

The rice immune receptor XA21 recognizes a tyrosine-sulfated protein from a Gram-negative bacterium

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Surveillance of the extracellular environment by immune receptors is of central importance to eukaryotic survival. The rice receptor kinase XA21, which confers robust resistance to most strains of the Gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is representative of a large class of cell surface immune receptors in plants and animals. We report the identification of a previously undescribed *Xoo* protein, called RaxX, which is required for activation of XA21-mediated immunity. *Xoo* strains that lack RaxX, or carry mutations in the single RaxX tyrosine residue (Y41), are able to evade XA21-mediated immunity. Y41 of RaxX is sulfated by the prokaryotic tyrosine sulfotransferase RaxST, but not nonsulfated, RaxX triggers hallmarks of the plant immune response in an XA21-dependent manner. A sulfated, 21-amino acid synthetic RaxX peptide (RaxX21-sY) is sufficient for this activity. *Xoo* field isolates that overcome XA21-mediated immunity encode an alternate *raxX* allele, suggesting that co-evolutionary interactions between host and pathogen contribute to RaxX diversification. RaxX is highly conserved in many plant pathogenic *Xanthomonas* species. The new insights gained from the discovery and characterization of the sulfated protein, RaxX, can be applied to the development of resistant crop varieties and therapeutic reagents that have the potential to block microbial infection of both plants and animals.

INTRODUCTION

Plasma membrane-localized receptors are critical components of the innate immune responses of animals and plants (1–4). Many of these receptors recognize and respond to the presence of conserved microbial molecules and are often referred to as pattern recognition receptors (PRRs). In animals, this recognition is carried out, in part, by Toll-like receptors (TLRs) (3). Humans have 10 characterized TLRs that recognize conserved microbial molecules such as lipopolysaccharide or flagellin (5, 6).

In plants, cell surface receptor kinases (RKs) and receptor-like proteins (RLPs) recognize microbial molecules in the apoplast (7–13). Well-characterized leucine-rich repeat (LRR)-RKs include *Arabidopsis* FLS2 that detects flagellin, or its peptide epitope flg22, and the elongation factor Tu receptor (EFR) that detects the bacterial elongation factor Tu, or its peptide epitope elf18 (10, 14). Lacking an adaptive immune system, plants have an extended array of innate immune receptors encoded in their genome. Rice, for example, has more than 300 RKs predicted to serve as innate immune receptors based on the

presence of a “non-RD” kinase domain, which lack the arginine-aspartate motif characteristic of most kinases (15, 16). Of the few non-RD RKs characterized to date, all have a role in innate immunity or symbiosis (4, 17).

The rice XA21 RK, one of the first innate immune receptors isolated, mediates recognition of the Gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causal agent of an agronomically important disease of rice (7, 18). Previous efforts to identify the microbial molecule that activates the XA21-mediated immune response led to the identification of a number of *Xoo* genes that are required for activation of XA21 (*rax* genes). These genes encode a tyrosine sulfotransferase, RaxST, and three components of a predicted type 1 secretion system (T1SS): a membrane fusion protein, RaxA; an adenosine triphosphate (ATP)-binding cassette (ABC) transporter, RaxB; and an outer membrane protein, RaxC. *raxST*, *raxA*, and *raxB* are located in a single operon (*raxSTAB*) (19). On the basis of these findings, we hypothesized that the activator of XA21-mediated immunity is a tyrosine-sulfated, type 1-secreted protein (19). However, the identity of this molecule has remained elusive (20–22). Here, we report the identification of the tyrosine-sulfated protein RaxX as the activator of XA21-mediated immunity.

RESULTS

RaxX is required for activation of XA21-mediated immunity

RaxB belongs to a subgroup of T1SS ABC transporters that contain a C39 peptidase domain (19). In other bacterial species, substrates for this class of secretion systems are often encoded in or near the T1SS operon (23). We therefore hypothesized that the RaxST/A/B substrate may be genetically linked to the *raxSTAB* operon. To test this hypothesis, we generated mutants in the *Xoo* strain PXO99 (24) with

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either a 1-kb deletion ~0.4 kb upstream from *raxST* (PXO99 Δ 1.0^{Sp}) or a 1.7-kb deletion immediately after *raxB* (PXO99 Δ 1.7^{Sp}) (Fig. 1A). The mutants were used to inoculate Taipei 309 (TP309), a rice variety lacking XA21, and XA21-TP309, a transgenic derivative expressing XA21 under control of its native promoter (7). On TP309, all strains formed long, water-soaked lesions typical of the disease (Fig. 1B). PXO99 Δ 1.7^{Sp} formed short lesions on XA21-TP309 similar to the control strain PXO99. In contrast, PXO99 Δ 1.0^{Sp} formed long lesions on XA21-TP309. These results suggested that the region upstream to the *raxSTAB* operon contains an element that is required for activation of XA21-mediated immunity.

Sequence analysis of this 1-kb region revealed the presence of a 183-nucleotide open reading frame (ORF) (covering the PXO99 genomic region 1,282,744–1,282,926, NC_010717). The ORF, which we named *raxX* (Fig. 1A), was not annotated in the original PXO99 genome (24), but has been recently noted as PXO_RS05990. We found that *raxX* is conserved among all sequenced *Xanthomonas* strains that encode the *raxSTAB* operon (fig. S1), but is not present in *Xanthomonas* species that lack the *raxSTAB* operon. RaxX shares no homology with other microbial proteins of known function.

To further investigate the role of RaxX, we transformed PXO99 Δ 1.0^{Sp} with a broad-range host vector expressing *raxX* under control of its native promoter (*praxX*). PXO99 Δ 1.0^{Sp}(*praxX*) regained the ability to activate XA21-mediated immunity and formed short lesions on XA21-TP309 (Fig. 1B). To confirm the importance of *raxX* in activation of XA21-mediated immunity, we generated a marker-free PXO99 mutant with the *raxX* ORF deleted (PXO99 Δ *raxX*). As observed for PXO99 Δ 1.0^{Sp}, PXO99 Δ *raxX* evades the XA21-mediated immune response, forming long lesions on XA21-TP309 (Fig. 1, B and C). PXO99 Δ *raxX*(*praxX*) also regained the ability to activate XA21-mediated immunity (Fig. 1, B and C). Similar results were obtained when these strains were inoculated on rice plants overexpressing XA21 in the Kitaake genetic background (Ubi::XA21) (fig. S2) (25). A variant of *praxX* carrying a mutation in the predicted start codon (*praxX*-atg>tga) failed to complement PXO99 Δ *raxX* (Fig. 1B). PXO99 Δ *raxX* bacterial populations accumulated to more than 30-fold higher levels in inoculated XA21-TP309 rice leaves than those of strains PXO99 and PXO99 Δ *raxX*(*praxX*) (Fig. 1D). These results indicate that RaxX is required for activation of XA21-mediated immunity.

The central tyrosine of RaxX is sulfated by RaxST

In addition to *raxST*, we have previously identified two other *rax* genes involved in microbial sulfation. These genes, *raxP* and *raxQ*, encode an ATP sulfurylase and an adenosine 5'-phosphosulfate kinase, and work in concert to produce the universal sulfuryl group donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (26). The requirement of these three genes for activation of XA21-mediated immunity by *Xoo* suggests that tyrosine sulfation plays a key functional role in this process.

To further investigate this possibility, we transformed a *raxST* mutant strain (PXO99 Δ *raxST*^{Sp}), which forms long lesions on XA21-TP309, with a plasmid expressing *raxST* under control of its native promoter (*praxST*). PXO99 Δ *raxST*^{Sp}(*praxST*) regained the ability to activate XA21-mediated immunity (Fig. 2A). RaxST carries a predicted PAPS binding motif conserved in mammalian sulfotransferases including the human tyrosine sulfotransferases TPST1 and TPST2 (fig. S3) (19). In TPST2, mutation of the conserved arginine in the PAPS binding motif impairs enzymatic activity (27). We generated a similar mutation in *raxST* and tested if the expression of this mutant variant on a plasmid (*praxST*-R35A) could complement the PXO99 Δ *raxST*^{Sp}

infection phenotype on XA21-expressing rice plants. The strain PXO99 Δ *raxST*^{Sp} (*praxST*-R35A) failed to activate XA21-mediated immunity (Fig. 2A), indicating that the sulfotransferase activity of RaxST is critical for its function.

On the basis of the genetic association of *raxX* with the *raxSTAB* operon, the importance of tyrosine sulfation for activation of the XA21-mediated immune response, and the presence of a single tyrosine residue in PXO99 RaxX (Y41) that is conserved among all available RaxX sequences (fig. S1), we hypothesized that RaxX Y41 is sulfated by RaxST and that this sulfation is required for RaxX function. To test this hypothesis, we transformed PXO99 Δ *raxX* with a plasmid carrying a derivative of RaxX with tyrosine 41 mutated to phenylalanine [PXO99 Δ *raxX*(*praxX*-Y41F)]. PXO99 Δ *raxX*(*praxX*-Y41F) failed to activate XA21-mediated immunity in XA21-TP309 (Fig. 2A and fig. S4). We also demonstrated that sulfated RaxX peptides, but not peptides carrying an Y41 to F substitution, are immunogenic on XA21-expressing rice plants (fig. S5 and next section). These results support the hypothesis that sulfation of RaxX Y41 is required for its activation of XA21-mediated immunity.

To determine whether RaxX Y41 is sulfated by RaxST in vitro, we incubated a chemically synthesized peptide covering the C-terminal region of RaxX (RaxX39, amino acids 22 to 60) with purified His-RaxST (fig. S6) in the presence of PAPS. Trypsin-digested peptides were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) in both negative and positive nanoelectrospray modes with ultraviolet photodissociation (UVPD) to generate informative a, b, c, x, y, and z product ions from cleavage of the peptide backbone. This method has previously been shown to facilitate the characterization of sulfated tyrosine residues within peptides by MS/MS (28, 29). The lability of the sulfation modification has proven problematic for other MS/MS methods. In negative ion mode, the sulfate group is retained on all product ions, thus allowing the sulfate modification to be unequivocally localized to Y41 of RaxX. MS/MS data showed fragment ions that account for 93% sequence coverage of peptide HVGGGDsYPPPGANPK (Fig. 2B). The high-resolution verification of the peptide mass in the negative mode MS1 (HVGGGDsYPPPGANPK) is displayed in fig. S7. The extracted ion chromatograms of the peptides of interest and positive mode UVPD mass spectrum are shown in figs. S8 and S9, respectively.

We next tested if RaxX is sulfated in vivo. Using selected reaction monitoring-MS (SRM-MS), we observed sulfation on tryptic peptides covering Y41 derived from RaxX-His purified from PXO99(*praxX*-His) (Fig. 2C and figs. S10 and S11). The sulfated version of the tryptic peptide covering Y41 of RaxX-His purified from PXO99 Δ *raxST*(*praxX*-His) was not detectable with multiple SRM transitions above the background noise level (~50 to 100 cycles per second) (Fig. 2C and fig. S11). In contrast, we did detect the corresponding nonsulfated peptide covering Y41 at high levels and with high confidence for RaxX-His isolated from both PXO99 (*praxX*-His) and PXO99 Δ *raxST*(*praxX*-His) (Fig. 2D and fig. S11). In combination, these results demonstrate that RaxST sulfates RaxX on Y41 in vivo and that sulfation of RaxX is required for its immunogenic activity on XA21-expressing rice plants.

RaxX is sufficient for activation of XA21-mediated immunity

Infection assays using bacterial mutants clearly demonstrate that RaxX is required for activation of XA21-mediated immunity. We next sought to determine whether sulfated RaxX can trigger XA21-mediated defense responses in the absence of *Xoo*. For this purpose, we produced full-length sulfated recombinant RaxX (RaxX60, 1 to 60 amino acids)

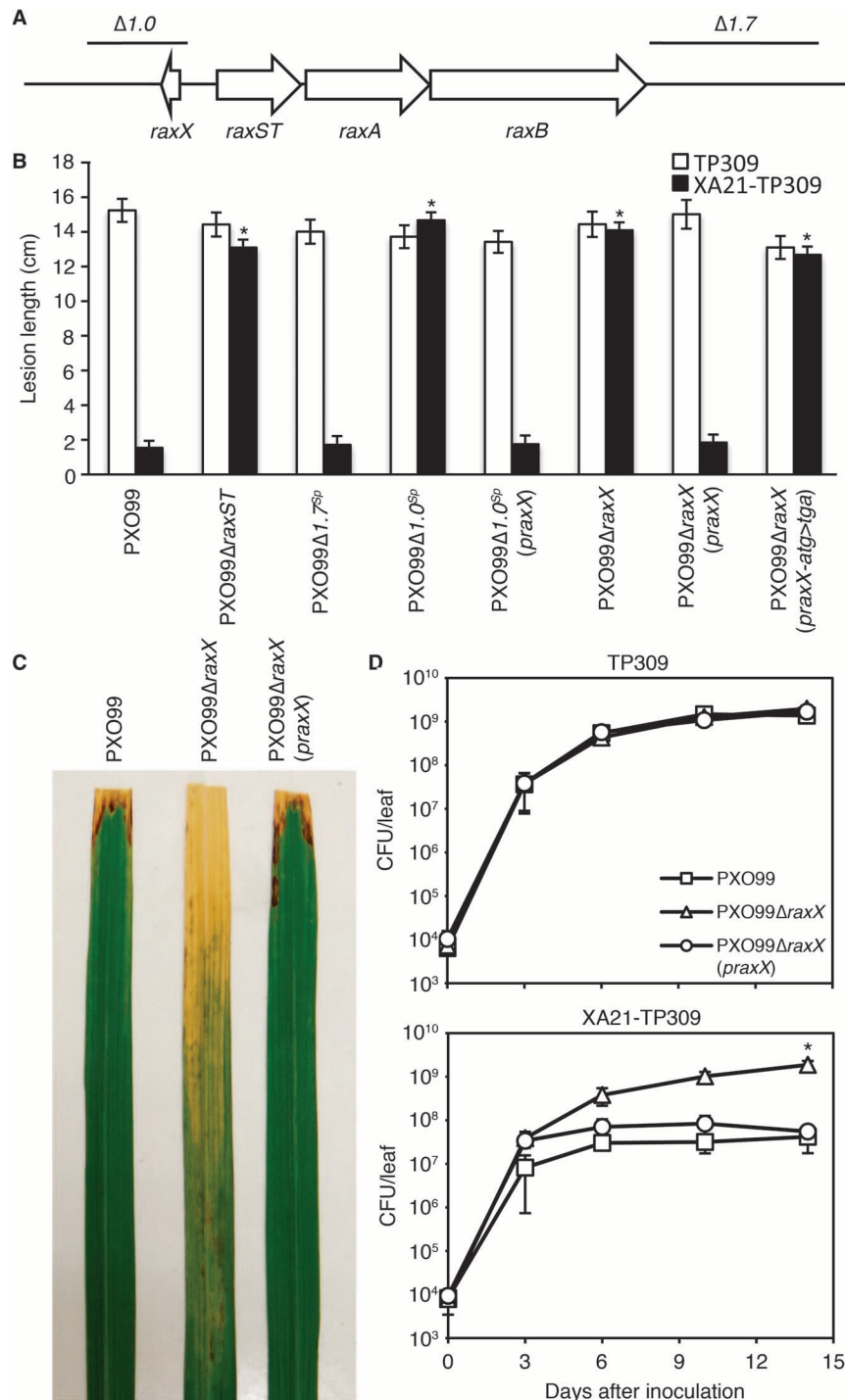


Fig. 1. *raxX*, a small ORF located upstream of the *raxSTAB* operon, is required for activation of XA21-mediated immunity. (A) *raxST*, *raxA*, and *raxB* are encoded in a single operon. A 1.0-kb region upstream of *raxST* and a 1.7-kb region downstream of *raxB* were deleted in PXO99 Δ 1.0^{sp} and PXO99 Δ 1.7^{sp}, respectively. The *raxX* ORF is located ~0.4 kb upstream of *raxST* and in the opposite orientation. **(B)** TP309 (open bars) or XA21-TP309 (black bars) were inoculated by clipping with scissors dipped in *Xoo* suspensions. Bars indicate the mean lesion length \pm SE measured 14 days after inoculation ($n \geq 14$). The "*" indicates statistically significant difference from PXO99 using Dunnett's test ($\alpha = 0.01$). No statistical differences in lesion length were observed on TP309 inoculated with the different strains. The experiment was repeated at least three times with similar results. **(C)** XA21-TP309 rice leaves display water-soaked lesions 2 weeks after inoculation with the indicated strains. **(D)** Growth of PXO99 (□), PXO99 Δ *raxX* (△), and PXO99 Δ *raxX*(*praxX*) (○) in rice leaves inoculated as in (B). In planta bacterial growth analysis was carried out as described (22). Bacterial quantification was determined as the number of colony-forming units (CFU) extracted per inoculated leaf. For the final data point, "*" indicates statistically significant difference from PXO99 using Dunnett's test ($\alpha = 0.01$, $n = 4$). The experiment was repeated twice with similar results.

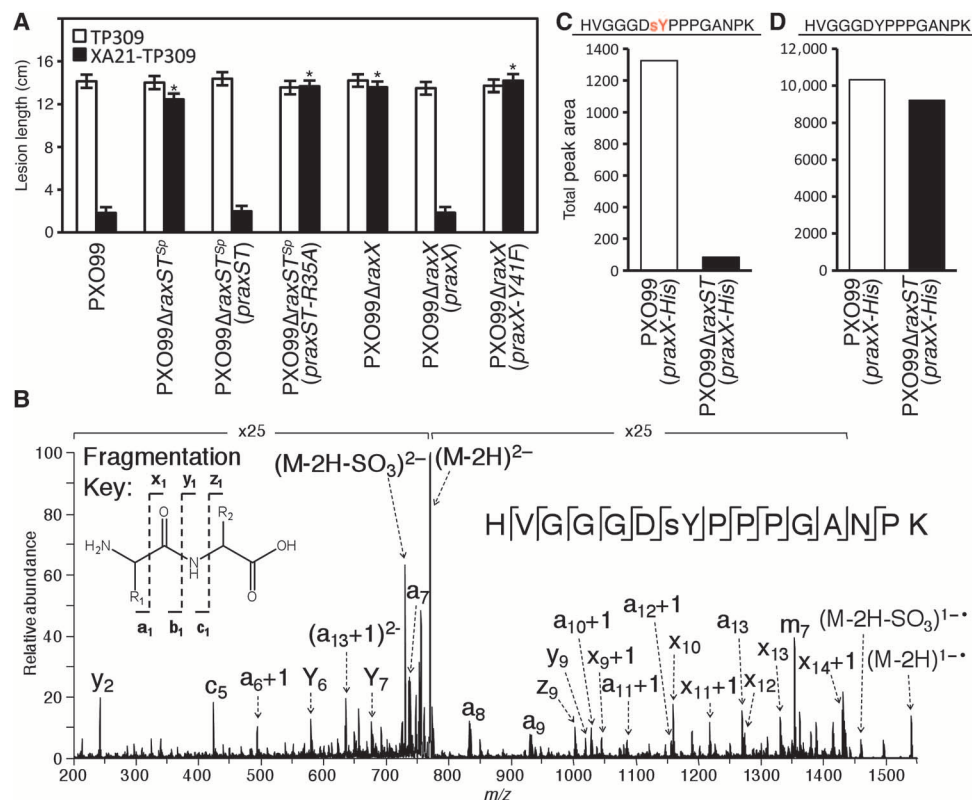


Fig. 2. RaxST sulfates RaxX on tyrosine 41. (A) The predicted PAPS binding residue R35 of RaxST and Y41 of RaxX are required for activation of XA21-mediated immunity. TP309 (open bars) and XA21-TP309 (black bars) were inoculated with the indicated *Xoo* strains, and lesion lengths were measured 14 days later as described in Fig. 1B. Bars indicate the mean lesion length \pm SE ($n \geq 14$). The “*” indicates statistically significant difference from PXO99 using Dunnett’s test ($\alpha = 0.01$). (B) Ultraviolet photodissociation (UVPD) mass spectrum of a tyrosine-sulfated peptide (HVGGGDsYPPPGANPK, 2-, m/z 770) from trypsin digestion of in vitro sulfated RaxX39. After incubation with *Escherichia coli*-expressed and purified His-RaxST, RaxX39 was digested with trypsin and analyzed by LC-UVPD-MS/MS in the negative nanoelectrospray mode to generate a, c, x, y, and z product ions, which are defined in the fragmentation key in the upper left corner of the spectrum. SO_3 is retained on all product ions, allowing the sulfate modification to be localized to Y41. Neutral losses of SO_3 from the precursor ion and charge reduced radical precursor ion are denoted as $(M-2H-SO_3)^{2-}$ and $(M-2H-SO_3)^{1-}$, respectively, in the spectrum. The ion labeled “m7” refers to the neutral loss of the sulfotyrosine side chain without additional fragmentation of the peptide backbone. Examination of extracted ion chromatograms of the sulfated and nonsulfated peptides suggests that the sulfated peptide is 100 \times lower in abundance than the nonsulfated peptide (see fig. S9). (C and D) RaxX-His proteins purified from PXO99(*praxX-His*) and PXO99 Δ raxST(*praxX-His*) were analyzed by selected reaction monitoring-MS (SRM-MS) (fig. S8). Total peak areas (arbitrary units) were quantitated for sulfated (C) and nonsulfated (D) tryptic RaxX peptides covering Y41.

using an expanded genetic code approach (30, 31). We heterologously expressed RaxX in *E. coli* together with an engineered aminoacyl-tRNA (transfer RNA) synthetase specific for sulfotyrosine, a cognate engineered amber suppressor tRNA, and the nonstandard amino acid sulfotyrosine (sY) (32). Nonsulfated RaxX was also expressed in *E. coli* without sulfotyrosine. We confirmed purity and tyrosine sulfation status of RaxX60 by gel-based assays and SRM-MS analysis (fig. S12). We tested if the resulting highly purified, full-length, sulfated RaxX60-sY and nonsulfated RaxX60-Y proteins could trigger defense gene expression in leaves of rice plants overexpressing XA21 (Ubi::XA21). As shown in fig. S13, sulfated RaxX60-sY, but not the nonsulfated form RaxX60-Y, triggered strong up-regulation of defense marker genes (table S1) in detached leaves of Ubi::XA21. Leaves from Kitaake rice plants, which lack the XA21 immune receptor, are insensitive to RaxX60-Y and RaxX60-sY. These results demonstrate that sulfated RaxX60-sY is sufficient to activate XA21-mediated defense gene expression in rice.

To identify a “minimal” epitope of RaxX that is sufficient to trigger these responses, we took biochemical and rational design approaches. We first tested whether chemically synthesized RaxX39 is sufficient to trigger XA21-dependent defense gene expression (Fig. 3A and fig. S14). We found that sulfated RaxX39-sY triggers defense gene expression in an XA21-dependent manner, whereas nonsulfated RaxX39 does not (fig. S14). To further narrow down the active region, we subjected RaxX39 to digestion with four site-specific proteases (GluC, ArgC, AspN, and trypsin). The predicted digestion patterns were confirmed by gel-based assays and by SRM-MS analysis for ArgC, AspN, and trypsin digests (fig. S15). We tested the resulting RaxX fragments for their ability to activate XA21-dependent signaling (fig. S16A). Only RaxX39-sY digestion products resulting from GluC and ArgC treatments retained activity on Ubi::XA21 plants. The ability of the ArgC fragment (32 to 55 amino acids) to activate XA21-mediated immunity was confirmed with chemically synthesized RaxX24-sY (32 to 55 amino acids) peptides (Fig. 3A and fig. S16B). Next, we tested N- and C-terminal

truncated versions of RaxX24-sY peptides. Chemically synthesized RaxX21-sY (35 to 55 amino acids) retained the ability to induce XA21-dependent signaling, whereas RaxX18-sY (32 to 49 amino acids) was compromised in this activity (Fig. 3A and fig. S16B). These results indicate that a chemically synthesized tyrosine-sulfated 21-amino acid derivative of RaxX, named RaxX21-sY, is sufficient to activate XA21-mediated defense responses.

In addition to activation of defense marker gene expression, the activation of PRR-triggered immunity in plants often involves the production of ethylene and reactive oxygen species (ROS) (11, 33). These responses are known to contribute to the final disease outcome in many plant pathogen interactions (11, 33). We therefore tested if RaxX21-sY can trigger these hallmarks of plant innate immune signaling in XA21-expressing rice leaves (Fig. 3, B to D, and figs. S17 to S19). As shown in Fig. 3 (B to D), only sulfated RaxX21-sY, but not RaxX21-Y, was able to activate defense marker gene expression and the production of ROS and ethylene in an XA21-dependent manner. These responses were most pronounced in rice plants overexpressing XA21 (Ubi::XA21). Nonetheless, we also observed significant RaxX21-sY-mediated up-regulation of defense gene expression and ROS production in Kitaake lines expressing XA21 from its native promoter (XA21::XA21) (figs. S18 and S19), with the highest levels of ROS production observed in rice leaves at later developmental stages (fig. S19).

To assess the sensitivity of XA21-expressing rice plants toward the different RaxX epitopes, we established the RaxX concentration needed to trigger the half maximal response (often referred to as the EC₅₀ value) using the fast and quantitative ROS assay. The EC₅₀ value in Ubi::XA21-expressing rice plants ranges from ~100 nM for RaxX60-sY, ~70 nM for RaxX39-sY, ~30 nM for RaxX24-sY, to ~20 nM for RaxX21-sY (Fig. 3A and fig. S20). In contrast, the further truncated peptide RaxX18-sY was completely inactive, even at high peptide concentrations (fig. S20). As an additional control, we tested an unrelated tyrosine-sulfated peptide called axY(S)22, derived from a PXO99 outer membrane protein (ACD57244), for its ability to activate XA21-mediated immunity (20, 22). Rice leaves overexpressing XA21 were insensitive to axY(S)22 at high peptide concentrations (1 μM) under conditions in which RaxX21-sY induced ROS production and defense gene expression changes (fig. S21). In summary, our data demonstrate that XA21-mediated immune signaling in rice is specifically induced by RaxX60-sY, and sulfated epitopes derived from the full RaxX60 protein, at low nanomolar concentrations.

A RaxX variant evades XA21-mediated immunity

When XA21 was first discovered, it was demonstrated to confer resistance to most tested strains of *Xoo* (18, 34). Since that time, several field strains that evade XA21-mediated immunity have been identified (35, 36). For most of these strains, the molecular basis of immune evasion is unknown. However, we previously demonstrated that two of these *Xoo* strains, DY89031 and CK89021, carry mutations in *raxA* and *raxST*, respectively (19). We hypothesized that other strains may evade XA21-mediated immunity because they encode variant *raxX* alleles. Indeed, we found that RaxX from the Indian field isolate IXO685, which evades XA21-mediated immunity (35), differs in nine amino acids as compared with RaxX from PXO99 (Fig. 4A). Four amino acid substitutions are located in the RaxX21 sequence.

We tested if *raxX* from IXO685 expressed under control of the native promoter of PXO99 *raxX* was able to complement the PXO99Δ*raxX* mutant phenotype on XA21-expressing rice plants. We found that

PXO99Δ*raxX*(*praxX*-IXO685) was unable to activate XA21-mediated immunity (Fig. 4B). Likewise, recombinant, highly purified RaxX60-sY-IXO685 produced using our expanded genetic code system was not able to trigger XA21-mediated ROS production (Fig. 4C and fig. S22). Expression of the PXO99 *raxX* allele in the IXO685 strain conferred the strain with the ability to activate XA21-mediated immunity (Fig. 4B). These results indicate that IXO685 can overcome XA21-mediated immunity because it has acquired several nonsynonymous substitutions in the *raxX* ORF. Two other Indian field isolates (IXO651 and IXO1221), which are also able to evade XA21-mediated immunity (35) and share the identical *raxX* sequence with IXO685, can also be complemented with *praxX* (fig. S23).

We carried out mutational analyses of PXO99 RaxX to determine if any of the four identified amino acid substitutions within the RaxX21 epitope of the XA21-evading strains are essential for XA21 activation. The four residues were mutated individually in *praxX*, and the mutated alleles were tested for functional complementation of the PXO99Δ*raxX* mutant by inoculation of XA21-expressing rice plants (Fig. 4D). PXO99Δ*raxX*(*praxX*-P44S) and PXO99Δ*raxX*(*praxX*-P48T) formed long lesions on XA21-TP309, PXO99Δ*raxX*(*praxX*-A46P) displayed an intermediate phenotype, and PXO99Δ*raxX*(*praxX*-R55P) formed short lesions. Consistent with this result, highly purified recombinant RaxX60-sY-P44S and RaxX60-sY-P48T proteins, produced using our expanded genetic code system, are unable to induce ROS production in Kitaake Ubi::XA21 (Fig. 4E and fig. S22). Thus, P44 and P48 are required for activation of XA21-mediated immunity. That is, the alterations of these specific amino acids in RaxX of IXO685, IXO651, and IXO1221 enable these strains to evade XA21-mediated immunity.

DISCUSSION

Our results indicate that the presence or absence of sulfation and the residues near Y41 are decisive for the ability of RaxX to trigger XA21-mediated immunity and that Y41 is a substrate for RaxST-mediated sulfation. We have identified RaxX in at least eight *Xanthomonas* species that infect maize, cassava, sugar cane, tomatoes, peppers, wheat, alfalfa, onions, banana, and citrus (fig. S1). In all of these strains, *raxX* is encoded upstream of *raxST*, *raxA*, and *raxB*. The colocalization of the *rax* genes suggests that they function in a common biological process and that the RaxX proteins in these species are also substrates for RaxST-mediated sulfation.

Although tyrosine sulfation has not been demonstrated in other prokaryotic species, it is a common posttranslational modification of eukaryotic proteins and plays important roles in regulating plant development and in mediating the interactions of plants and animals with microbes. For example, in humans, sulfation of the C-C chemokine receptor type 5 (CCR5) is critical for binding of the envelope glycoprotein gp120 by the HIV (37). In plants, the sulfated peptide signaling factors PSK (phytosulfokine) and PSY1 (plant peptide containing sulfated tyrosine 1) have been implicated in xylem trachea development, root growth, suppression of plant defense, and promotion of plant health (38, 39).

Although RaxX shares no homology with microbial proteins of known function, we have noted a remarkable similarity between the sulfated, secreted *Arabidopsis* 18-amino acid peptide signaling factor PSY1, four predicted rice PSY1 orthologs, and RaxX residues 40 to 55

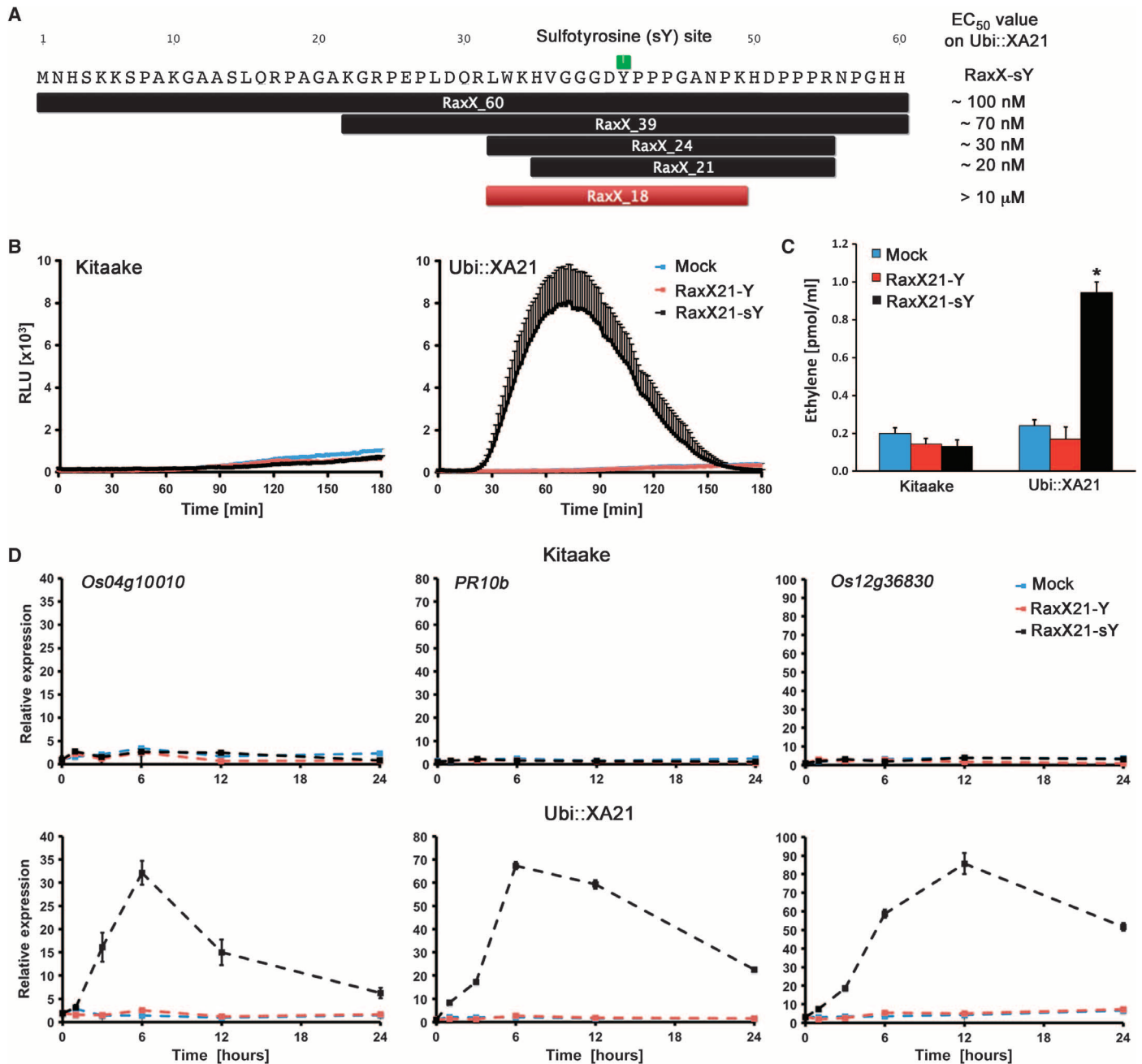


Fig. 3. Sulfated RaxX triggers XA21-mediated defense responses. (A) Amino acid sequence of RaxX from PXO99. RaxX derivative peptides of varying lengths were tested for their ability to activate XA21-dependent signaling. Sulfated peptides shown in black trigger XA21-mediated defense responses, whereas nonsulfated peptides and the sulfated peptide RaxX_18 shown in red do not (figs. S13 to S21). “EC₅₀ value on Ubi::XA21” refers to the EC₅₀ values determined by monitoring total ROS production over 3 hours after application of the RaxX protein and peptide derivatives (fig. S20) at different concentrations on Kitaake rice expressing XA21 under the control of the ubiquitin promoter (Ubi::XA21) (*n* = 6). (B) ROS production in leaves of Kitaake and Ubi::XA21 rice plants treated with H₂O (mock), RaxX21-Y, or RaxX21-sY (250 nM) (*n* = 6). RLU, relative light units. (C) Ethylene production in leaves of Kitaake and Ubi::XA21 rice plants after 4 hours of treatment with H₂O (mock), RaxX21-Y, or RaxX21-sY (1 μM). The “*” indicates statistically significant difference from mock treatment using Dunnett’s test (α = 0.01, *n* = 3). (D) Temporal changes in defense marker gene (*Os04g10010*, *PR10b*, and *Os12g36830*) expression in leaves of Kitaake and Ubi::XA21 rice plants treated with H₂O (mock), RaxX21-Y, or RaxX21-sY (500 nM, *n* = 3). All data points depict means ± SE. These experiments were repeated at least three times with similar results.

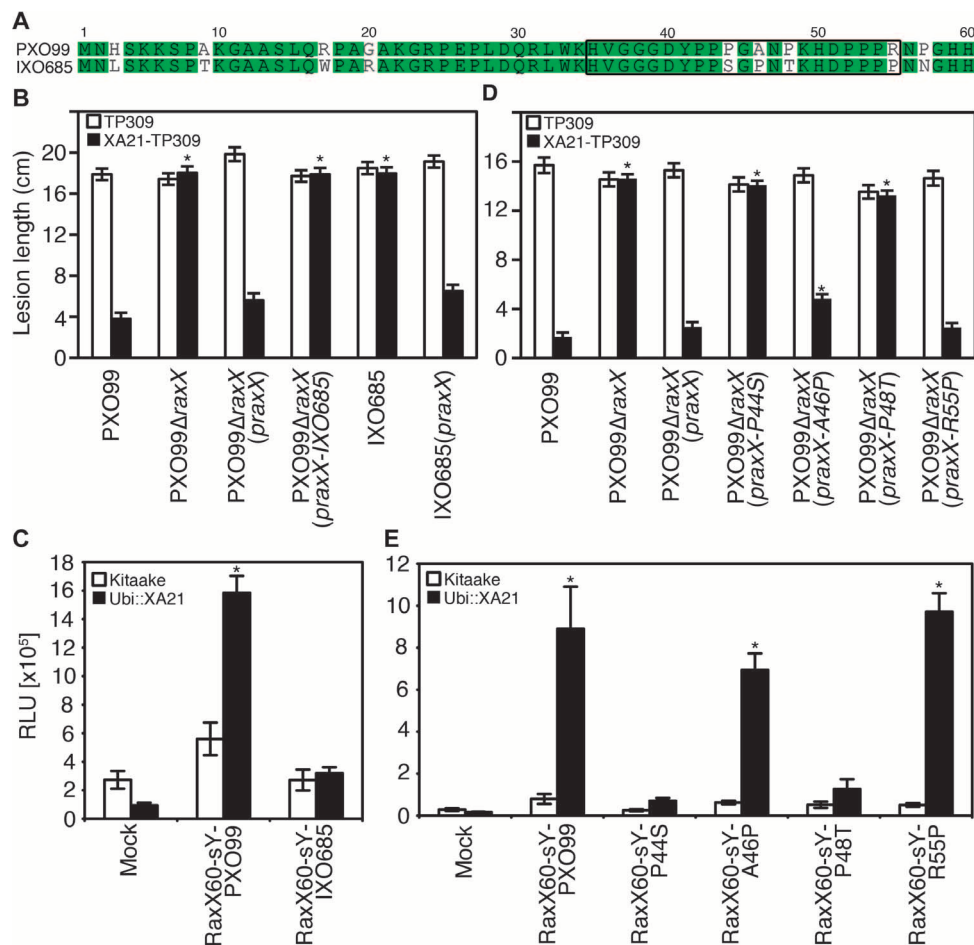


Fig. 4. Comparative genomics and mutational analyses identify key amino acids required for RaxX activity. (A) Alignment of amino acid sequences of RaxX from PXO99 and IXO685. The region corresponding to RaxX21 is boxed. (B and C) RaxX from the field strain IXO685 does not trigger XA21-mediated immune response. (B) TP309 (open bars) and XA21-TP309 (black bars) were inoculated with the indicated *Xoo* strains, and lesion lengths were measured 14 days later as described in Fig. 1B. Bars indicate the mean lesion length \pm SE ($n \geq 14$). (C) ROS production in leaves of Kitaake (open bars) and Ubi::XA21 (black bars) rice plants treated with the indicated peptides (250 nM) or mock treatment. Bars depict mean RLU over 3 hours \pm SE ($n = 6$). (D and E) RaxX point mutation analysis reveals that P44 and P48 are required for activation of XA21-mediated immune response. (D) TP309 (open bars) and XA21-TP309 (black bars) were inoculated with the indicated *Xoo* strains, and lesions were measured 14 days later as described in Fig. 1B. Bars indicate the mean lesion length \pm SE ($n \geq 14$). (E) ROS production in leaves of Kitaake (open bars) and Ubi::XA21 (black bars) rice plants treated with the indicated peptides (250 nM) or mock treatment. Bars depict average RLU \pm SE ($n = 6$). For (B) to (D), the “*” indicates statistically significant difference from mock treatments using Dunnett’s test ($\alpha = 0.01$). These experiments were repeated at least three times with similar results.

(fig. S24). These similarities suggest the exciting possibility that in the absence of XA21, *Xoo* and other Xanthomonads use sulfated RaxX to mimic PSY1-like peptides to suppress host defense responses, facilitate infection, or enhance plant health, maintenance of which is critically important for propagation and survival of biotrophic pathogens (39–41).

The robust immunity conferred by XA21 to most *Xoo* strains is a highly valued agronomic trait, and therefore, XA21 has been introgressed into diverse rice varieties grown by rice farmers (42–47). The discovery of *raxX* and allelic variants that can evade XA21-mediated recognition can be used to screen *Xoo* field populations for the presence of potentially virulent variants. Such an anticipatory breeding approach would alert farmers of the need to plant different varieties or mixtures before the breakdown of XA21-mediated immunity.

The knowledge gained from these studies can also be used to expand the spectrum of XA21-mediated immunity to additional strains of *Xoo*. For example, new synthetic receptors of XA21 with novel en-

gineered recognition specificities such as those that can recognize RaxX from IXO685 would be of great interest. Because *Xanthomonas* species affect virtually all crop plants, it is expected that future studies will lead to new strategies for engineering resistance to non-rice pathogens. For example, *X. axonopodis* pv. *citrumelo*, *X. euvesicatoria*, and *X. oryzae* pv. *oryzicola*, which cause serious diseases on citrus, tomato, and rice, carry alternate *raxX* alleles (fig. S1). This knowledge provides an opportunity to engineer new XA21 variants that recognize these alleles and use the novel variants for engineering plants resistant to *Xanthomonas* infections that cannot be controlled by more conventional genetic methods.

The sulfated RaxX/XA21 interaction represents a new biological system for studies of the interaction of sulfated epitopes and their receptors. A recent report of the generation of a potent new antiviral drug (48), which was based on more than 10 years of work on a single family of sulfated peptides, indicates the clinical relevance of studies of

sulfopeptides. Thus, the studies reported here are applicable to generation of therapeutic reagents with the potential to block microbial infection of both plants and animals.

MATERIALS AND METHODS

Bacterial culture and growth conditions

Lists of plasmids and bacterial strains used in this study are provided in tables S2 and S3. *Xoo* was cultured on peptone sucrose agar (PSA) plates at 28°C with the appropriate antibiotic(s). YEB medium [yeast extract (5 g/liter), tryptone (10 g/liter), NaCl (5 g/liter), sucrose (5 g/liter), MgSO₄ (0.5 g/liter), pH 7.3] was used for liquid culture at 28°C with shaking at 230 rpm. *E. coli* was cultured at 37°C and 230 rpm in lysogenic broth (LB) for cloning or M9T (per 1 liter: M9 plus 10 g of tryptone, 5 g of NaCl; after autoclaving, 1 ml of 1 M MgSO₄ and 1 ml of 0.02% biotin were added). Kanamycin was used at 50 µg/ml, carbenicillin at 50 µg/ml, spectinomycin at 50 µg/ml, and cephalixin at 20 µg/ml.

Xoo mutation and complementation

Xoo mutants were generated in Philippine race 6 strain PXO99Az, a derivative of strain PXO99 (referred to simply as PXO99 in this manuscript) (24, 49). PXO99Δ*raxST*^{SP}, PXO99Δ*l.0*^{SP}, and PXO99Δ*l.7*^{SP} were generated by marker exchange mutagenesis using the suicide vector pJP5603 (50). About 500-bp (base pair) sequences flanking both sides of the region to be deleted were sequentially cloned into the multiple cloning site using the restriction enzyme sites Kpn I/Bam HI and Bam HI/Sal I. A spectinomycin resistance cassette was ligated between the two flanking sequences using the Bam HI site. PXO99-competent cells were transformed with the suicide plasmids by electroporation and plated to PSA with spectinomycin (50 µg/ml). Colonies with the expected antibiotic resistance phenotype for double crossover (spectinomycin-resistant, kanamycin-sensitive) were validated by polymerase chain reaction (PCR).

Marker-free *raxX* (PXO99Δ*raxX*) and *raxST* (PXO99Δ*raxST*) mutants were generated using the vector pUFR80 (51). About 500-bp sequences flanking were sequentially cloned into the multiple cloning site of pUFR80 using the sites Eco RI/Bam HI and Bam HI/Hind III. *praxX*-KO-MF and *praxST*-KO-MF were transformed into PXO99-competent cells by electroporation, and the cells were plated to nutrient agar (NA) (CM0001, OXOID) with kanamycin (50 µg/ml). Transformants were grown in nutrient broth without kanamycin and plated to NA with 5% sucrose to select for a second crossover event. Colonies were analyzed by PCR to identify deletion mutants.

Strains were transformed using derivatives of the broad host range vector pVSP61 (52). The *raxX* and *raxST* sequences along with their predicted promoter region (300 bp upstream of the predicted start codons) were cloned into pVSP61 using the Eco RI/Hind III sites. Point mutations were generated by QuikChange mutagenesis. For *praxX*-*IXO685*, the *raxX* promoter from PXO99 was first introduced into pVSP61 using the sites Eco RI/Bam HI to generate *praxXprom*. The *raxX* sequence from *IXO685* was amplified from genomic DNA and cloned into *praxXprom* behind the PXO99 *raxX* promoter using the restriction sites Bam HI/Hind III.

Rice and rice growth conditions for inoculations

Oryza sativa ssp. *japonica* rice varieties TP309, a 106-17-derived transgenic line of TP309 carrying *XA21* driven by its own promoter

(XA21-TP309) (7), Kitaake, and Ubi::Myc::XA21-Kitaake line 7-A-8 (Ubi::XA21) (25) were used for rice inoculations. Native TP309 and Kitaake do not contain *XA21*. Seeds were germinated in distilled water at 28°C for 1 week and then transplanted into sandy soil (80% sand, 20% peat from Redi-Gro) in 5.5-inch square pots (two seedlings per pot). Plants were grown in tubs in a greenhouse. The plants were top-watered daily with fertilizer water [N, 58 ppm (parts per million); P, 15 ppm; K, 55 ppm; Ca, 20 ppm; Mg, 13 ppm; S, 49 ppm; Fe, 1 ppm; Cu, 0.06 ppm; Mn, 0.4 ppm; Mo, 0.02 ppm; Zn, 0.1 ppm; B, 0.4 ppm] for 4 weeks, followed by water for 2 weeks. Six weeks after planting, the rice was transferred to a growth chamber set to 28°C/24°C, 80%/85% humidity, and 14/10-hour lighting for the day/night cycle.

Plants were inoculated 2 to 3 days after transfer using the scissors clipping method (53). Bacteria for inoculation were taken from PSA plates and resuspended in water at a density of 10⁸ CFU/ml. Water-soaked lesions were measured 14 days after inoculation. In planta bacterial growth analysis was carried out as described (22). Four biological replicates were measured for each data point. Each biological replicate was the mean number of colonies from four technical replicates.

Generation of RaxX peptides

Sulfated (sY) and nonsulfated (Y) versions of RaxX39 (KGRPEP-LDQRLWKHVGGGDYPPPGANPKHDPPRNPNGHH), RaxX24 (LWKHVGGGDYPPPGANPKHDPPPR), RaxX21 (HVGGGDYPPPGANPKHDPPPR), RaxX18 (LWKHVGGGDYPPPGANPK), and ax22(S)Y (20) were ordered from Pacific Immunology. The peptides were checked for purity by high-performance liquid chromatography and MS analysis by the company. All peptides were resuspended in ddH₂O. No other solvents were required to dissolve the peptide.

For the expanded genetic code approach, full-length RaxX (RaxX60) from PXO99 was expressed as MBP-3C-RaxX-His fusion protein in *E. coli* C321.Δ*A.exp* (31). *E. coli* C321.Δ*A.exp* was cotransformed with SYP29_pULTRA_SY, made by cloning the sY-specific aminoacyl-tRNA synthetase from (32) into the pULTRA system (54) and pBAD/MBP-3C-RaxX-His (Amber) to generate sulfated RaxX or pBAD/MBP-3C-RaxX-His to generate nonsulfated RaxX. Transformed bacteria were grown in 0.5 liter of M9T medium. For expression of sulfated RaxX, sulfotyrosine (sY), synthesized as described in (32), was added to a final concentration of 3 mM. Cultures were grown at 37°C with shaking at 230 rpm. Expression was induced at an optical density of 0.6 to 0.7 by addition of 1 mM isopropyl-β-D-thiogalactopyranoside and 0.25% (w/v) arabinose for 5 hours. MBP-3C-RaxX-His was purified from intracellular total protein extracts over a 5-ml Ni-NTA column by fast protein liquid chromatography (FPLC) (ÄKTA, GE Healthcare). The MBP-tag was removed by overnight digestion with 3C protease at a concentration of 1:200 (w/w) at 4°C. The MBP was separated from full-length RaxX-His over a 1-ml SP-XL column, a strong cation exchanger, on an FPLC (ÄKTA, GE Healthcare). Nonsulfated RaxX was expressed and purified in the same way, except pBAD/MBP-3C-RaxX-His plasmids without Amber mutations were used, and no sulfotyrosine was added to the cultures. The yield for sulfated and nonsulfated highly purified RaxX-His was approximately 1 mg of protein per 1 liter of culture. All RaxX allelic variants were prepared according to the same protocol.

In vitro sulfation of RaxX by His-RaxST

RaxST was expressed as a full-length fusion protein with an N-terminal His-tag in BL21(DE3) cells and purified using Ni-NTA agarose beads (Qiagen). His-RaxST expression was confirmed by SDS-polyacrylamide

gel electrophoresis with Coomassie blue staining and Western blot analysis with Penta-His antibody (Qiagen) (fig. S4). The in vitro sulfation reaction was carried out as described by Han *et al.* (28). Purified RaxST (20 μ g) was incubated with 170 μ g of RaxX39 in the presence of sulfation buffer [25 mM tris (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, 480 μ M PAPS] for 4 hours at 30°C.

Nano-LC-UVPD-MS

Proteins from in vitro sulfation reactions were buffer-exchanged into 50 mM tris-HCl (pH 8.5), 1 M urea using a 3-kD molecular weight cutoff (MWCO) filter and digested with trypsin overnight at 37°C using a 1:25 enzyme to substrate ratio. After digestion, samples were passed through a 10K MWCO filter to separate RaxX39 peptides from trypsin.

Tryptic peptides of RaxX39 were separated by reversed-phase LC at 300 nl/min using a Dionex Ultimate 3000 NSLC system configured for preconcentration. Mobile phases A and B were water and acetonitrile, respectively, both containing 0.1% formic acid. A solution of 2% acetonitrile and 0.1% formic acid was used for sample loading onto a New Objective IntegraFrit 100- μ m \times 3-cm trap column packed in-house with 5- μ m, 100-Å Michrom Magic C18 AQ. After 5 min of preconcentration at 7 μ l/min, the trap column was switched in-line with a New Objective PicoFrit analytical column (75 μ m \times 15 cm) packed with Waters 3.5- μ m, 130-Å XBridge BEH C18. A 23-min linear gradient starting at 2% B and ending at 40% B was used for separation.

LC-MS/MS analysis was performed on a Thermo Scientific Velos Pro mass spectrometer equipped with a Coherent ExciStar XS Excimer laser operated at 193 nm with a pulse frequency of 500 Hz (55). Data acquisition was carried out by first performing a full MS1 scan from mass/charge ratio (m/z) 300 to 2000 followed by three MS/MS UVPD events to activate the doubly charged sulfopeptide of interest, HVGGGDsYPPPGANPK, at m/z 769.8 in negative mode and m/z 771.8 in positive mode. A single laser pulse was used for ion activation in negative mode during a 2.0-ms activation period. Two laser pulses were used for ion activation in the positive mode. In both polarities, the isolation width was 3 daltons and the activation q value was 0.10. MS/MS fragmentation patterns were manually interpreted. For the UVPD mass spectrum shown in Fig. 2B, the neutral loss of SO_3 and CO_2 from the precursor ion and/or charge reduced precursor ion are denoted as “ $-SO_3$ ” and “ $-CO_2$,” respectively, in the spectrum. Product ions $a + 1$ and $x + 1$ correspond to homolytic cleavage of the α -carbonyl bond of the peptide backbone without subsequent transfer of hydrogen radicals to produce even electron a and x ions, which are the diagnostic N- and C-terminal ions used to identify the peptide.

High-resolution mass measurement of the diagnostic tryptic peptide corresponding to HVGGGDsYPPPGANPK from the RaxX39 sample sulfated by RaxST in vitro was undertaken on a Thermo Fisher Scientific Instruments Orbitrap Elite mass spectrometer operated at an FT resolution of 120,000 at m/z 400.

Isolation of RaxX from PXO99 for SRM-MS

Briefly, RaxX-His proteins were purified from *Xoo* strains and their sulfation was analyzed by SRM-MS. PXO99(*praxX-His*) and PXO99 Δ *raxX* (*praxX-His*) were cultured in 1 liter of XVM2 (56) liquid medium for 40 hours at 28°C. The cells were harvested by centrifugation at 10,000 rpm for 20 min at 4°C. RaxX-His was purified from intracellular total protein extract over a 5-ml Ni-NTA column on an FPLC (ÄKTA, GE Healthcare). Isolation of Rax-His was confirmed by Coomassie blue staining and Western blot analysis with Penta-His antibody (Qiagen) (fig. S6).

Selected reaction monitoring–mass spectrometry

Purified RaxX-His was digested with 1 μ g of trypsin in the presence of 5 mM dithiothreitol (DTT). Peptide digests were analyzed using an Agilent 1260 LC system operating in normal flow mode at 400 μ l/min coupled to an Agilent 6460QQQ Mass Spectrometer. Peptides were separated on an Ascentis Express Peptide C18 column [2.7- μ m particle size, 160-Å pore size, 5-cm length \times 2.1-mm inside diameter (ID)], coupled to a 5-mm \times 2.1-mm ID guard column with similar particle and pore size, operating at 60°C; Sigma-Aldrich] using a 43-min method with the following gradient: initial conditions were 98% buffer A (0.1% formic acid), 2% buffer B (99.9% acetonitrile, 0.1% formic acid), after which B was increased to 35% over 35.70 min, followed by an increase to 90% B in 18 s, where it was held for 2 min. Buffer B was then decreased to 5% in 30 s, where it was held for 2.5 min, then further decreased to 2%, and held for 2 min to reequilibrate the column.

The eluted peptides were ionized via an Agilent Jet Stream ESI source operating in positive ion mode with the following source parameters: gas temperature = 250°C, gas flow = 13 liters/min, nebulizer pressure = 35 psi, sheath gas temperature = 250°C, sheath gas flow = 11 liters/min, capillary voltage = 3500 V, nozzle voltage = 0 V. The data were acquired using Agilent MassHunter version B.06.00. Data were acquired in MRM mode with transitions generated via Skyline (57) software (version 2.6). In silico SRM transitions were selected with 2+ and 3+ charge states, no missed tryptic cleavages, sulfated and nonsulfated tyrosine modifications, and singly charged fragment ions from both the y and b series. The Skyline-generated method was refined to three transitions per peptide and set to Agilent 6460QQQ for instrument-specific collision energies. The final method consists of 36 transitions, with a 25-ms dwell time per transition and MS1 and MS2 resolution set to unit. Acquired SRM data were imported into Skyline where confident peptide quantification was based on multiple concurrent SRM transitions above background signal and peak areas were refined with the mProphet algorithm (58).

Rice and rice growth conditions for plant defense assays

Kitaake, Ubi::Myc::XA21-Kitaake line 7-A-8 (Ubi::XA21) (25), and XA21::XA21-Kitaake (25) were grown in growth chambers at 24° or 28°C with a 14-hour/10-hour light-dark cycle at 80% humidity. Rice plants were grown in hydroponic system using A-OK Starter Plugs (Grodan) and watered with Hoagland's solution twice a week.

Assays for ROS

Leaves of 4- to 6-week-old hydroponically grown rice plants were cut longitudinally along the mid vein and then transversely into 1- to 1.5-mm-thick leaf pieces. These leaf pieces were floated on autoclaved ddH₂O overnight. The next morning, two leaf pieces were transferred into one well of a 96-well white plate containing 100 μ l of excitation solution [5 to 20 mM L-012 (Wako) and horseradish peroxidase (0.5 to 2 mg/ml; Sigma)]. Leaf pieces were treated with the indicated concentration of peptides or mock-treated (ddH₂O or a peptide buffer solution). Chemiluminescence was measured for 3 hours with 0.5 s per reading with a high-sensitivity plate reader (TriStar, Berthold).

Gene expression assays

Rice leaf tissue was treated with peptides as described previously with slight adaptations (59). Leaves of 4-week-old hydroponically grown rice plants were cut into 2-cm-long strips and incubated for at least 12 hours in ddH₂O to reduce residual wound signal. Leaf strips were

treated with the indicated peptides and for the indicated time. Leaf tissue was snap-frozen in liquid nitrogen and processed appropriately. *PR10b* and *Os04g10010* were previously described as defense marker genes in rice (59). Other defense marker genes, *Os12g36830* and *Os06g37224*, were identified in RNAseq experiments as up-regulated genes after elf18 treatment of rice leaves expressing EFR::XA21::GFP (60) (<http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=OryzaspilotREDO>). For quantitative reverse transcription PCRs (qRT-PCRs), the Bio-Rad SsoFast EvaGreen Supermix was used. The genes and qRT-PCR primer pairs are listed in table S1. qRT-PCRs were run for 40 cycles with annealing and amplification at 62°C for 5 s and denaturation at 95°C for 5 s. The expression levels of all defense marker genes were normalized to the actin gene expression level and to the respective mock-treated control at 0 or 1 hour.

Ethylene production

Leaves of 3- to 4-week-old hydroponically grown rice plants were cut in small horizontal strips (~0.3 to 0.5 mm) and floated on water in petri dishes overnight at room temperature. Ethylene biosynthesis was assayed by placing leaf samples in 6-ml tubes with 500 µl of water or the indicated peptide at a concentration of 1 µM as indicated. Tubes were sealed with rubber caps, and ethylene accumulating in the headspace within 4 hours of incubation was determined by gas chromatography as described (61).

RaxX39 peptide digestion

RaxX39-Y or RaxX39-sY (10 µg) was digested in buffer D (50 mM tris-HCl, 1 mM DTT) with 1 µg of one of the following site-specific proteases: GluC, ArgC, AspN, or trypsin. Digestions or mock reactions were performed at 25°C for 16 hours.

Statistical analysis

Statistical analyses were performed using JMP software (SAS Institute Inc.).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/1/6/e1500245/DC1>

Fig. S1. Sequence alignment of 15 *raxX* alleles.

Fig. S2. *raxX* is required for activation of XA21-mediated immunity in the Ubi::XA21 Kitaake (Ubi::XA21) genetic background.

Fig. S3. Alignment of the PAPS binding motif of RaxST from PXO99, TPST1 from human, and TPST2 from human.

Fig. S4. RaxX Y41 is required for activation of XA21-mediated immunity.

Fig. S5. RaxX Y41F does not trigger the XA21-mediated immune response.

Fig. S6. Purification of the full-length RaxST protein carrying an N-terminal His-tag.

Fig. S7. Expanded MS1 spectrum of HVGGGDsYPPPGANPK from trypsin digestion of in vitro sulfated RaxX39.

Fig. S8. Extracted ion chromatograms (XIC) showing the difference in ion abundances for the sulfated and nonsulfated peptide HVGGGDYPPPGANPK (2- charge state).

Fig. S9. Positive UVPD mass spectrum of HVGGGDsYPPPGANPK from trypsin digestion of in vitro sulfated RaxX39.

Fig. S10. RaxX-His proteins were purified from PXO99(*praxX-His*) and PXO99Δ*raxST*(*praxX-His*).

Fig. S11. SRM-MS analysis of the tryptic RaxX peptide HVGGGDYPPPGANPK from PXO99(*praxX-His*) and PXO99Δ*raxST*(*praxX-His*).

Fig. S12. Expression and sulfation of RaxX60-Y and RaxX60-sY.

Fig. S13. Heterologously expressed, full-length RaxX60-sY activates XA21-mediated defense gene expression.

Fig. S14. Chemically synthesized RaxX39-sY activates XA21-mediated defense gene expression.

Fig. S15. Digestion of RaxX39-sY by the four site-specific proteases GluC, trypsin, ArgC, and ApsN.

Fig. S16. A 21-amino acid sulfated peptide derived from RaxX is sufficient to activate XA21-mediated defense gene expression and the production of ROS.

Fig. S17. Chemically synthesized RaxX21-sY is sufficient to activate XA21-mediated defense gene expression.

Fig. S18. Chemically synthesized RaxX21-sY activates gene expression in Kitaake lines expressing XA21 from its native promoter (XA21::XA21).

Fig. S19. Chemically synthesized RaxX21-sY activates the production of ROS in Kitaake lines expressing XA21 from its native promoter (XA21::XA21).

Fig. S20. ROS production dose-response curves in Ubi::XA21 leaves treated with chemically synthesized RaxX sulfated peptides.

Fig. S21. The tyrosine-sulfated peptide axY(S)22, derived from Ax21, does not trigger XA21-mediated immune responses in rice.

Fig. S22. Expression and purification of different RaxX alleles.

Fig. S23. Complementation of *Xoo* isolates IXO651, IXO685, and IXO1221 with *praxX* confers the ability to activate XA21-mediated immunity.

Fig. S24. RaxX is similar to the *Arabidopsis thaliana* peptide signaling factors PSY1 and to rice PSY1 homologs.

Table S1. Marker genes and primers used for qPCR.

Table S2. Plasmids used in this study.

Table S3. Bacterial strains used in this study.

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