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Rice OsFLS2-Mediated Perception of Bacterial Flagellins Is Evaded by *Xanthomonas oryzae* pvs. *oryzae* and *oryzicola*

Shanzhi Wang[1,2,6], Zhe Sun[1,2,6], Huiqin Wang[1,2], Lijuan Liu[1,2], Fen Lu[1,2], Jun Yang[1,3], Min Zhang[4], Shiyong Zhang[3], Zejian Guo[1,2], Andrew F. Bent[5] and Wenxian Sun[1,2,*]

[1]Department of Plant Pathology, China Agricultural University, 2 West Yuanmingyuan Road, Haidian District, Beijing 100193, China

[2]Key Laboratory of Plant Pathology, Ministry of Agriculture, China Agricultural University, Beijing 100193, China

[3]Rice Research Institute, Shandong Academy of Agricultural Science, Jinan 250100, Shandong Province, China

[4]College of Bioscience and Biotechnology, Hunan Agricultural University, Changsha 410128, Hunan Province, China

[5]Department of Plant Pathology, University of Wisconsin, Madison, WI 53706, USA

[6]These authors contributed equally to this article.

***Correspondence: Wenxian Sun (wxs@cau.edu.cn)**

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ABSTRACT

Bacterial flagellins are often recognized by the receptor kinase FLAGELLIN SENSITIVE2 (FLS2) and activate MAMP-triggered immunity in dicotyledonous plants. However, the capacity of monocotyledonous rice to recognize flagellins of key rice pathogens and its biological relevance remain poorly understood. We demonstrate that ectopically expressed OsFLS2 in *Arabidopsis* senses the eliciting flg22 peptide and *in vitro* purified *Acidovorax avenae* (*Aa*) flagellin in an expression level-dependent manner, but does not recognize purified flagellins or derivative flg22^{Xo} peptides of *Xanthomonas oryzae* pvs. *oryzae* (*Xoo*) and *oryzicola* (*Xoc*). Consistently, the flg22 peptide and purified *Aa* flagellin, but not *Xoo*/*Xoc* flagellins, induce various immune responses such as defense gene induction and MAPK activation in rice. Perception of flagellin by rice does induce strong resistance to *Xoo* infection, as shown after pre-treatment of rice leaves with *Aa* flagellin. OsFLS2 was found to differ from AtFLS2 in its perception specificities or sensitivities to different flg22 sequences. In addition, post-translational modification of *Xoc* flagellin was altered by deletion of glycosyltransferase-encoding *rbfC*, but this had little effect on *Xoc* motility and *rpfC* mutation did not detectably reduce *Xoc* virulence on rice. Deletion of flagellin-encoding *fliC* from *Xoo*/*Xoc* blocked swimming motility but also did not significantly alter *Xoo*/*Xoc* virulence. These results suggest that *Xoo*/*Xoc* carry flg22-region amino acid changes that allow motility while evading the ancient flagellin detection system in rice, which retains recognition capacity for other bacterial pathogens.

Key words: OsFLS2, flagellin, perception specificity, *Xanthomonas oryzae*

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INTRODUCTION

One of the major molecular strategies that plants have developed to defend against pathogens is to use plasma membrane-bound pattern recognition receptors (PRRs) to respond to microbe-associated molecular patterns (MAMPs), in a process that has been termed MAMP-triggered immunity (MTI) (Ausubel, 2005; Chisholm et al., 2006; Jones and Dangl, 2006; He et al., 2007). MAMPs, designated as general elicitors previously, have drawn intensive attention since bacterial flagellins were first characterized as elicitors of plant defenses (Felix et al., 1999). Additional types of MAMPs that subsequently have been characterized include elongation factor Tu (EF-Tu), cold shock proteins, peptidoglycan and lipopolysaccharide from bacteria, glucans, arachidonic acid and ergosterol from oomycete, and chitin from fungi (Boller and Felix, 2009). Correspondingly, PRRs

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that recognize the corresponding MAMPs have been discovered, such as FLAGELLIN SENSING2 (FLS2), elongation factor Tu receptor (EFR), chitin elicitor receptor kinase 1 (CERK1), and LysM-containing proteins (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006; Miya et al., 2007; Liu et al., 2012).

FLS2, first characterized as a transmembrane leucine-rich repeat (LRR) kinase in *Arabidopsis*, senses bacterial flagellin and thus triggers plant innate immunity (Gómez-Gómez and Boller, 2000; Zipfel et al., 2004). FLS2 orthologs have since been identified in tomato, *Nicotiana benthamiana*, *Brassica*, and rice (Dunning et al., 2007; Hann and Rathjen, 2007; Robatzek et al., 2007; Takai et al., 2008). Putative *FLS2* coding sequences were found in other plant genomes such as poplar and grape (Tuskan et al., 2006; Jaillon et al., 2007). Wide distribution of FLS2 in different species suggests the perception of bacterial flagellin through FLS2 in plants is evolutionarily ancient (Boller and Felix, 2009). The flg22 region in a conserved domain near the N terminus of bacterial flagellins carries the elicitation determinant of bacterial flagellin that is recognized by FLS2 in many plant species (Felix et al., 1999; Sun et al., 2006; Zou et al., 2012). The synthetic flg22 peptide used by default in most studies carries a 22-amino-acid sequence derived from a *Pseudomonas aeruginosa* flagellin (Felix et al., 1999). In *Arabidopsis*, flg22 directly binds to the concave surface of FLS2 LRR in the region from the extracellular LRR3 to LRR16 (Sun et al., 2013). Another study showed that LRRs 7–10 and LRRs 19–24 of the LeFLS2 ectodomain are both involved in ligand perception in tomato (Mueller et al., 2012). FLS2 orthologs exhibit characteristically different perception specificity in several cases. For example, LeFLS2 strongly responded to $flg15^{E\ coli}$, a shortened version of the flg22 peptide derived from *Escherichia coli*, while *Arabidopsis* AtFLS2 and *Nicotiana* NbFLS2 did not (Robatzek et al., 2007). In addition, a new flgII-28 region distinct from flg22 in the flagellin of *Pseudomonas syringae* pv. *tomato* (*Pst*) has been recently characterized as a MAMP, which may be specifically recognized by Solanaceae species (Cai et al., 2011; Clarke et al., 2013).

Takai et al. (2008) characterized OsFLS2 as the FLS2 ortholog in rice and demonstrated that rice cultured cells with OsFLS2 overexpression exhibited evident cell death in response to flg22 stimulation. Constitutive expression of flagellin of *Acidovorax avenae*, an important rice pathogen, in the transgenic rice plants also triggered immune responses and conferred disease resistance to rice blast caused by a fungal pathogen *Magnaporthe oryzae* (Takakura et al., 2008). These studies suggest that rice possesses a conserved flagellin perception system utilizing the FLS2 receptor. However, previous studies also showed that suspension-cultured rice cells were insensitive or only weakly sensitive to flg22, but strongly sensitive to full-length flagellin from incompatible strains of *A. avenae* (Felix et al., 1999; Takai et al., 2008). This finding suggests that rice might perceive *A. avenae* flagellin in additional or different ways. Other recent studies demonstrated that a rapid oxidative burst was detected in rice leaves upon flg22 treatment (Ding et al., 2012). A better understanding is needed of FLS2 function in monocot plants such as rice. It is also of interest to investigate whether the perception of bacterial flagellins contributes to plant resistance to bacterial infection in rice.

Bacterial blight and leaf streak, caused by Gram-negative bacteria *Xanthomonas oryzae* pvs. *oryzae* (*Xoo*) and *oryzicola* (*Xoc*), respectively, are among the most important bacterial diseases in rice (Mew, 1987; Nino-Liu et al., 2006). The interaction between rice and *Xoo*/*Xoc* has become a model pathosystem to study bacterial pathogenicity (Nino-Liu et al., 2006). It has been demonstrated that extracellular polysaccharides, secreted proteases, xylanase, cellobiosidase, esterase, small diffusible factors, and type III effectors are important virulence factors in *Xoo* and/or in *Xoc* (Ray et al., 2000; Rajeshwari et al., 2005; Jha et al., 2007; Aparna et al., 2009; Büttner and Bonas, 2010; Zhang et al., 2013). Bacterial flagellin has been shown to serve as an important type of virulence factor in some phytopathogenic bacteria (Panopoulos and Schroth, 1974; Haefele and Lindow, 1987), such as *Ralstonia solanacearum* (Tans-Kersten et al., 2001), but in many others the flagellin is recognized as a MAMP by host plants and triggers host immunity (Felix et al., 1999; Boller and Felix, 2009). So far, it is unknown which role(s) *Xoo* and *Xoc* flagellins play in the *Xoo*/*Xoc* infection of rice plants. Previous studies revealed that flagellins of different *X. campestris* pv. *campestris* (*Xcc*) strains exhibit distinct abilities to elicit host innate immunity (Sun et al., 2006), that other bacteria also make flagellins that fail to elicit *Arabidopsis* or tomato FLS2 (Felix et al., 1999; Pfund et al., 2004), and that *P. aeruginosa* expresses an extracellular protease that degrades defense-eliciting flagellin fragments (Bardoel et al., 2011). Therefore, it is of interest to investigate whether the important pathogens *Xoo* and *Xoc* have evolved to evade the flagellin perception system in rice.

Flagellin glycosylation has an important role in plant–pathogen interactions (Taguchi et al., 2009; Hirai et al., 2011). Although flagellins derived from *P. syringae* pv. *glycinea* race 4 (Pgl 4) and *P. syringae* pv. *tabaci* (Pta 6605) share absolute amino acid sequence identity, they have distinct abilities to induce hypersensitive response (HR) in tobacco plants, suggesting that post-translational modification of flagellins is important for the HR-inducing activity and virulence of *P. syringae* (Taguchi et al., 2003a; 2003b). Consistently, a deglycosylated flagellin from the compatible *A. avenae* strain K1 induced immune responses in cultured rice cells while the native K1 flagellin did not. Site-directed mutagenesis of glycosylated amino acid residues ^{178}Ser and ^{183}Ser in K1 flagellin suggested that the glycans attached to these residues cover elicitation determinants and prevent the perception by the rice flagellin surveillance system (Hirai et al., 2011). Based on genome information of *Xoo* and *Xoc*, a glycosylation island containing multiple genes is inserted in flagellar gene cluster, between the flagellar gene *fliE* and flagellar regulatory gene *fleQ* (Supplemental Figure 1). This suggests that these genes are involved in flagellin glycosylation. One of the genes in the operon, *rbfC*, encodes a glycosyltransferase with Glycos_transf_2 conserved domain, suggesting the testable hypothesis that RbfC is a key component for flagellin glycosylation that may impact the interaction of rice and *Xoo*/*Xoc*.

In this study, amino acid polymorphisms in *Xoo* and *Xoc* flagellins were investigated using multiple strains. We subsequently determined the perception specificity and sensitivity of rice OsFLS2 to various flg22 peptides, to *in vitro* purified bacterial flagellins derived from *Xoo*, *Xoc*, and *A. avenae*, and to swapped or

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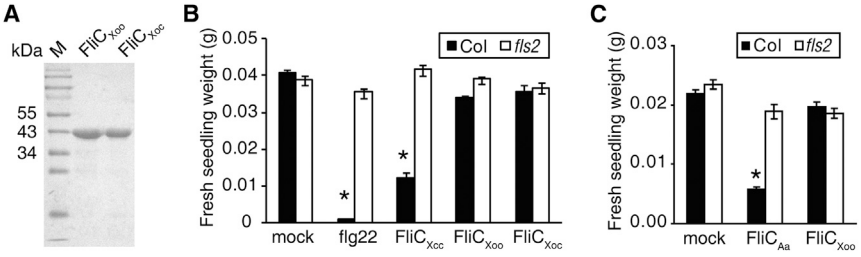

Figure 1. *FLS2*-Dependent Elicitation by the Different His-Tagged Flagellins of *Xoo* PXO99, *Xoc* RS105, *Xcc* B305, and *A. avenae* R2 in *Arabidopsis*.

(A) His-tagged flagellin proteins shown on SDS–PAGE gels stained by Coomassie blue. M indicates pre-stained protein markers; FliC$_{Xoo}$ and FliC$_{Xoc}$ indicate His-tagged *Xoo* and *Xoc* flagellins after elution from nickel-nitrilotriacetic acid agarose columns, respectively.

(B) Purified His-tagged *Xoo* and *Xoc* flagellins were non-eliciting in *Arabidopsis* Col-0, assayed by SGI. *Arabidopsis* Col-0 (black bars) and *fls2-101* (white bars) seedlings were treated with water as a negative control, 5 μM flg22 as a positive control, or 5 μM purified flagellin of *Xcc* B305 (FliC$_{Xcc}$), *Xoo* PXO99 (FliC$_{Xoo}$), and *Xoc* RS105 (FliC$_{Xoc}$).

(C) His-tagged *A. avenae* flagellin (FliC$_{Aa}$), but not FliC$_{Xoo}$, inhibited seedling growth in *Arabidopsis* Col-0. Fresh seedling weights (mean ± SE) are for 12 seedlings per treatment, measured 10–14 days after treatment.

Asterisks in **(B)** and **(C)** indicate statistical significance between flg22/flagellin treatments and mock controls ($P < 0.05$, Tukey's honest significance test).

mutated variants of *Xoo* and *Aa* flagellins. Furthermore, *ΔfliC$_{Xoo}$*, *ΔfliC$_{Xoc}$*, and *ΔrbfC$_{Xoc}$* mutants were constructed and their virulence to rice was investigated. Multiple defense assays indicated that some of the *in vitro* purified bacterial flagellins, such as those from *A. avenae*, can be recognized by OsFLS2 and trigger immunity against bacterial infection, while others including *Xoo* and *Xoc* flagellins have evaded the OsFLS2-mediated surveillance system through sequence diversification at multiple amino acid residues in the flg22 region that are essential for perception.

RESULTS

X. oryzae pvs. *oryzae* and *oryzicola* Strains Have Low Levels of Flagellin Polymorphism

Flagellin proteins from different bacterial species, pathovars, and even strains can exhibit different levels of variations in amino acid sequences (Beatson et al., 2006; Sun et al., 2006). To determine amino acid polymorphism of *X. oryzae* flagellins, we isolated and sequenced the *fliC* genes from 15 *Xoo* and 5 *Xoc* strains using the primers designed according to the published *fliC* sequences of *Xoo* PXO99 and *Xoc* BLS256 (Supplemental Tables 1 and 2). Alignment of the derived amino acid sequences showed that the flagellins of PXO99 and RS105 have only three amino acid polymorphisms including A181G, A282T, and S366T. Only one amino acid residue in PXO61 and two in PXO112 flagellin are different among these *Xoo* strains, and all of five *Xoc* strains share the identical flagellin protein sequences (Supplemental Figure 2), indicating very low levels of amino acid polymorphisms in flagellins among *Xoo* and *Xoc* strains.

In Vitro Purified Flagellins of Different Rice Bacterial Pathogens Have Distinct Abilities to Elicit Growth Inhibition in *Arabidopsis* Seedlings

To investigate whether *Xoo* and *Xoc* flagellins have an elicitation activity to trigger plant defense responses, full-length flagellin proteins derived from the representative strain PXO99 for *Xoo* (FliC$_{Xoo}$) and RS105 for *Xoc* (FliC$_{Xoc}$) were purified as His6 fusion proteins (Figure 1A). The ability of purified proteins to elicit plant defenses was first evaluated using seedling growth inhibition (SGI) assays in *Arabidopsis* (Figure 1B). The results showed that the growth of *Arabidopsis* Col-0 seedlings was not significantly affected after the treatment of purified FliC$_{Xoo}$ and FliC$_{Xoc}$ but was strongly inhibited by flg22 and the *in vitro* purified flagellin derived from *Xcc* B305 (Figure 1B). All these purified flagellins and flg22 failed to inhibit the growth of *fls2-101* seedlings (Figure 1B). For comparison, the His6-tagged flagellin derived from the rice-compatible *Acidovorax avenae* strain R2 (FliC$_{Aa}$) was also purified and used in the SGI assays. The data demonstrated that the growth of Col-0 seedlings, but not of *fls2* seedlings, was strongly inhibited by FliC$_{Aa}$ treatment (Figure 1C). These results indicate that the most common *Xoo* and *Xoc* flagellins expressed in *E. coli* have no elicitation activity while *A. avenae* flagellin can be perceived by FLS2 in *Arabidopsis*.

Heterologous Expression of OsFLS2 in *Arabidopsis* Confers Recognition of the flg22 Peptide but Not *Xoo* and *Xoc* Flagellins

OsFLS2-overexpressing rice cells exhibited obvious cell death in response to flg22 stimulation while only weak defense responses were induced in wild-type rice cells, suggesting that the expression level of OsFLS2 is critical for flg22-dependent elicitation of rice immunity (Takai et al., 2008). To investigate if *Xoo* and *Xoc* flagellins are recognized by OsFLS2, we developed *OsFLS2* transgenic *Arabidopsis fls2-101* plants with OsFLS2 expression driven by a 35S or *OsFLS2* endogenous promoter. Six transgenic lines, OE-1, OE-HA-1, NE-2, NE-8, NE-13, and EV-C-4, representing different levels of *OsFLS2* expression, were identified for further assays (Supplemental Figure 3A). Western blot analysis demonstrated that OsFLS2 in the transgenic line OE-1 with the 35S promoter was highly expressed while it was hardly detected in the transgenic line NE-8 with the native promoter (Figure 2A). SGI assays showed that transgenic lines with higher expression exhibited much stronger inhibition in response to flg22 than the lines with lower expression. In particular, the transgenic lines NE-8 and NE-13, in which OsFLS2 expression is relatively low, were not altered in growth after flg22 treatment (Supplemental Figure 3B). In addition, expression of defense-related genes including *AtWRKY40*, *AtWRKY53*, and *AtCuBP* was up-regulated by flg22 in the OsFLS2-overexpressing transgenic line OE-1, but not in the transgenic lines EV-C-4 and NE-8 (Supplemental Figure 3C). In line with ligand-induced FLS2 degradation in *Arabidopsis* (Smith et al., 2014), OsFLS2 accumulation in the OsFLS2-overexpressing transgenic *Arabidopsis* plants was significantly reduced at 90 min after flg22 treatment, but not by *Xoo*/*Xoc* flg22 (flg22^{Xo}) (Supplemental Figure 3D). Bacterial infection assays showed that the growth of *Pst* DC3000 in OsFLS2-overexpressing plants was

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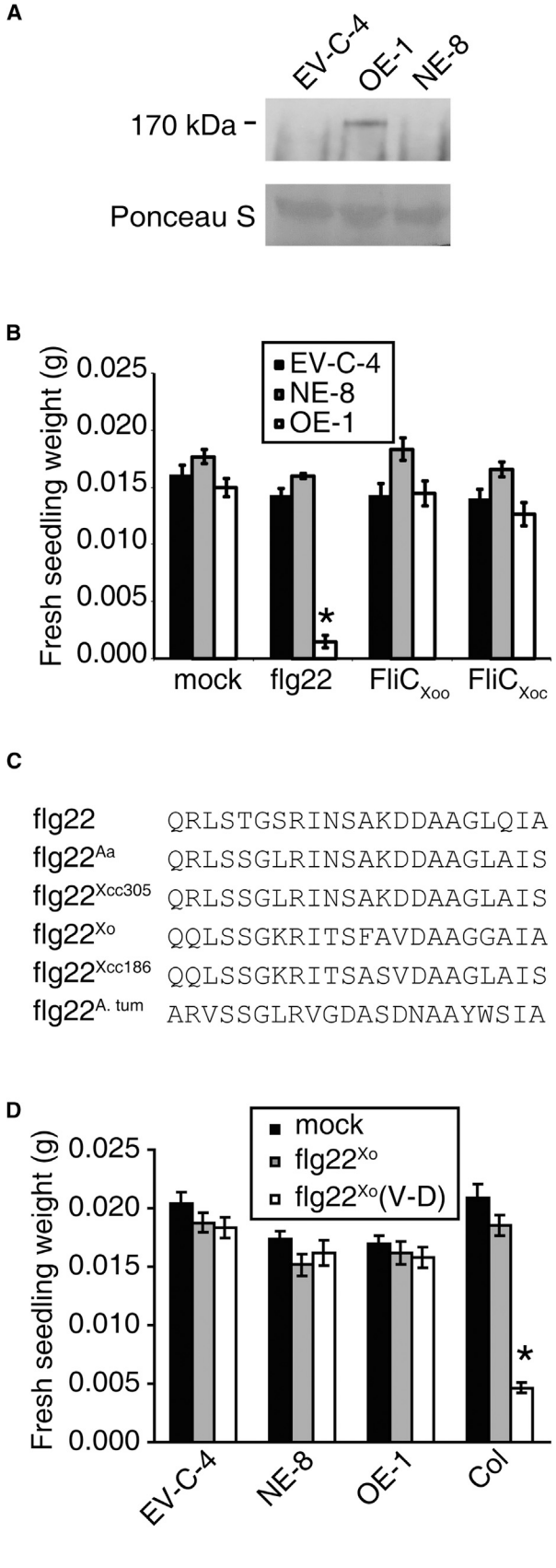

Figure 2. SGI Assays of Transgenic *Arabidopsis fls2-101* with Different Expression Levels of OsFLS2 Treated with FliC$_{Xoo}$, FliC$_{Xoc}$, flg22^{Xo}, and Its Derivative flg22^{Xo}(V-D).

(A) OsFLS2 expression was detected via immunoblotting in transgenic *Arabidopsis* plants. Total protein extracts of the transgenic lines EV-C-4, NE-8, and OE-1 were probed with the anti-OsFLS2 polyclonal antibody. Bottom: Ponceau S staining of the same blot to detect protein loading.

(B) OsFLS2 ectopically expressed in *Arabidopsis* hardly recognized purified FliC$_{Xoo}$ and FliC$_{Xoc}$, even when OsFLS2 was overexpressed in *Arabidopsis*. The seedlings of transgenic lines EV-C-4, NE-8, and OE-1 were treated with 5 μM *in vitro* purified *Xoo* and *Xoc* flagellins, water (mock), and 5 μM flg22 as controls in SGI assays. The flg22 peptide, but not FliC$_{Xoo}$ and FliC$_{Xoc}$, strongly inhibited seedling growth in OsFLS2 overexpression transgenic plants. The inhibition is dependent on the expression level of OsFLS2 in transgenic plants. Asterisk indicates statistical significance between flg22 treatment and mock control ($P < 0.05$).

(C) Alignment analysis showed variations in the flg22 region among several flagellins from different species including *X. oryzae* and *A. avenae*.

(D) AtFLS2 and OsFLS2 in *Arabidopsis* exhibited different recognition specificity or sensitivity to flg22^{Xo}(V-D) in which the Val residue was replaced by the Asp residue in flg22^{Xo} peptide. The wild-type and transgenic *Arabidopsis* seedlings (EV-C-4, NE-8, and OE-1 lines) were treated with water, 5 μM flg22^{Xo}, or flg22^{Xo}(V-D) for 10 days and then weighed (mean ± SE). Asterisk indicates statistical significance in seedling weights of Col and the transgenic line EV-C-4 ($P < 0.05$, Tukey's honest significance test).

significantly inhibited after flg22 pre-treatment compared with that in the EV-C-4 transgenic plants (Supplemental Figure 3E). Taken together, these data demonstrated that flg22 perception by OsFLS2 was dependent on its expression level in *Arabidopsis* and only activated strong defenses in OsFLS2-overexpressing *Arabidopsis* plants.

To investigate if the expression level of OsFLS2 in *Arabidopsis* affects perception of *Xoo* and *Xoc* flagellins, the EV-C-4, NE-8, and OE-1 seedlings were treated with mock buffer, flg22, purified FliC$_{Xoo}$, and FliC$_{Xoc}$ (Figure 2B). The results showed that *Xoo* and *Xoc* flagellins did not elicit SGI of these transgenic plants regardless of the expression level of OsFLS2. By contrast, strong SGI was observed when the OsFLS2-overexpressing transgenic line OE-1 was treated with flg22 and FliC$_{Aa}$. No evident growth inhibition was detected for the transgenic line NE-8 in response to these purified proteins and peptides. More importantly, the microscale thermophoresis assay showed a significant change in thermophoresis when fluorescently labeled FliC$_{Aa}$, but not fluorescently labeled FliC$_{Xoo}$, was added with different concentrations of microsomal membranes isolated from the OsFLS2-overexpressing line, indicating that OsFLS2 ectopically expressed in *Arabidopsis* may bind to FliC$_{Aa}$ but not to FliC$_{Xoo}$ (Supplemental Figure 4B). Furthermore, OsFLS2 in the transgenic *Arabidopsis* line OE-1 was significantly degraded within 90 min after FliC$_{Aa}$ treatment, but not by FliC$_{Xoo}$ (Supplemental Figure 4C). These results suggest that there is minimal or no recognition of purified *Xoo* and *Xoc* flagellins mediated by OsFLS2 in *Arabidopsis*, even when it is overexpressed.

To determine if residue polymorphisms in flg22 region are responsible for different elicitation abilities, flg22 sequences in different bacterial flagellins were aligned and compared (Figure 2C). Multiple residues are different between the eliciting flg22 and non-eliciting flg22^{Xo}. Based on previous reports (Sun et al., 2006), we speculated that the D-V polymorphism at the

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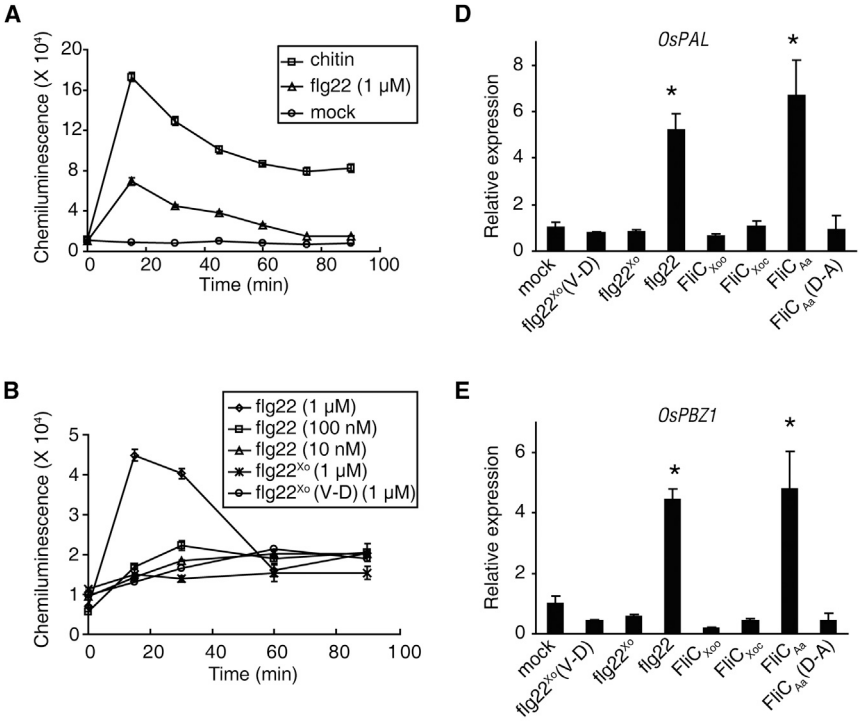

Figure 3. Different Defense Responses Were Triggered in Rice Suspension-Cultured Cells in Response to Various flg22 Peptides, Purified *X. oryzae*, and *A. avenae* Flagellins.

(A) An evident ROS burst was generated after flg22 and chitin treatments in rice cells.

(B) ROS generation in response to flg22 in rice cells is dose dependent. flg22^{Xo}(V-D) can hardly be recognized in rice cells.

(C) *In vitro* purified FliC$_{Aa}$, but not FliC$_{Xoo}$, induced a ROS burst in rice cells.

(D and E) Expression of *OsPAL* **(D)** and *OsPBZ1* **(E)** was significantly up-regulated in rice cells by the treatment of flg22 and FliC$_{Aa}$, but not by flg22^{Xo}, flg22^{Xo}(V-D), FliC$_{Xoo}$, and FliC$_{Xoc}$, revealed by quantitative RT–PCR analyses. Means ± SE are shown. Significantly up-regulated expression after flg22/flagellin treatments relative to mock controls is marked by asterisks ($P < 0.05$, Tukey's honest significance test).

(F) An MAPK was activated in rice cells after the treatment of flg22 and FliC$_{Aa}$ detected by immunoblotting analysis with an anti-pMAPK antibody. Arrowheads indicate phosphorylated MAPK bands. Bottom: Coomassie brilliant blue (CBB) staining to detect total protein loading.

position 14 might be partially responsible for loss of elicitation activity of *Xoo*/*Xoc* flagellins. SGI assays showed that both *Arabidopsis* wild-type and OE-1 transgenic plants were not responsive to flg22^{Xo}. By contrast, flg22^{Xo}(V-D) moderately inhibited the growth of Col-0 seedlings but did not alter the growth of the OE-1 transgenic seedlings (Figure 2D). The data suggest that AtFLS2 and OsFLS2 may have different recognition specificities for flg22 peptides.

Purified Bacterial Flagellins Exhibit Different Elicitation Abilities in Rice Cell Cultures

To investigate whether rice cells sense *Xoo* or *Xoc* flagellin, a highly sensitive approach was developed to detect reactive oxygen species (ROS) generation in rice cell cultures. As shown in Figure 3, ROS burst was clearly detected in rice cell cultures in response to flg22, although the intensity of induced ROS burst was lower than that upon chitin treatment (Figure 3A). In addition, the intensity of the flg22-induced ROS burst was dose dependent (Figure 3B). The method was subsequently used to detect ROS burst in response to treatments of different purified flagellins. In contrast to purified *Aa* flagellin, which triggered an evident ROS burst similar to that after flg22 treatment, *Xoo* flagellin caused no ROS generation in rice cell suspensions (Figure 3C). Consistently, the flg22^{Xo} and flg22^{Xo}(V-D) peptides hardly caused ROS burst in rice cell suspensions while flg22 elicited a rapid ROS generation (Figure 3B). Expression of defense marker genes including *OsPAL* and *OsPBZ1* was significantly induced in cultured rice cells by flg22 and FliC$_{Aa}$, but not by FliC$_{Xoo}$, FliC$_{Xoc}$, and the flg22^{Xo} and flg22^{Xo}(V-D) peptides (Figure 3D and 3E). Furthermore, a mitogen-activated protein kinase (MAPK) in rice was rapidly activated by flg22 and FliC$_{Aa}$ (Figure 3F). Together with the data from *OsFLS2* transgenic *Arabidopsis*, the results suggest that purified *Xoo* and *Xoc* flagellins are not recognized by rice OsFLS2.

The flg22 Region Is the Dominant Recognition Site of OsFLS2 in the Purified *A. avenae* Flagellin

In vitro purified *A. avenae* flagellin has a strong elicitation activity, in contrast to *X. oryzae* flagellins. To investigate whether other domains besides the flg22 region in *A. avenae* flagellin induce OsFLS2-mediated immunity, we constructed hybrid flagellins by swapping segments between the *fliC* genes of *Xoo* and *A. avenae* as shown in Figure 4A. The chimeric His6-tagged flagellins were purified and tested for elicitation of defense responses in *OsFLS2*-overexpressing *Arabidopsis* seedlings (Figure 4B). SGI assays showed that FliC$_{Aa/Xo}$80, FliC$_{Aa/Xo}$157, and FliC$_{Aa/Xo}$412 that contain the flg22 region of *Aa* flagellin strongly inhibited the growth of wild-type and *OsFLS2*-overexpressing *Arabidopsis* seedlings just as *Aa* flagellin did. By contrast, *Xoo* flagellin and the chimeric FliC$_{Xo/Aa}$58, FliC$_{Xo/Aa}$138, FliC$_{Xo/Aa}$280, and FliC$_{Xo/Aa}$356 that contain the flg22 region of *Xoo* flagellin had no obvious inhibition effect on these transgenic

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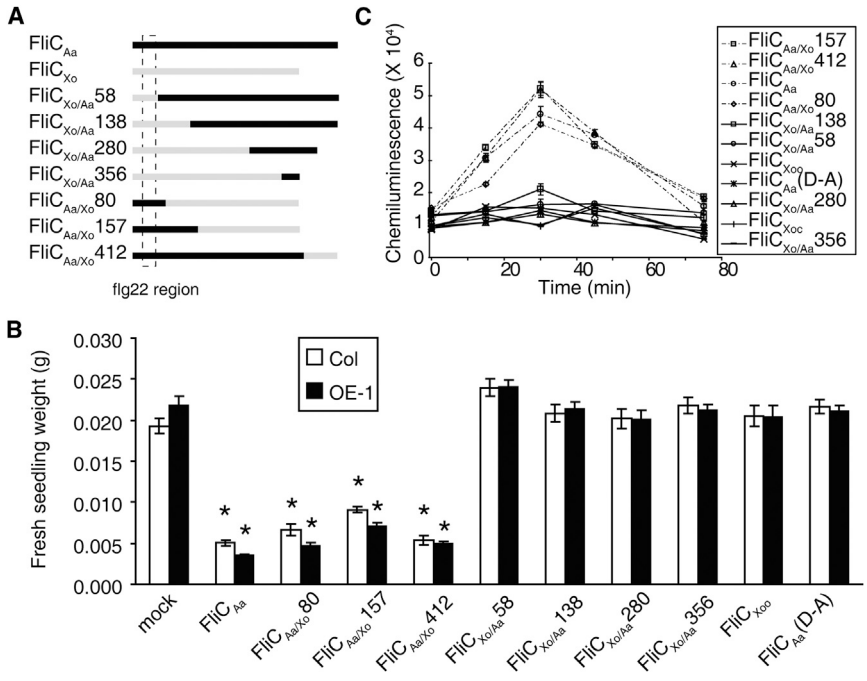

Figure 4. The flg22 Region in *A. avenae* Flagellin Is the Major Elicitation Determinant for OsFLS2 Recognition Revealed by Domain Swaps Between *X. oryzae* and *A. avenae* Flagellins and Site-Directed Mutagenesis.

(A) Schematic diagram of domain swaps between *X. oryzae* and *A. avenae* flagellins. Black areas represent *A. avenae* flagellin sequences and gray areas indicate *X. oryzae* flagellin sequences. The flg22 region is also shown.

(B) Elicitation of *Arabidopsis* defense responses by *in vitro* purified FliC$_{Xoo}$, FliC$_{Aa}$, and domain-swapped fusion flagellins, assayed as SGI. *Arabidopsis* Col-0 and transgenic OE-1 seedlings were treated with PBS (mock), 5 μM purified *X. oryzae* and *A. avenae* His-tagged flagellin, or the hybrid flagellins described in **(A)**. Means ± SE are shown. Asterisks indicate statistical significance of different treatments relative to mock controls ($P < 0.01$, Tukey's honest significance test).

(C) Elicitation of defense responses in rice suspension-cultured cells, assayed as the generation of ROS burst. Rice cells were treated with 5 μM purified *X. oryzae* and *A. avenae* His-tagged flagellin, or the hybrid flagellins described in **(A)**. Means ± SE are shown.

Arabidopsis seedlings. Accordingly, only the chimeric proteins carrying flg22-containing N terminus of *A. avenae* flagellin elicited a ROS burst in rice cultured cells just as *A. avenae* flagellin did. The chimeric proteins with the *Xoo* flg22 region did not induce a ROS burst in rice cells (Figure 4C). The results indicate that the first 80-amino-acid region is the only dominant determinant in *A. avenae* flagellin that is recognized by OsFLS2 and elicits the inhibition of seedling growth. To confirm the essential role of the flg22 region in elicitation activity, two presumed key residues for elicitation in the region, the Asp codons in the 43rd and 44th positions, were changed to Ala codons in the *A. avenae fliC* gene through site-directed mutagenesis. The FliC$_{Aa}$(D-A) variant largely lost the elicitation activity in the *Arabidopsis* SGI assay and did not induce ROS burst or expression of the defense marker genes *OsPBZ1* and *OsPAL*, or activate MAPKs in rice cells (Figures 3D–3F, 4B, and 4C). These results indicate that the flg22 region of *A. avenae* flagellin is the dominant elicitation determinant that can be recognized by OsFLS2.

OsFLS2 and AtFLS2 Have Different Flagellin Perception Specificities

To reveal how *X. oryzae* evades OsFLS2-mediated flagellin surveillance, the recognition specificities of OsFLS2 and AtFLS2 were compared using the wild-type and *OsFLS2* transgenic *Arabidopsis* plants. Sequence alignment showed that seven amino acid residues are different in the flg22 region between *Aa* and *Xoo* flagellins: R31Q, L36K, N39T, A41F, K42A, D43V, and L48G (Figure 2C). Flagellin variants with different point mutations were constructed and purified for determining their elicitation abilities by SGI assays (Figure 5A). The three FliC$_{Aa}$ mutants N39T, A41F, and D43V completely lost the ability to inhibit the growth of OsFLS2-overexpressing transgenic seedlings, and the inhibition ability of the L48G variant was also significantly attenuated. By contrast, only the D43V variant, but not the N39T, A41F, and L48G, lost the ability to inhibit the growth of wild-type *Arabidopsis* seedlings (Figure 5A). Meanwhile, the *Xoo* flagellin variant FliC$_{Xo}$-M3 with T39N, F41A, and V43D mutations partially restored the elicitation activity while FliC$_{Xo}$-M5 with five point mutations gained full ability to inhibit OE-1 seedling growth. Consistent with previous reports (Sun et al., 2006), SGI assays showed that single V43D mutation of *Xoo* flagellin can partially restore, and FliC$_{Xo}$-M1 to FliC$_{Xo}$-M5 can fully restore, AtFLS2-mediated elicitation activity on Col-0 seedlings (Figure 5A). MAPK activation was detected in rice leaves after flagellin treatments, which revealed the same defense-triggering activity of these flagellin variants as SGI assays did (Figure 5B and 5C; Supplemental Figure 5). Together, the results further confirmed that OsFLS2 exhibited flagellin recognition specificities or sensitivities different from those of AtFLS2.

Pre-Treatment with *A. avenae* Flagellin, but Not *X. oryzae* Flagellin, Induces Strong Resistance against *Xoo* PXO99^A

To verify that purified flagellins induce defense responses in rice plants, expression of defense marker genes was detected in rice leaves after flagellin treatment. In line with the results in cultured cells, *OsPBZ1* expression was greatly induced in rice leaves after treatment of *Aa* flagellin (Figure 6A). MAPK was also rapidly activated by flg22 and FliC$_{Aa}$, but not by FliC$_{Xoo}$ and FliC$_{Xoc}$ (Figure 6B). We also demonstrated that the protein level of OsFLS2 in rice leaves was significantly reduced within 90 min by the treatments of flg22 and FliC$_{Aa}$, but not by FliC$_{Xoo}$, although OsFLS2 gene expression was induced by eliciting flagellin (Supplemental Figure 6). To investigate whether defense responses induced by *A. avenae* flagellin contribute to rice resistance against pathogen infection, *X. oryzae* pv. *oryzae* PXO99^A was inoculated into rice leaves pre-treated with different bacterial flagellins. Disease symptom observations showed that the pre-treatment of FliC$_{Aa}$ and flg22 significantly reduced the length of disease lesions caused by PXO99^A infection. By

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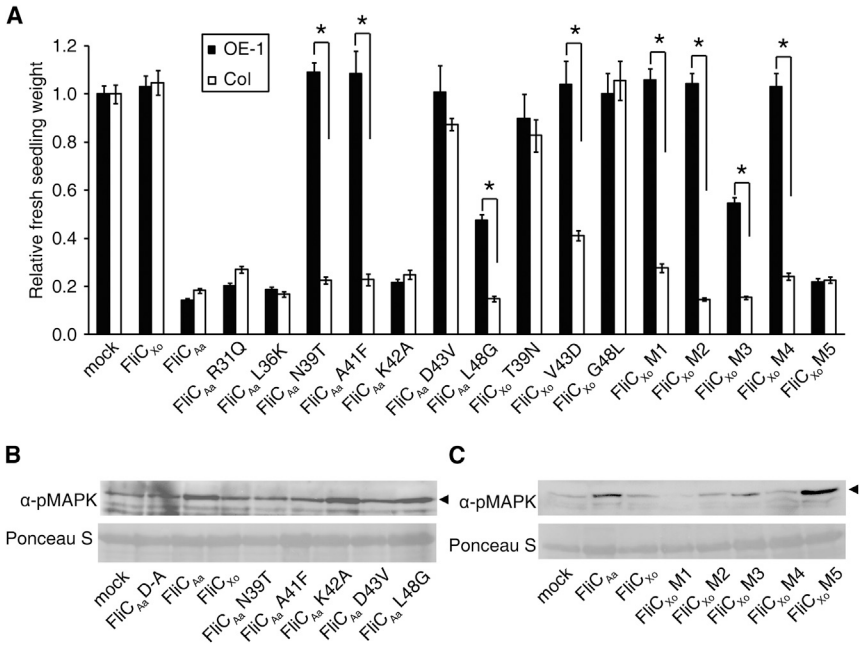

Figure 5. OsFLS2 and AtFLS2 Exhibited Different Perception Specificities for the flg22 Region of Flagellin.

(A) SGI assays for the wild-type and transgenic OE-1 lines upon the treatments of different *Aa* and *Xoo* flagellin variants. $FliC_{Aa}$R31Q indicates *in vitro* purified *A. avenae* flagellin with single point mutation (R to Q) at the 31st residue; $FliC_{Xo}$M1–M5 represent *Xoo* flagellins with different point mutations A42K/V43D, F41A/A42K/V43D, T39N/F41A/A42K/V43D, F41A/A42K/V43D/G48L, and T39N/F41A/A42K/V43D/G48L, respectively. Fresh seedling weights (mean ± SE) are for 12 seedlings per treatment, measured after 10 days of growth in 0.5× MS medium supplemented with PBS (mock) and 1 μM *in vitro* purified His6-tagged flagellin variant proteins. Asterisks indicate significant difference in seedling weights of Col-0 and the transgenic line OE-1 ($P < 0.05$, *t* test).

(B and C) MAPK activation in rice leaves after the treatment of *A. avenae* and *Xoo* flagellins with single and multiple point mutations was detected by immunoblot analysis using an anti-pMAPK antibody. Arrowheads indicate phosphorylated MAPK bands. Bottom: Ponceau S staining of the same blot to detect total protein loading.

contrast, PXO99^A caused severe disease symptoms on the rice leaves pre-treated with $FliC_{Xoo}$ and mock-treated (Figure 6C). These results indicate that OsFLS2-mediated defense responses triggered by flg22 and $FliC_{Aa}$ strongly enhance plant immunity against bacterial infection in rice.

Construction and Phenotype Identification of *Xoo*Δ*fliC* and *Xoc*Δ*fliC* Mutants

To further investigate if *Xoo* and *Xoc* flagellins are recognized *in vivo* and subsequently trigger host immunity, the $fliC_{Xoo}$ and $fliC_{Xoc}$ gene-deletion mutants were generated through homologous recombination. The Δ$fliC_{Xoo}$ and Δ$fliC_{Xoc}$ mutants were confirmed using two alternative approaches. First, swimming motility of the wild-type, Δ$fliC_{Xoo}$, and Δ$fliC_{Xoc}$ strains was tested on semi-solid agar plates. It was demonstrated that both Δ$fliC_{Xoo}$ and Δ$fliC_{Xoc}$ mutants lost swimming motility completely (Figure 7A and 7B). Second, crude flagellin extracts were isolated from the wild-type and mutant strains and subjected to Western blot analysis using a polyclonal antibody raised in a rabbit against purified *Xoc* flagellin. The Δ$fliC_{Xoo}$ and Δ$fliC_{Xoc}$ mutants produced no flagellin proteins (Figure 7C and 7D). Moreover, both complementation strains of Δ$fliC_{Xoo}$ and Δ$fliC_{Xoc}$ restored swimming motility and the ability to produce the flagellins (Figure 7A–7D). Notably, no significant difference in the growth rate was observed between the wild-type *Xoo* or *Xoc* strains and the respective *fliC* mutants in liquid medium (Supplemental Figure 7A and 7B).

Experimental data also showed that the Δ$fliC_{Xoo}$ mutant had a cellulase activity similar to that of the wild-type and complemented strain C-Δ$fliC_{Xoo}$, as indicated by the diameter of transparent circles formed by the degradation of carboxymethyl cellulose (Supplemental Figure 8A). The absence of a clearing zone formed on *Xoo*-culturing plates with skim milk indicates that *Xoo* cannot secrete proteases under this experimental condition (data not shown). By contrast, the Δ$fliC_{Xoc}$ mutant secreted an amount of proteases similar to that of the wild-type and complemented strain C-Δ$fliC_{Xoc}$, indicated by the similar size of clearing zones caused by proteolytic degradation of skim milk (Supplemental Figure 8B). In addition, no significant difference was found in the amount of extracellular polysaccharides (EPS) produced by the wild-type and Δ*fliC* mutant strains (Supplemental Figure 8C and 8D). These findings indicate that the loss of flagella in *Xoo* and *Xoc* imposes little effect on the production of extracellular cellulases, proteases, and polysaccharides.

Construction and Phenotype Identification of the *Xoc* Δ*rbfC* Mutants

The Δ*rbfC* mutant of *Xoc* was constructed to study RbfC function in flagellin glycosylation. Western blot analyses demonstrated that the apparent molecular weight of flagellin produced by the Δ$rbfC_{Xoc}$ mutant was very similar to that of the *in vitro* purified flagellin expressed in *E. coli*, but evidently lower than that of the wild-type strain (Figure 7E). Plasmid-born full-length *rbfC* gene restored the molecular weight of flagellin back to that of the wild-type (Figure 7E). These results indicate that RbfC plays an important role in the post-translational modification, most likely glycosylation, of *Xoc* flagellin. Therefore, it is of interest to determine whether altered modification caused by *rbfC* deletion affects the swimming motility of *Xoc*. Similar diameters of motility halos formed by the *Xoc* wild-type and Δ*rbfC* mutant strains indicate that the Δ$rbfC_{Xoc}$ mutant has the same swimming ability as the wild-type strain (Figure 7F). These results suggest that flagellin modification mediated by RbfC in *Xoc* has no effect on bacterial swimming. Furthermore, the Δ$rbfC_{Xoc}$ mutant, like the Δ$fliC_{Xoc}$ mutant, produced a similar amount of EPS and had a comparable level of extracellular protease activity relative to the wild-type strain (Supplemental Figure 8E and 8F).

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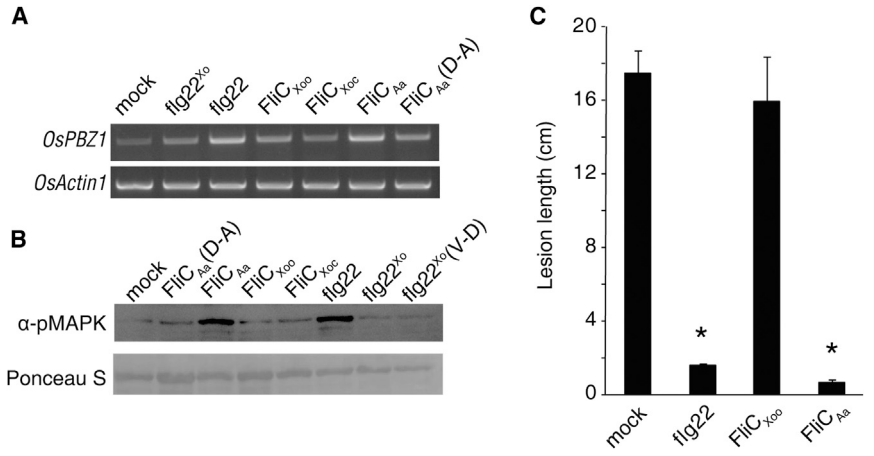

Figure 6. *A. avenae* Flagellin and flg22 Triggered Immune Responses in Rice Leaves and Strong Resistance against the Infection of *Xoo* PXO99^{A}.

(A) *OsPBZ1* expression was significantly up-regulated in rice leaves after the treatment of flg22 and FliC$_{Aa}$, but not by flg22^{Xo}, FliC$_{Xoo}$, and FliC$_{Xoc}$, revealed by RT–PCR analyses. *OsActin1* was used as an internal reference gene.

(B) An MAPK was activated in rice leaves after the treatment of flg22 and FliC$_{Aa}$, detected by immunoblot analysis using an anti-pMAPK antibody. Bottom: Ponceau S staining of the same blot to show total protein loading.

(C) The length of disease lesions caused by *Xoo* PXO99^{A} inoculation on *Oryzae sativa* cv. Jingang 30 leaves pre-treated with flg22, FliC$_{Xoo}$, and FliC$_{Aa}$. Lesion lengths were measured for at least 10 leaves per treatment at 14 days after inoculation. Means ± SE are shown. Asterisks indicate statistical significance relative to the mock control ($P < 0.05$, Tukey's honest significance test).

No Significant Alteration in Virulence of the *Xoo*Δ*fliC*, *Xoc*Δ*fliC*, and *Xoc*Δ*rbfC* Mutants to Rice

Bacterial flagellin plays double-sided roles in virulence. The flagellin was previously demonstrated as a virulence factor (Panopoulos and Schroth, 1974; Haefele and Lindow, 1987; Tans-Kersten et al., 2001), but it is also recognized as a conserved molecular signature that induces plant innate immunity (Felix et al., 1999; Boller and Felix, 2009). Here, the wild-type PXO99^{A}, Δ*fliC*$_{Xoo}$ mutant, and complementation strain C-Δ*fliC*$_{Xoo}$ were inoculated into the leaves of 6-week-old rice plants by the leaf-clipping method. Near equal length of disease lesions on inoculated leaves indicates that the mutant exhibits no difference in virulence to rice compared with the wild-type strain (Figure 8A). These results suggest that the *fliC*$_{Xoo}$ gene is not essential for *Xoo* virulence to rice in the tested stages. The wild-type *Xoc*, Δ*fliC*$_{Xoc}$, Δ*rbfC*$_{Xoc}$, and the corresponding complementation strains were also investigated for virulence via pressure inoculation. The equal lesion length of infected leaves indicates that all of the strains exhibit similar abilities to cause disease in this rice leaf assay (Figure 8B and 8C).

DISCUSSION

Flagellin-triggered immunity plays an important role in defense against bacterial infection in many plant species. In this study, we investigated and compared elicitation activities of flagellins derived from several important rice bacterial pathogens including *A. avenae* and *X. oryzae* pathovars. Experimental data indicate that rice plants possess a highly efficient OsFLS2-mediated flagellin detection system, which can be triggered by eliciting flagellins and thus defend against bacterial infection. However, the surveillance system has been circumvented by *X. oryzae* through mutating multiple key recognition residues in flagellin.

Perception of Flagellin in Rice Constitutes a Sensitive Surveillance System

Earlier studies showed that flg22 can hardly trigger a ROS burst and induces only weak immune responses in cultured rice cells (Takai et al., 2008). Here, a clearly visible ROS burst was observed in rice cell cultures in response to flg22 treatment when a highly sensitive ROS detection method was used in our assays (Figure 3A) (Schwacke and Hager, 1992; Perez and Rubio, 2006). The intensity of ROS burst in response to flg22 and *in vitro* purified *A. avenae* flagellin was similar, despite it being much weaker than the ROS burst induced by chitin treatment (Figure 3). The inconsistency might be caused by cell cultures with distinct genetic background. Different expression levels of OsFLS2 in *Arabidopsis* exhibit significantly different abilities to sense flg22, indicating that the perception of flagellin is dependent on OsFLS2 protein level (Supplemental Figure 3). Low protein accumulation in ectopic expression of FLS2 driven by the native promoter in *Arabidopsis* has been reported previously. For example, *LeFLS2* expression driven by the native promoter in transgenic *Arabidopsis fls2* plants did not complement flagellin perception due to no or low LeFLS2 accumulation (Robatzek et al., 2007). We showed that OsFLS2 sensed flg22 in transgenic *Arabidopsis* when it was highly expressed. ROS burst, defense gene expression, and MAPK activation assays demonstrated that rice cultured cells responded to flg22 and *in vitro* purified *A. avenae* flagellin efficiently (Figure 3). More importantly, pre-treatment of rice leaves with FliC$_{Aa}$ and flg22 triggered strong resistance to *Xoo* infection and inhibited bacterial multiplication (Figure 6). Microscale thermophoresis (MST) is a novel technology that can be used to detect protein–protein interaction (Broghammer et al., 2012; Lin et al., 2012). Here, a significant difference in thermophoresis was detected when FliC$_{Aa}$ was mixed with different concentration of OsFLS2-containing microsomal membranes by MST assays, suggesting the binding of OsFLS2 to FliC$_{Aa}$ (Supplemental Figure 4B). As expected, OsFLS2 in rice and in transgenic *Arabidopsis* plants was subjected to ligand-induced degradation shortly after ligand treatment (Supplemental Figures 3D, 4C, and 6A). The phenomenon has been reported for FLS2 in *Arabidopsis* (Smith et al., 2014). Collectively, these data indicate that rice possesses a sensitive flagellin surveillance system mediated by OsFLS2, which potentially plays an important role in rice defense against bacterial pathogens.

Xoo and *Xoc* Might Have Evolved to Escape the Flagellin Surveillance System in Rice

Bacterial pathogens have developed different molecular mechanisms to avoid and/or suppress the host immune system. It is

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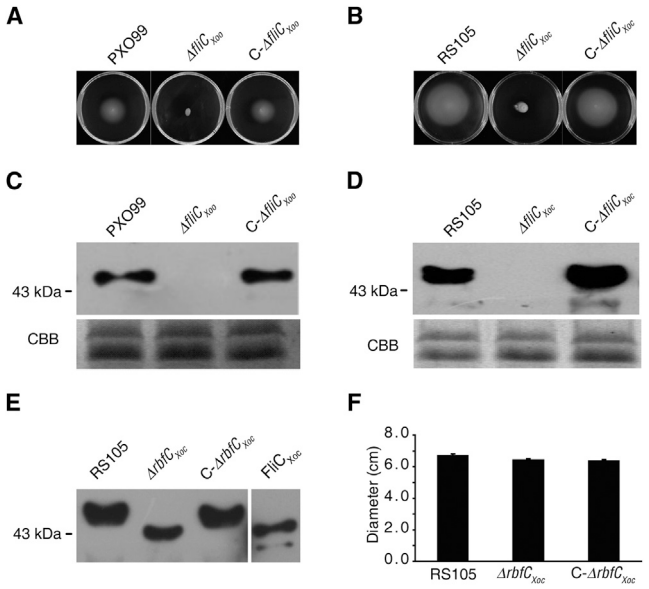

Figure 7. Flagellin Biosynthesis and Swimming Motility of the *ΔfliC*$_{Xoo}$, *ΔfliC*$_{Xoc}$, and *ΔrbfC*$_{Xoc}$ Mutants.

(A and B) The swimming motility of *ΔfliC*$_{Xoo}$ **(A)** and *ΔfliC*$_{Xoc}$ **(B)** mutants was completely lost, but was restored in the complementation strains C-*ΔfliC*$_{Xoo}$ **(A)** and C-*ΔfliC*$_{Xoc}$ **(B)** in semi-solid agar plates.

(C and D) Flagellin biosynthesis in the *ΔfliC*$_{Xoo}$ **(C)** and *ΔfliC*$_{Xoc}$ **(D)** mutants was disrupted, but was restored in the complementation strains C-*ΔfliC*$_{Xoo}$ **(C)** and C-*ΔfliC*$_{Xoc}$ **(D)** demonstrated by Western blot analyses with an anti-flagellin antibody. Bottom: Coomassie brilliant blue (CBB) staining to detect total protein loading.

(E) The deletion of *rbfC* altered the apparent molecular weight of *Xoc* flagellin, indicated by Western blot analysis with an anti-flagellin antibody. All lanes are from same blot.

(F) The motility of the *ΔrbfC*$_{Xoc}$ mutant was similar to the wild-type strain, indicated by almost equal diameters of swimming halos.

well known that type III effectors secreted by phytopathogenic bacteria target essential components in the plant immune signaling pathways and suppress plant immunity (Kay and Bonas, 2009; Feng and Zhou, 2012; Lindeberg et al., 2012; Lee et al., 2013; Xin and He, 2013). Phytopathogenic bacteria also passively evade plant immunity through losing or mutating type III effectors that have been recognized by host R proteins to trigger HRs (Jones and Dangl, 2006). Since most MAMPs are conserved and often essential for bacterial pathogenicity and fitness, pathogenic bacteria have evolved more diversified strategies to avoid host surveillance of these molecular signatures. Post-translational modifications of protein MAMPs, such as flagellin glycosylation, were demonstrated to be an effective approach to disguise (Hirai et al., 2011). Some *Pseudomonas* species secrete alkaline proteases to degrade monomeric flagellins and thus escape FLS2 recognition (Bardoel et al., 2012; Pel et al., 2014). In addition, site mutation(s) in key recognition sites of PRRs is a more common evasion method (Felix et al., 1999; Pfund et al., 2004; Sun et al., 2006).

In this study, our results indicate that the flagellin perception system in rice does not recognize *Xoo* and *Xoc* flagellins either *in vitro* or *in vivo*. First, both the wild-type and transgenic *Arabidopsis fls2* seedlings with different OsFLS2 levels did not respond to purified *Xoo* and *Xoc* flagellins in SGI assays, indicating that these flagellins cannot be recognized by AtFLS2 or OsFLS2 (Figures 1B and 2B). Notably, the purified *Aa* flagellin, similarly to flg22, caused strong SGI in *OsFLS2*-overexpressing transgenic *fls2* plants (Figures 2B and 4B). Second, *Xoo*, *Xoc*, and *A. avenae* flagellins purified from *E. coli* had significantly different elicitation abilities in rice cultured cells. *In vitro* purified *A. avenae* flagellin triggered an oxidative burst, and enhanced defense gene expression and MAPK activation in rice cultured cells while *Xoo* and *Xoc* flagellins did not (Figures 3 and 4). More convincingly, pre-treatment of rice leaves with purified *Xoo* and *Xoc* flagellins did not induce immunity while *A. avenae* flagellin triggered strong resistance to the infection of *Xoo* PXO99^{A} in rice (Figure 6). Moreover, both *Xoo* and *Xoc fliC* gene-deletion mutants caused disease lesions in rice leaves similar to those caused by the wild-type strains (Figure 8). Flagellin glycosylation in *Xoo* and *Xoc* might mask recognition epitopes so that rice cannot initiate self-defense in response to these flagellins. We investigated this possibility by making the *rbfC* gene-deletion mutant in *Xoc*. Western blot analysis demonstrated that post-translational modification of flagellin was altered when *rbfC* was deleted in *Xoc*. Bacterial inoculation assays showed that virulence of the *rbfC* mutant was not altered compared with the wild-type strain. In addition, *in vitro* purified underglycosylated *Xoo*/*Xoc* flagellins did not induce OsFLS2 degradation and defense responses. These data suggest that flagellin glycosylation might not be involved in flagellin recognition in *Xoo* and *Xoc*. Taken together, our findings indicate that *Xoo* and *Xoc* have evolved to skip the flagellin surveillance system in rice.

The flg22 Region in *A. avenae* Flagellin Is the Major Elicitation Determinant for OsFLS2 Recognition

Previous studies showed that the native flagellin purified from a rice-incompatible *A. avenae* strain N1141, and deglycosylated flagellin of the compatible K1 strain that shares identical flagellin protein sequences with the strain R2, induced similar levels of plant immunity that was much stronger than flg22-triggered defenses in Oc rice cells (Takai et al., 2008; Hirai et al., 2011). It is of interest to determine elicitation epitopes in *A. avenae* flagellin and to investigate whether other regions rather than flg22 induce OsFLS2-mediated immunity. Defense responses triggered by domain swaps between *A. avenae* and *Xoo* flagellins indicate that domains other than flg22 in the *A. avenae* flagellin hardly contribute to defense elicitation (Figure 4). The fusion flagellins containing the flg22 region of *A. avenae* flagellin triggered a ROS burst in rice cells and inhibited the growth of the wild-type and OsFLS2-overexpressing transgenic *Arabidopsis* seedlings. By contrast, all fusions containing the flg22 region of *Xoo* flagellin have no evident elicitation ability in ROS burst and SGI assays (Figure 4). More convincingly, site-directed mutagenesis in the flg22 region of *Aa* and *Xoo* flagellins showed that the regions determined elicitation activity in *A. avenae* flagellin and *X. campestris* pv. *campestris* flagellin (Figures 4 and 5) (Sun et al., 2006). Consistently, we demonstrated that flg22 elicited immune responses as strong as those produced by *in vitro* purified underglycosylated R2 flagellin in rice plants and cell cultures (Figures 3 and 5). These data indicate that the flg22 region in *A. avenae* flagellin is the major elicitation determinant for OsFLS2 recognition in rice. However, the possibility that

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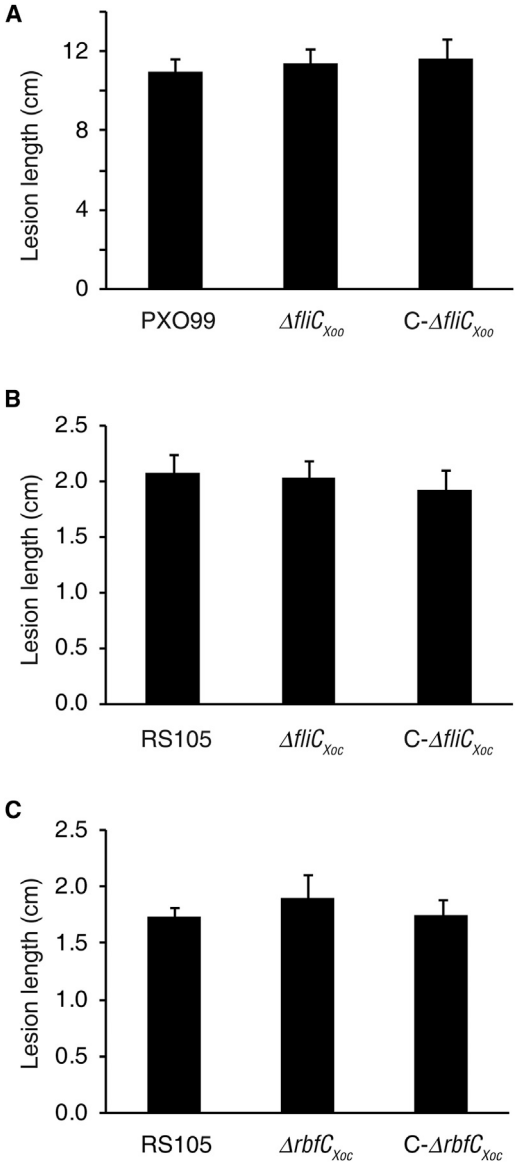

Figure 8. Virulence Assays on Rice of the *ΔfliC*$_{Xoo}$, *ΔfliC*$_{Xoc}$, and *ΔrbfC*$_{Xoc}$ Mutants.

(A) The length of disease lesions caused by *Xoo* PXO99^{A}, *ΔfliC*$_{Xoo}$ mutant, and the complementation strain C-*ΔfliC*$_{Xoo}$ was measured at 2 weeks after leaf-clipping inoculation.

(B) The length of disease lesions caused by *Xoc* RS105, *ΔfliC*$_{Xoc}$ mutant, and the complementation strain C-*ΔfliC*$_{Xoc}$ was measured at 2 weeks after pressure infiltration.

(C) The length of disease lesions caused by *Xoc* RS105, *ΔrbfC*$_{Xoc}$ mutant and the complementation strain C-*ΔrbfC*$_{Xoc}$ was measured at 2 weeks after pressure infiltration. No significant difference was detected in lesion length ($P < 0.05$, Tukey's honest significance test).

other flagellin domains or post-translational modifications, including flagellin glycosylation, function as elicitors of defense responses in rice or other plant species cannot be ruled out. Recently, the flgII-28 region in *Pst* flagellin specifically recognized by Solanaceae species and a new domain other than elf18 in EF-Tu recognized by rice were reported as new MAMPs (Cai et al., 2011; Clarke et al., 2013; Furukawa et al., 2013).

OsFLS2 May Have a Lower Functional Plasticity to Respond to Various Flagellins Than AtFLS2

Although both AtFLS2 and OsFLS2 can recognize the conserved flg22 region of eliciting flagellins, the two receptors have recognition specificity for certain amino acid residues in the flg22 region. SGI and MAPK activation assays indicate that both receptors can no longer recognize the eliciting flagellin when the key recognition site D43 is mutated (Figure 5). However, the perception specificity of the two receptors can be differentiated by other mutated flagellins. The wild-type *Arabidopsis* seedlings were still elicited by the N39T and A41F mutant *Aa* flagellins, while the *OsFLS2* transgenic *fls2* line OE-1 had no ability to recognize these mutated proteins (Figure 5). On the other hand, site-directed mutagenesis on non-eliciting flagellin revealed that the single V43D mutation can partially restore the eliciting ability of *Xoo* flagellin on Col-0 seedlings, while only FliC$_{Xo}$M3 and FliC$_{Xo}$M5 with multiple point mutations can be recognized by OsFLS2 (Figure 5 and Supplemental Figure 5). Referring to the crystal structure of FLS2-BAK1-flg22 (Sun et al., 2013), residue D43 in the flg22 region directly binds to two positively charged pockets of AtFLS2 and is important for immunogenic activities. The V43 residue of FliC$_{Xoo}$ carries a nonpolar side chain and fails to form electrostatic attraction to OsFLS2 or AtFLS2. By contrast, the N39 and A41 residues do not interact with AtFLS2 directly, but function as a kink to connect two AtFLS2-interacting fragments. Structure modeling predicted that the T39 and F41 residues might alter the spatial structure of flg22^{Xo} (Supplemental Figure 9), which causes *Xoo* flagellin not to be recognized by OsFLS2. These results indicate that OsFLS2 and AtFLS2 have different flagellin perception specificities, or perhaps sensitivities, and that OsFLS2 exhibits less functional plasticity to respond to various flagellin variants than does AtFLS2.

In summary, OsFLS2 was demonstrated to mediate a sensitive flagellin surveillance system in rice through defense assays in rice and in transgenic *Arabidopsis* with ectopic expression of OsFLS2, although OsFLS2 and AtFLS2 have different perception specificities for bacterial flagellins. OsFLS2-mediated immunity induced by flg22 and *A. avenae* flagellin conferred strong resistance against *Xoo* infection in rice plants. These results demonstrate biological relevance and significance of OsFLS2-mediated flagellin perception in rice resistance to some bacterial diseases, but at least some *X. oryzae* pathovars have evolved to elude flagellin detection. The findings open new avenues for dissection and manipulation of disease resistance pathways through flagellin perception in rice.

METHODS AND MATERIALS

All experiments were repeated at least three times with similar results if not specifically noted.

Plant Materials and Bacterial Strains

Arabidopsis thaliana ecotype Col-0 and *fls2-101* mutant (a T-DNA insertion line) plants were used in this study. *Arabidopsis* plants were grown in a growth room maintained at 22°C with a 10 h photoperiod. Rice plants (*Oryza sativa* cvs. Zhonghua 17, Jingang 30, and Nipponbare) were grown in greenhouse or in the growth chamber. Bacterial strains and plasmids used in this study are listed in Supplemental Table 2. The

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flagellin-derived peptides flg22 (QRLSTGSRINSAKDDAAGLQIA), flg22^{Xo} (QQLSSGKRITSFAVDAAGGAIA), and flg22^{Xo}(V-D) (QQLSSGKRITSFADDAAGGAIA) were synthesized at Science Peptide Biological Tech. Co. (Shanghai, China).

Development of Suspension-Cultured Rice Cells

Compact calli with vigorous growth initiated from rice cv. Zhonghua 17 were used to establish cell-suspension cultures following the described procedure with minor modifications (Ozawa and Komamine, 1989; Lu et al., 2015). Induced calli were inoculated into liquid N6 medium supplemented with 1 mg/l 2,4-D, 300 mg/l casein hydrolysate, and 10 mM proline. The suspension cultures were incubated at 26°C on a rotary shaker at 140 rpm. Sub-culturing was performed every 14 days in the first month and every week thereafter.

Expression and Purification of His-Tagged Flagellin Proteins

The *fliC* loci of *A. avenae* and *Xoo*/*Xoc* were amplified using the PCR primer sets, FliC$_{Aa}$-F/FliC$_{Aa}$-R and FliC$_{Xoo/Xoc}$-F/FliC$_{Xoo/Xoc}$-R (Supplemental Table 2). PCR products containing *fliC* coding sequences were subcloned into the expression vector pQE30 (Qiagen, Hilden, Germany). The *fliC* nucleotide sequences were determined by sequencing at least three independent clones. Derived amino acid sequences were then aligned using DNAman (Lynnon Biosoft, Quebec, Canada). His-tagged flagellin proteins were expressed in *E. coli* XL1-Blue cells and purified as described by Sun et al. (2006). The concentration of proteins was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). The purity of flagellin proteins was verified by SDS–PAGE gels stained with Coomassie brilliant blue G 250.

Site-Directed Mutagenesis and Domain Swapping between *Xoo* PXO99^{A} and *A. avenae* R2 Flagellin Proteins

A series of domain swaps between the *fliC* alleles of *Xoo* PXO99 and *A. avenae* R2 were generated by overlap extension PCR as described previously (Sun et al., 2006). In brief, different 5′ and/or 3′ fragments of the PXO99 and R2 *fliC* genes were amplified using the respective primer sets (e.g., for the FliC$_{Aa/Xo}$80 construct, the primer pairs FliC$_{Aa}$-F/FliC$_{Aa/Xo}$80-R and FliC$_{Aa/Xo}$80-F/FliC$_{Xoo/Xoc}$-R were used for 5′ and 3′ fragment amplification, respectively) (Supplemental Table 2). The gel-purified PCR products were added together into a fusion PCR. The resulting PCR products were gel-purified and subcloned into pQE30 for protein expression after digestion by BamHI and HindIII. Site-directed mutagenesis was generated by circular PCR with DpnI digestion to eliminate background wild-type plasmid pQE30-*fliC* as described previously (Sun et al., 2006). The resultant products were then transformed into XL1-Blue supercompetent cells for protein expression after mutations were confirmed by sequencing.

Construction of OsFLS2 Binary Vectors and Plant Transformation

The open reading frames of *OsFLS2* with or without the stop codon were amplified from genomic DNA isolated from *Oryza sativa* cv. Nipponbare with *Pfu* TURBO DNA polymerase using the primer sets OsFLS2-GW-F/OsFLS2-GW-R and OsFLS2-GW-F/OsFLS2-GW-R-tag, respectively (Supplemental Table 2). PCR products were gel-purified and subcloned into pENTR/D TOPO vector (Invitrogen, Carlsbad, CA, USA). The pENTR/D TOPO-*FLS2* plasmids were then recombined into pGWB14 (Nakagawa et al., 2007) using LR clonase II enzyme mix (Invitrogen, Carlsbad, CA). The full-length *OsFLS2* gene was amplified via PCR with native-OsFLS2-F/native-OsFLS2-R and subcloned into pCAMBIA1200. After confirmation by sequencing, these constructed plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90) by the freeze–thaw method and then transferred into *Arabidopsis fls2-101* plants by floral dip transformation. To select transformed plants with hygromycin resistance, T1 seeds were surface-sterilized and plated on 0.5× Murashige and Skoog (MS) plates with 200 mg/l cefotaxime and 25 mg/l hygromycin. Healthy etiolated seedlings were picked from selection plates that were kept in the dark at room temperature for 4–5 days after cold treatment and further grown under 12:12 h light/dark regimen for flg22 treatment in SGI assays, or grown out for other studies.

SGI Assays

SGI assays were performed as described with some minor modifications (Sun et al., 2012). In brief, *Arabidopsis* seedlings were transferred to a 24-well plate (one seedling per well), with each well carrying 500 μl of 0.5× MS salts and 5 μM flg22 peptide or purified His-tagged flagellin proteins. After 10–14 days of further growth, 10–12 seedlings for each treatment were blotted dry and weighed.

Oxidative Burst Assays

ROS assays for rice cultured cells were performed following the optimized method based on a Co(II)-catalyzed oxidation of luminol (5-amino-2,3 dihydro-1,4-phthalazinedione) instead of the ferricyanide-catalyzed oxidation (Perez and Rubio, 2006). Rice cell cultures were prepared as described by Schwacke and Hager (1992). In brief, cultured cells were washed three times and then pre-incubated with 3 ml of fresh media in 20 ml vials for 3 h at 26°C. The cells were further incubated for variable periods after the addition of elicitors. Following incubation, the supernatant (10 μl) was mixed with 1 ml of a Co(II)-luminol reagent and chemiluminescence was measured immediately by an Infinite F200 reader (Tecan, Mannedorf, Switzerland) with the count time set as 1 s.

Protein Binding Assays by MST

The microsomal membranes of the *OsFLS2* transgenic line OE-1 and empty vector transgenic line EV-C-4 were prepared by two-step centrifugation after homogenization (Santoni, 2007). The pellets were resuspended in resuspension buffer (5 mM KH_2PO_4, 330 mM sucrose, 10 mM NaF, pH 7.8). The concentration of microsomal membranes was determined by measuring optical density at 600 nm (OD_{600}). The interaction of OsFLS2 and FliC$_{Aa}$ or FliC$_{Xoo}$ was detected by MST using a NanoTemper (Munich, Germany) Monolith™ NT.115 instrument. *In vitro* purified FliC$_{Aa}$ and FliC$_{Xoo}$ were fluorescently labeled according to the manufacturer's protocol. The microsomal membranes extracted from the OE-1 and EV-C-4 lines were mixed with a series of different ratios and then added with 200 nM of fluorescently labeled FliC$_{Aa}$ and FliC$_{Xoo}$. The samples were loaded into standard-treated silica capillaries (NanoTemper) after incubation at room temperature for 10 min. Fluorescence was measured on 20% LED power and 20% IR-laser power.

Semi-Quantitative and Quantitative RT–PCR

Six-day-old *Arabidopsis* seedlings were treated with 5 μM flg22 or His-tagged *Xoo*/*Xoc* flagellins in 0.5× MS medium for 24 h and rice cultured cells were treated with 0.5 μM peptides or proteins for 6 h. After treatment, the samples were collected for RNA isolation using Trizol reagent according to the manufacturer's protocol (Invitrogen). Complementary DNA (cDNA) was synthesized by reverse transcriptase Superscript III (Invitrogen) using total RNA as template. The resultant cDNA was used as template for PCR performed with *Easy Taq* DNA polymerase (TransGen, Beijing, China). Quantitative RT–PCR was performed using an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). The gene expression levels were calculated based on three technical repeats and were normalized against the *OsActin1* (Os03g0718100) reference gene. The primers used to track the expression of *AtActin, AtWRKY40/53, AtCuBP*, *OsFLS2*, *OsPBZ1* (Os12g0555500), and *OsPAL* (Os02g0626100) genes are listed in Supplemental Table 2.

Construction of the Mutant and Complementation Strains

The *ΔfliCxoo* mutant of *Xoo* PXO99 was generated by homologous recombination with the suicide plasmid pUFR80 as described previously (Castañeda et al., 2005; Sun et al., 2011). In brief, upstream and downstream fragments of the *fliC$_{Xoo}$* coding sequence were amplified separately from *Xoo* genomic DNA using the primer sets FliC$_{Xoo}$-Bam HI-F/FliC$_{Xoo}$-del-R and FliC$_{Xoo}$-del-F/FliC$_{Xoo}$-HindIII-R (Supplemental

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Table 2). PCR products were gel-purified and added together into a fusion PCR. The resultant PCR fragment was cloned into pUFR80. The construct pUFR80-Δ*fliC*$_{Xoo}$ was conjugated into the PXO99 strain by tri-parental mating. The gene-deletion mutants were screened through colony PCR and then confirmed by sequencing and Southern blot analyses (Cannon et al., 1979). The *ΔfliC*$_{Xoc}$ and *ΔrbfC*$_{Xoc}$ mutants of *Xoc* RS105 were constructed using the same strategy and primer sets listed in Supplemental Table 2.

For complementation, an approximately 3 kb DNA fragment containing the full-length *fliC*$_{Xoo}$ gene was amplified using the primer set FliC$_{Xoo}$-BamHI-F/FliC$_{Xoo}$-HindIII-R (Supplemental Table 2). The PCR product was cloned into pMD18-T vector (Takara, Dalian, China) and then subject to sequencing. The sequence-verified *fliC* gene fragment was subcloned into the wide host-range vector pVSP61 (Loper and Lindow, 1987) and then transferred into the *ΔfliC*$_{Xoo}$ mutant via tri-parental mating. Complementation strains of the *ΔfliC*$_{Xoc}$ and *ΔrbfC*$_{Xoc}$ mutants were also generated using the respective primer sets (Supplemental Table 2).

Motility Assays, Secretion Assays of Extracellular Proteases and Cellulase, and Quantitative Determination of EPS

Swimming ability, the secretion of extracellular proteases, and EPS production were evaluated as described by Zhang et al. (2013). The secretion of cellulase was detected on agar plates with 0.5% carboxymethyl cellulose (CMC). Two microliters of bacteria (OD_{600} = 0.5) were spotted onto CMC agar plates. The plates were incubated at 28°C for 48 h and then flooded with 0.1% Congo red for 30 min. The stained plates were de-stained with 1 M NaCl for 20 min twice. The activity of cellulase of *Xoo* strains was quantified by measuring the diameter of transparent zones that were formed after cellulase degradation of CMC.

Bacterial Growth Assays in *Arabidopsis* after flg22 Protection

The leaves of 5- to 6-week-old *Arabidopsis* plants were syringe-infiltrated with 1 μM flg22 or water as mock controls. *Pst* DC3000 (OD_{600} = 0.0002) was pressure infiltrated into the pre-treated leaves at 24 h after pre-treatment. Bacterial population sizes were determined at indicated time points as described. Each data point consisted of at least four replicates.

Antibody Generation and Western Blot Analysis

The polyclonal antibodies were generated in rabbits by immunization with keyhole limpet hemocyanin-conjugated OsFLS2 C-terminal peptide (CLKMSKLVGED) and *in vitro* purified *Xoc* flagellin, respectively (Takai et al., 2008). The anti-OsFLS2 antibody was affinity purified using the antigen peptide-conjugated resin at GenScript Corp. (Nanjing, China). Crude proteins were extracted from *Xoo* and *Xoc* cultures following the procedures as described by Felix et al. (1999). In brief, protein extracts were prepared by boiling the overnight cultured cells for 20 min and removing cell debris by centrifugation at 12 000 *g* for 10 min. For OsFLS2 detection, transgenic *Arabidopsis* seedlings (10 days old) or sliced rice leaves were treated with 5 μM flg22, FliC$_{Xoo}$, or FliC$_{Aa}$ for indicated times. The samples were then collected and ground into powder with an oscillating mixer mill MM400 (Retsch, Haan, Germany) before addition of 200 μl extraction buffer (0.35 M Tris–HCl pH 6.8, 30% glycerol, 10% SDS, 0.6 M dithiothreitol, 0.012% bromophenol blue). Protein gel blotting was performed using standard procedures. Anti-OsFLS2 antibody was used at a 1:400 dilution. For detection of MAPK activation, rice cells were treated with 0.5 μM peptides or flagellin proteins for 15 min. The extraction of crude proteins and detection of MAPK phosphorylation were performed as described previously (Bartels et al., 2013). The phosphorylated MAPKs were detected by Western blot with an antibody against phospho-p44/42 MAP kinase (Cell Signaling Technologies, Danvers, MA, USA).

Virulence Assays for *X. oryzae* on Rice

Virulence to rice of *Xoo* and *Xoc* strains was investigated on rice cultivars Nipponbare and Jingang 30. *Xoo* and *Xoc* cultures were resuspended with 10 mM $MgCl_2$ to an OD_{600} of 0.8 (~8 × 10^8 cfu/ml) and 0.3 (~3 × 10^8 cfu/ml) for inoculation, respectively. Rice leaves were inoculated with *Xoo* and *Xoc* strains using leaf-clipping (Kauffman et al., 1973) and needleless syringe pressure inoculation (Schaad et al., 1996), respectively. The inoculated plants were kept at 100% humidity for 24 h following inoculation and then maintained under growth conditions described above. The lesion length was measured at 14 days after inoculation. At least 10 leaves were scored for each treatment.

Statistical Analysis

Statistical analyses were performed by Tukey's honest significance test or by *t* test for pairwise comparisons using SPSS software.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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