlings. Error bars represent  $\pm$  SD of the mean. Essentially identical results were obtained in at least three independent experiments.

in the range of 1–100  $\mu$ M are also necessary to trigger immunity in rice (9).

To determine whether the recognition of Ax21 peptides by *Arabidopsis* results in a biologically significant response, we tested whether Ax21-derived peptides trigger enhanced resistance in seedlings to *P. syringae*, similarly to Flg22-elicited protection of seedlings against *P. syringae* (13). By using a *P. syringae* pv. *maculicola* (*Psm*) strain expressing the *LUX* operon from *Photobacterium luminescens*, we were able to monitor bacterial growth by measuring light emission in a scintillation counter (adapted from refs. 13 and 14). For this MAMP-elicited protection assay, seedlings were grown in 12-well plates (20–30 seedlings per well) for 10 d and elicited with various MAMPs, including Ax21 peptides, for a period of 24 h before inoculation with *Psm-LUX*. To assess bacterial growth inside seedlings, washed seedlings were ground at different times after inoculation and luminescence was measured with a scintillation counter. Bacterial growth was estimated by converting light emission into CFUs (using an experimentally determined CPMs/CFUs conversion table). Although relatively high concentrations of the Ax21 peptides were necessary to detect protection activity (100  $\mu$ M), as in the case of the oxidative burst (Fig. 2), peptides that are either active (axY<sup>S22</sup>) or inactive (axY22 and axY22A) in rice were equally capable of triggering enhanced resistance against *P. syringae* in *Arabidopsis* (Fig. 3A). Similarly to the concentration of Flg22 required to elicit an oxidative burst in the seedling assay, 1  $\mu$ M Flg22, but not lower concentrations, elicited protection against *Psm-LUX*. Importantly, both Flg22 and axY<sup>S22</sup> peptides also elicited enhanced resistance against *X. campestris* (Fig. 3B), which suggests that perception of Ax21-derived peptides could be part of a natural *Arabidopsis* defense mechanism against *Xcc*.

In an effort to determine which amino acids in the 17-aa synthetic axY<sup>S22</sup> peptide are important for perception by *Arabidopsis*, we tested a previously described collection of 17 peptides, each one carrying an alanine in place of the original amino



**Fig. 3.** Ax21-derived peptides trigger enhanced resistance against bacteria. (A) Ten-day-old seedlings were grown in 12-well plates, elicited with 1  $\mu$ M Flg22 or 1  $\mu$ M, 10  $\mu$ M, or 100  $\mu$ M axY22, axY<sup>S22</sup>, or axY22A for 24 h, and then infected with *Psm-LUX*. Bacterial titer was assessed as CFU/mg fresh weight seedling tissue 36 h after inoculation: axY<sup>S22</sup> is the sulfated 17-mer that is active in rice; axY22 is the nonsulfated version of axY<sup>S22</sup>; axY22A contains a Tyr→Ala substitution. Neither axY22 nor axY22A can trigger rice XA21-mediated immunity. (B) Seedlings were elicited with 100  $\mu$ M axY<sup>S22</sup> for 24 h and then inoculated with *Xcc*. Each column represents the bacterial titer 24 h after inoculation as CFU/mg fresh weight seedling tissue and is the mean of three wells containing 20 seedlings each. Error bars represent  $\pm$  SD of the mean. Essentially identical results were obtained in at least three independent experiments. \* $P < 0.001$  compared with mock (*t* test).

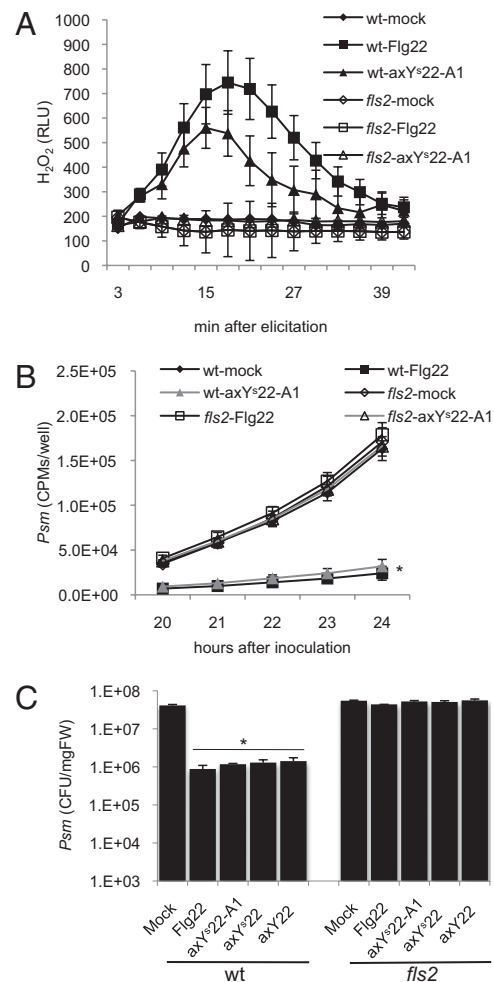
acid, except for residue #1 (which normally contains an Ala at that position) that was replaced by Gly (9). Surprisingly, all of these modified peptides exhibited a similar level of activity as axY22 or axY<sup>s</sup>22 in the *P. syringae* protection assay when tested at 100  $\mu$ M (Fig. S14). However, replacing Ala by Gly at position #1 (axY<sup>s</sup>22-A1), or Glu by Ala at position #2 (axY<sup>s</sup>22-A2), increased the activity of the peptide by at least 10-fold (Fig. S1 B and C). That is, the two peptides containing substitutions at one of the two N-terminal residues were as active at 10  $\mu$ M as axY22 or axY<sup>s</sup>22 were at 100  $\mu$ M.

One explanation for the unexpected result that all of the substituted Ax21 peptides had activity at 100  $\mu$ M is that all peptides at such a relatively high concentration would non-specifically elicit a robust defense response or would directly inhibit the growth of *P. syringae*. To test this possibility, we pretreated seedlings with high concentrations of peptides that have been reported to be inactive in *Arabidopsis*. A Flg22-like peptide derived from *Agrobacterium tumefaciens* (Flg22<sup>A.tum</sup>), or a Flg22-derived peptide Flg22 $\Delta$ 2, both previously shown to be inactive in adult *Arabidopsis* plants (15), were also inactive at 100  $\mu$ M in the seedling protection assay (Fig. S2), suggesting that a high concentration of any peptide does not trigger nonspecific activation of MAMP-elicited responses. To further test the specificity of peptide perception in seedlings, we tested Flg22 and Elf26 perception in *fls2* and *efr* receptor mutants, respectively. High concentrations of active Flg22 or active Elf26 did not trigger a measurable response in the corresponding receptor mutants (Fig. S3), suggesting that high concentrations of peptides do not bypass the requirement for a specific receptor. Finally, to further assess whether the Ax21 peptide response is receptor-specific, we performed a saturation experiment with the axY<sup>s</sup>22 peptide. We found that at concentrations greater than 100  $\mu$ M of peptide the response was saturated, indicating that Ax21 perception is most likely mediated by a specific receptor or receptors (Fig. S4).

Because the axY<sup>s</sup>22-A1 peptide is 10-fold more active than the wild-type peptide in the *Arabidopsis* assays described in this section, we used 10  $\mu$ M axY<sup>s</sup>22-A1 for most of the experiments described below.

**FLS2 Is Required for Perception of Ax21-Derived Peptides.** Most of the known MAMP receptors in plants, including FLS2, EFR, and rice XA21, belong to the IRAK family of non-RD kinases. In an effort to identify *Arabidopsis* Ax21 peptide receptor(s), we used the axY<sup>s</sup>22-A1 peptide in the seedling oxidative burst and protection assays to test a collection of 71 mutant lines consisting of one or two (when available) T-DNA insertion mutations corresponding to each of the 47 non-RD IRAK kinase genes. Surprisingly, only two independent T-DNA insertions in the *fls2* gene compromised the H<sub>2</sub>O<sub>2</sub> burst (Fig. 4A) or the protection against *P. syringae* (Fig. 4B) after axY<sup>s</sup>22-A1 elicitation (Table S1). Similar results were obtained with these two *fls2* mutants in larger scale assays in 12-well plates, where the two *fls2* mutants also failed to respond to axY<sup>s</sup>22-A1, axY<sup>s</sup>22 or axY22 (Fig. 4C).

**Ax21 Peptides Elicit the Same Responses as Flagellin.** To further characterize FLS2-mediated perception of axY<sup>s</sup>22-A1, we tested whether the NADPH oxidase RBOHD (respiratory burst oxidase homolog D), an enzyme that has been shown to be required for reactive oxygen species production after elicitation with Flg22 (16), is also necessary for the axY<sup>s</sup>22-A1 triggered H<sub>2</sub>O<sub>2</sub> burst. Indeed, an *AtrbohD* mutant did not exhibit an H<sub>2</sub>O<sub>2</sub> burst following treatment with axY<sup>s</sup>22-A1 (Fig. 5A). Moreover, we found that mutations in the FLS2 adaptor protein BAK1, which is required for Flg22-elicited signaling (17), partially compromised the enhanced resistance triggered by axY<sup>s</sup>22-A1 (Fig. 5B). Finally, we found that in the seedling assay, 5  $\mu$ M axY<sup>s</sup>22-A1 activates the expression of a set of genes previously shown to be up-regulated by 1  $\mu$ M Flg22 (18) in an FLS2-dependent manner (Fig. 5C). These data show that axY<sup>s</sup>22-A1 triggers a similar cascade of downstream events as those triggered by Flg22, which



**Fig. 4.** FLS2 is necessary for Ax21-derived peptide perception. (A) A hydrogen peroxide burst was elicited in 10-d-old Col-0 wild-type or *fls2* mutant (SAIL\_691\_C04) seedlings in 96-well plates with axY<sup>s</sup>22-A1 (10  $\mu$ M) or Flg22 (1  $\mu$ M). Each datapoint represents the mean of six seedlings (six wells). Error bars represent  $\pm$  SD of the mean. (B) Ten-day-old Col-0 wild-type or *fls2* (SAIL\_691\_C04) seedlings were grown in 96-well plates, elicited with axY<sup>s</sup>22-A1 (10  $\mu$ M) or Flg22 (1  $\mu$ M) for 24 h and then infected with *Psm-LUX*. Bacterial titer was determined with a 96-well plate scintillation counter each hour between 20 and 24 h after inoculation. Each datapoint represents the mean of six seedlings (six wells). Error bars represent  $\pm$  SD of the mean. (C) Ten-day-old Col-0 wild-type or *fls2* mutant (SAIL\_691\_C04) seedlings were grown in 12-well plates and elicited with various Ax21 peptides and infected with *Psm-LUX* as in Fig. 3A. A second *fls2* insertion mutant (Salk\_121477) showed identical results. Each column represents the mean of three wells containing 20 seedlings each. Error bars represent  $\pm$  SD of the mean. Essentially identical results were obtained in at least three independent experiments. \* $P$  < 0.001 compared with mock ( $t$  test).

is consistent with the hypothesis that FLS2 is the axY<sup>s</sup>22-A1 receptor.

**Flg22 Binding Domain of FLS2 Is also Required for Ax21 Peptide Activity.** The FLS2 receptor is a 1,173-aa protein that consists of an intracellular kinase domain, a hydrophobic membrane-spanning domain, and an extracellular domain composed of 25 LRRs (19). A single nucleotide change in LRR #10 (G  $\rightarrow$  R 318, referred to as the *fls2-24* allele) abolishes the binding of Flg22 without compromising the accumulation or stability of FLS2 protein (15, 20), as do other nearby mutations in the proposed binding domain for Flg22 (21). To initiate studies to determine whether Flg22 and axY<sup>s</sup>22-A1 use the same binding domain to activate





MAMPs that an individual plant could recognize without devoting a large fraction of the genome to PRRs. In addition, because MAMPs are likely to be present in low concentrations in a natural infection, the simultaneous recognition of multiple MAMPs may help to strengthen MAMP-mediated signaling. The promiscuity of FLS2 in recognizing both flagellin and the Ax21-derived peptides produced by *Xanthomonas* suggests that the *Arabidopsis* immune system has evolved to maximize the utility of a limited number of PRRs.

MAMP recognition in mammals is carried out by Toll-like receptors (TLRs), transmembrane receptors with LRR external domains, and associated non-RD cytoplasmic kinases, which together are functionally equivalent to FLS2 or XA21. The mammalian receptor TLR2 was initially thought to bind MAMPs as different in structure as LPS, peptidoglycan, and lipoproteins from diverse bacteria and parasites. Later studies, however, demonstrated that LPS and peptidoglycan preparations were contaminated with lipoproteins or lipopeptides (22), which are now thought to be the actual ligands of TLR2. Because all of the peptides used in our study were synthetic (HPLC-purified and subjected to mass spectrometry to confirm mass and sequence and potential contamination with Flg22) (Fig. S6), the apparent promiscuity of FLS2 that we observe cannot be explained as an artifact caused by flagellin or Flg22 contamination.

Mammalian TLR receptors, such as TLR2 and TLR6, can form heterodimers, which broaden the range of specificity of these receptors. TLR2-TLR6 heterodimers recognize 2-acyl-lipoproteins, whereas TLR6 homodimers recognize lipoteichoic acid and zymosan and TLR2 homodimers seem to be inactive (23). A similar mechanism may operate in plants and is a viable hypothesis for the apparent promiscuity of FLS2, although partner MAMP receptors for FLS2 have not been identified. Alternatively, because Flg22 and Ax21 peptides do not show any obvious sequence similarity, it is possible that FLS2 may function as a coreceptor for another protein that is the actual receptor for the Ax21-derived peptides. Consistent with the idea that MAMP receptors function in complexes with transmembrane receptor partners, FLS2 and EFR function in concert with the coreceptor kinase BAK1, which is also required for BRI1-mediated perception of brassinolide (17, 24). However, there is no evidence that BAK1 interacts directly with brassinolide or MAMPs.

In contrast to the coreceptor model, our data suggest that the Flg22- and Ax21-derived peptides directly bind to FLS2 because an FLS2 mutation that affects the binding of Flg22 to FLS2 blocked Ax21-derived peptide elicited responses (Fig. 6A). Furthermore, a deleted version of Flg22, Flg22 $\Delta$ 2, which functions as a suppressor of Flg22 responses (15), partially suppressed the axY<sup>S22</sup>-A1-mediated oxidative burst (Fig. 6B), again suggesting that Flg22- and Ax21-derived peptides may compete for the same binding site on FLS2 (Fig. 6B). Whether FLS2 is the only receptor for Ax21-derived peptides or whether FLS2 is recruited in a receptor complex together with other receptors and adaptors that may modulate its specificity, remains to be determined.

If FLS2 is not the direct or the only receptor providing the binding site for Ax21 peptides, it is unlikely than any of the other 34 *Arabidopsis* non-RD RLKs function as an Ax21 peptide receptor, because T-DNA insertions in the corresponding genes do not affect the ability of Ax21 peptides to activate an *Arabidopsis* immune response, unless they have redundant functions in partnering with FLS2 for Ax21 perception. We limited our search for potential Ax21 receptors to those with the non-RD types of kinase domain (Table S1) because genomic analysis indicated that the presence of the non-RD motif is highly predictive of a function in the innate immune response in both plants and animals (6). For example, all plant non-RD kinases that have been assigned a physiological function serve a key role in innate immunity, including three of the best-studied plant PRRs, FLS2, EFR, and XA21. Because XA21 is closely related to *Arabidopsis* non-RD RLKs of the subfamily LRR-XII, which includes FLS2 and EFR (6, 25), the identification of *AtFLS2* as

an *Xa21* functional homolog is perhaps somewhat predictable. Nevertheless, because the extracellular LRRs of FLS2 and XA21 have no compelling similarity and FLS2 was thought to be specific for flagellin perception, the result that perception of Ax21-derived peptides requires FLS2 is striking.

Given the significant level of identity between FLS2 and XA21, and in light of our data suggesting that FLS2 may be the receptor for Ax21-derived peptides, it is interesting to note that rice cultivars that lack *Xa21* but that still encode functional OsFLS2 do not respond to Ax21-expressing *Xanthomonas* strains (8) and do not perceive Ax21 peptides (9). These latter data suggest that OsFLS2 does not have the broad ligand specificity exhibited by AtFLS2, or that the experimental protocols used in the rice experiments were not sensitive enough to detect this broad specificity.

One explanation for the current divergent specificities of OsFLS2 and AtFLS2 is to postulate the existence of an ancient progenitor of OsFLS2 and AtFLS2 that had the broad ligand specificity of AtFLS2. In the *Arabidopsis* lineage, a broad ligand-specificity of the ancient receptor may have been preserved, but in the rice lineage, a gene duplication may have allowed one paralog (*FLS2*) to lose its capacity to perceive Ax21 peptides as the other paralog (*Xa21*) evolved a high level of specificity for sulfated Ax21 peptides in the wild species *Oryza longistaminata*. One line of evidence in support of this model is that AtFLS2 recognizes many different variants of the axY<sup>S22</sup> 17-mer in addition to Flg22 (Figs. 1 and 2, and Fig. S1). This proposed evolutionary course of events makes sense in light of the vast expansion of predicted PRRs in rice compared with dicots. With nearly 10-fold fewer predicted PRRs, [328 non-RD RLKs in rice versus 35 in *Arabidopsis* (1, 7)], *Arabidopsis* PRRs need to have broad specificity to detect as many MAMPs as rice.

Although microbes can potentially produce a very large number of conserved signatures, only a few MAMPs have been identified, and most of these trigger a similar set of responses. MAMP responses include alkalization of the apoplast, transcriptional up-regulation of defense genes, activation of MAP kinases, deposition of callose, and the transient production of reactive oxygen species (oxidative burst). It is not known, however, if these responses are universal. We therefore suggest that the assessment of MAMP-enhanced resistance, which is likely to be the ultimate consequence of MAMP recognition, may be the most appropriate way to assess MAMP perception. In this article, we describe the development of a high throughput assay for detecting enhanced resistance against bacterial infection (Fig. 4B), which may assist in the identification of novel MAMPs.

The lack of physical information to conclusively define the ligand binding sites of FLS2 makes it difficult to understand how a single receptor mediates the perception of MAMPs with no apparent structural similarity. However, in light of our functional data showing that FLS2 mediates perception not only of flagellin but also of Ax21-derived peptides, it is possible that FLS2 (as well as XA21 and other PRRs) may mediate recognition of other ligands as well. Hence priorities for future research include not only identification of the binding sites of known ligands for well-characterized PRRs, but also discovery of additional putative ligands and potential partner proteins that may alter plant PRR extracellular domain configuration and ligand specificity.

## Methods

**Plant Growth.** Adult plants were grown in climate-controlled growth rooms (Conviron MTP5144) on Metro-Mix 360 soil (Sun Agro) at 22 °C, 75% humidity, and a 16 h photoperiod at 100  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  of light (for genotyping of T-DNA lines and seed propagation) or a 12-h photoperiod (for GUS staining). SALK\_ and SAIL\_ T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (<http://abrc.osu.edu/>). GABI\_Kat lines were obtained from the Arabidopsis Biological Resource Center or from NASC (<http://arabidopsis.info/>). An *Arabidopsis atrbohD* mutant was obtained from M. A. Torres (Universidad Politécnica de Madrid, Spain). The *fls2-24* mutant was obtained from T. Boller (Botanical Institute, University of Basel, Switzerland). Genotyping was carried out by PCR of genomic DNA using PCR primers designed by SIGNAL T-DNA Express and following the recommended combi-

nation of primers (<http://signal.salk.edu/tdnaprimers.2.html>). A complete list of PCR primers is shown in Table S2. For detailed information on genotyping see *SI Methods*.

**Seedling Liquid Culture.** For growing seedlings in liquid medium in 12-well assay plates (BD Falcon; 353043), seeds were sterilized in 20% bleach (2 min), washed three times with sterile water, and 20 to 30 seeds were dispensed into wells containing 1 mL MS 1× medium (Murashige and Skoog basal medium with vitamins from Phytotechnology Laboratories supplemented with 0.5 g/L Mes hydrate and 0.5% sucrose at pH 5.7). Seedlings were grown for 10 d (replacing medium at day 8) at 22 °C, 95% humidity (to prevent medium evaporation) in a plant growth chamber (Conviro; E7/2) under 100  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and a 16-h photoperiod.

For growing seedlings in 96-well plates (Greiner Bio-One; 655083), seeds were sterilized as above and several seeds were dispensed into each well containing 125  $\mu\text{L}$  MS 1× medium (see above). At day 8, the medium was replaced using an eight-well vacuum manifold connected to a vacuum line and seedlings were thinned to leave a single seedling per well. Seedlings were grown for 2 more days at 22 °C, 95% humidity in a plant growth chamber (Conviro; E7/2) under 100  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and a 16-h photoperiod.

**Synthetic Peptides.** Flg22, Flg22<sup>A.tum</sup>, Flg22 $\Delta$ 2 and Elf26 were synthesized by the Massachusetts General Hospital-Peptide/Protein Core Facility. Ax21-derived peptides were synthesized by Pacific Immunology Corporation. For a complete list of Ax21-derived peptides, see ref. 9. Lyophilized peptides were resuspended in sterile water. Mass spectrometry was used to determine the purity of the axY<sup>5</sup>22-A1 peptide as described in *SI Methods* and Fig. S6.

**Luminol Chemiluminescence Assay for H<sub>2</sub>O<sub>2</sub> Detection in 96-Well Plates.** Ten-day-old seedlings were removed from the growth chamber 4 h after the beginning of the light period and kept in the dark for 30 min before elicitation. For the rest of the assay, plates were kept in the dark. Every plate contained 12 wells containing Col-0 wild-type seedlings in row A. Each plate also contained seven different T-DNA insertion lines in rows B to H, (12 seedlings per row; 1 seedling per well). In columns 1 to 6, seedlings were treated with water. In columns 7 to 12, seedlings were treated with peptides (10  $\mu\text{L}$ , in water). A second plate containing the same distribution of wild-type and T-DNA lines was elicited with peptides (10  $\mu\text{L}$ ) in columns 1 to 6 and water in columns 7 to 12. After the addition of 10  $\mu\text{L}$  of water or peptides, plates were centrifuged briefly at 30 × g (Beckman Coulter Allegra x22 swinging arms centrifuge) to ensure that the added peptides were distributed into the medium and that seedlings were exposed to peptide. Immediately after centrifugation, 10  $\mu\text{L}$  of a Luminol-HRP solution in 100 mM K<sub>2</sub>/KPO<sub>4</sub> buffer pH 7.9 [0.5  $\mu\text{g}/\text{mL}$  Luminol (A4685) plus 0.5  $\mu\text{g}/\text{mL}$  Type VI-A HRP from Sigma (P6782)] was added to each well and the plates were briefly

centrifuged again. Plates were placed into the 96-well scintillation reader and light emission was monitored using a 96-well scintillation counter (1450 Microbeta Wallac TriLux Scintillation/Luminescence counter). Every well was read for a total of 5 s in noncoincidental mode. Every plate was read in full 20 to 25 times (every 2.5 min) for a total of 40 to 50 min. Kinetics of H<sub>2</sub>O<sub>2</sub> production were determined by integration of data for every well over the reading period. Every time point is the mean value of six seedlings (either mock or peptide elicited).

**MAMP-Triggered Enhanced Resistance Assay in 96-Well Plates.** Ten-day-old seedlings in 96-well assay plates were grown and arranged in the assay plates and either mock-treated or elicited with peptides as described above for the oxidative burst assays. Seedlings were grown for an additional 24 h after the peptide or mock treatment and then inoculated with 10  $\mu\text{L}$  of *P. syringae* pv. *maculicola* strain ES4326 (OD<sub>600</sub> = 0.0002) carrying the LUX operon from *P. luminescens* (*Psm-LUX*) (14). Bacterial growth was carried out as indicated in *SI Methods*. Inoculated seedlings were grown for 20 h and then transferred hourly to the 96-well scintillation counter for light quantification, as described above. Reads were repeated every hour for a total of 6 h. Kinetics of bacterial growth was determined by integration of data for each well. Each time point is the mean value of six seedlings (either mock or peptide elicited).

**MAMP-Triggered Enhanced Resistance Assay in 12-Well Plates.** Ten-day-old seedlings, germinated and grown in 12-well plates, as described above, were mock-treated with water or treated with peptides for 24 h, inoculated with 100  $\mu\text{L}$  of *Psm-LUX* (OD<sub>600</sub> = 0.002), and incubated an additional 36 h, after which seedlings from each well were removed, quickly dried on paper towels, and transferred to a sterile 2-mL Eppendorf tube. Samples were weighed (to calculate fresh weight) and 400  $\mu\text{L}$  of sterile water plus one 5-mm stainless steel bead was added to each tube. Seedlings were ground with a TissueLyser at 25 shakes per second for 3 min. Aliquots of 100  $\mu\text{L}$  from the ground seedlings were transferred to 96-well plates for light quantification, as described above. Enhanced resistance against *X. campestris* pv. *campestris* (*Xcc* strain 33919) was assessed by eliciting seedlings with Flg22 (1  $\mu\text{M}$ ) or axY<sup>5</sup>22-A1 (10  $\mu\text{M}$ ) for 24 h and then inoculating with 10  $\mu\text{L}$  of *Xcc* (OD<sub>600</sub> = 0.002). Bacterial growth was carried out as indicated in *SI Methods*. Seedlings were blotted dry and ground 36 h after inoculation as described above. Serial dilutions were plated on LB agar to determine CFUs.

**ACKNOWLEDGMENTS.** We thank to J. Bush for plant care. This work was supported by National Institutes of Health Grant R37 GM48707 and National Science Foundation Grant MCB-0519898 (to F.M.A.), National Institutes of Health Grant R01 GM599962 and National Science Foundation Grant MCB-0817738 (to P.C.R.), and by Department of Energy Basic Energy Sciences Grant DE-FG02-02ER15342 (to A.F.B.).

- Ronald PC, Beutler B (2010) Plant and animal sensors of conserved microbial signatures. *Science* 330:1061–1064.
- Gomez-Gomez L, Felix G, Boller T (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J* 18:277–284.
- Felix G, Duran JD, Volko S, Boller T (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J* 18:265–276.
- Asai T, et al. (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* 415:977–983.
- Zipfel C, et al. (2004) Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature* 428:764–767.
- Dardick C, Ronald P (2006) Plant and animal pathogen recognition receptors signal through non-RD kinases. *PLoS Pathog* 2:e2.
- Park CJ, Han SW, Chen X, Ronald PC (2010) Elucidation of XA21-mediated innate immunity. *Cell Microbiol* 12:1017–1025.
- Song WY, et al. (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science* 270:1804–1806.
- Lee SW, et al. (2009) A type I-secreted, sulfated peptide triggers XA21-mediated innate immunity. *Science* 326:850–853.
- Lee SW, Han SW, Bartley LE, Ronald PC (2006) From the Academy: Colloquium review. Unique characteristics of *Xanthomonas oryzae* pv. *oryzae* AvrXa21 and implications for plant innate immunity. *Proc Natl Acad Sci USA* 103:18395–18400.
- Sun W, Dunning FM, Pfund C, Weingarten R, Bent AF (2006) Within-species flagellin polymorphism in *Xanthomonas campestris* pv. *campestris* and its impact on elicitation of *Arabidopsis* FLAGELLIN SENSING2-dependent defenses. *Plant Cell* 18:764–779.
- Millet YA, et al. (2010) Innate immune responses activated in *Arabidopsis* roots by microbe-associated molecular patterns. *Plant Cell* 22:973–990.
- Songnan W, et al. (2008) A Seedling Assay for MAMP Signaling and Infection Studies (International Society for Molecular Plant-Microbe Interactions, St. Paul, MN).
- Fan J, Crooks C, Lamb C (2008) High-throughput quantitative luminescence assay of the growth in planta of *Pseudomonas syringae* chromosomally tagged with *Photorhabdus luminescens* luxCDABE. *Plant J* 53:393–399.
- Bauer Z, Gómez-Gómez L, Boller T, Felix G (2001) Sensitivity of different ecotypes and mutants of *Arabidopsis thaliana* toward the bacterial elicitor flagellin correlates with the presence of receptor-binding sites. *J Biol Chem* 276:45669–45676.
- Zhang J, et al. (2007) A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. *Cell Host Microbe* 1:175–185.
- Chinchilla D, et al. (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448:497–500.
- Denoux C, et al. (2008) Activation of defense response pathways by OGs and Flg22 elicitors in *Arabidopsis* seedlings. *Mol Plant* 1:423–445.
- Gómez-Gómez L, Boller T (2000) FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol Cell* 5:1003–1011.
- Gómez-Gómez L, Bauer Z, Boller T (2001) Both the extracellular leucine-rich repeat domain and the kinase activity of FLS2 are required for flagellin binding and signaling in *Arabidopsis*. *Plant Cell* 13:1155–1163.
- Dunning FM, Sun W, Jansen KL, Helft L, Bent AF (2007) Identification and mutational analysis of *Arabidopsis* FLS2 leucine-rich repeat domain residues that contribute to flagellin perception. *Plant Cell* 19:3297–3313.
- Zähringer U, Lindner B, Inamura S, Heine H, Alexander C (2008) TLR2—promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity. *Immunobiology* 213:205–224.
- Akira S, Takeda K (2004) Toll-like receptor signalling. *Nat Rev Immunol* 4:499–511.
- Li J, et al. (2002) BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* 110:213–222.
- Shiu SH, et al. (2004) Comparative analysis of the receptor-like kinase family in *Arabidopsis* and rice. *Plant Cell* 16:1220–1234.