

# UC Davis

## UC Davis Previously Published Works

### Title

The rice XA21 ectodomain fused to the Arabidopsis EFR cytoplasmic domain confers resistance to *Xanthomonas oryzae* pv. *oryzae*

### Permalink

<https://escholarship.org/uc/item/38z8v70w>

### Journal

PeerJ, 6(5)

### ISSN

2167-8359

### Authors

Thomas, Nicholas C

Oksenberg, Nir

Liu, Furong

et al.

### Publication Date

2018

### DOI

10.7717/peerj.4456

### Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

# The rice XA21 ectodomain fused to the Arabidopsis EFR cytoplasmic domain confers resistance to *Xanthomonas oryzae* pv. *oryzae*

Nicholas C. Thomas<sup>1,2,\*</sup>, Nir Oksenberg<sup>1,\*</sup>, Furong Liu<sup>1</sup>, Daniel Caddell<sup>1</sup>, Alina Nalyvayko<sup>1</sup>, Yen Nguyen<sup>1</sup>, Benjamin Schwessinger<sup>1,3</sup> and Pamela C. Ronald<sup>1</sup>

<sup>1</sup> Department of Plant Pathology and the Genome Center, University of California, Davis, Davis, CA, USA

<sup>2</sup> Current affiliation: Department of Plant Pathology and Microbiology, University of California, Riverside, Riverside, CA, USA

<sup>3</sup> Current affiliation: Research School of Biology, Australian National University, Acton, MA, Australia

\* These authors contributed equally to this work.

## ABSTRACT

Rice (*Oryza sativa*) plants expressing the XA21 cell-surface receptor kinase are resistant to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) infection. We previously demonstrated that expressing a chimeric protein containing the ELONGATION FACTOR Tu RECEPTOR (EFR) ectodomain and the XA21 endodomain (EFR:XA21) in rice does not confer robust resistance to *Xoo*. To test if the XA21 ectodomain is required for *Xoo* resistance, we produced transgenic rice lines expressing a chimeric protein consisting of the XA21 ectodomain and EFR endodomain (XA21:EFR) and inoculated these lines with *Xoo*. We also tested if the XA21:EFR rice plants respond to a synthetic sulfated 21 amino acid derivative (RaxX21-sY) of the activator of XA21-mediated immunity, RaxX. We found that five independently transformed XA21:EFR rice lines displayed resistance to *Xoo* as measured by lesion length analysis, and showed that five lines share characteristic markers of the XA21 defense response (generation of reactive oxygen species and defense response gene expression) after treatment with RaxX21-sY. Our results indicate that expression of the XA21:EFR chimeric receptor in rice confers resistance to *Xoo*. These results suggest that the endodomain of the EFR and XA21 immune receptors are interchangeable and the XA21 ectodomain is the key determinant conferring robust resistance to *Xoo*.

**Subjects** Agricultural Science, Genetics, Molecular Biology, Plant Science

**Keywords** Rice, XA21, EFR, Disease resistance, *Xanthomonas*, Immune receptor, Chimeric receptor

## INTRODUCTION

Plant cell-surface immune receptors confer defense against pathogen infection. Cell-surface mediated immunity in plants is mainly conferred by receptor-like proteins (RLPs) and receptor-like kinases (RLKs) that recognize pathogen associated molecular patterns

Submitted 19 April 2017  
Accepted 15 February 2018  
Published 9 May 2018

Corresponding authors  
Benjamin Schwessinger,  
benjamin.schwessinger@anu.edu.au  
Pamela C. Ronald,  
pcronald@ucdavis.edu

Academic editor  
Sheila McCormick

Additional Information and  
Declarations can be found on  
page 12

DOI 10.7717/peerj.4456

© Copyright  
2018 Thomas et al.

Distributed under  
Creative Commons CC-BY 4.0

**OPEN ACCESS**

(Jones & Dangl, 2006; Macho & Zipfel, 2014). Three well-studied cell-surface RLKs that confer resistance to bacterial pathogens include FLAGELLIN SENSING2 (FLS2; At5G6330) (Gómez-Gómez & Boller, 2000), EF-TU RECEPTOR (EFR; At5g20480) (Zipfel et al., 2006) from *Arabidopsis* and XA21 (U37133) from *Oryza longistaminata* (Song et al., 1995). The identification of the microbial molecules recognized by these three receptors have enhanced in depth characterization of their functional properties. The FLS2 receptor binds the flg22 peptide derived from bacterial flagellin (Felix et al., 1999; Gómez-Gómez & Boller, 2000; Chinchilla et al., 2006). EFR recognizes the elf18 peptide derived from the bacterial Elongation Factor Thermo-unstable protein (EF-Tu) (Kunze et al., 2004; Zipfel et al., 2006). XA21 recognizes the sulfated required for activation of Xa21-mediated immunity X (RaxX) protein produced by *Xanthomonas oryzae* pv. *oryzae* (Xoo; Pruitt et al., 2015). Although these receptors specifically recognize different molecules, they share similar domain structures including ectodomains containing leucine rich repeats and endodomains containing intracellular kinases of the non-arginine aspartate (non-RD) class (Dardick & Ronald, 2006).

Domain swap studies between cell-surface receptors have led to the hypothesis that the nature of the endodomains is the primary determinant dictating the specific disease resistance outcome. For example, studies of a chimeric receptor generated by fusion of the *Arabidopsis* BRASSINOSTEROID-INSENSITIVE1 (BRI1) receptor, which recognizes brassinosteroid hormones, to the XA21 endodomain (BRI1:XA21) (Li & Chory, 1997) indicated that the chimeric receptor could be activated by brassinosteroid treatment. Rice cells expressing BRI1:XA21 (NRG-1 in the original publication) and treated with brassinosteroid initiated cell death, produced reactive oxygen species (ROS), and expressed stress-related genes. The stress-related symptoms were attributed to the activation of the XA21 endodomain because the full-length BRI1 receptor does not induce the same stress-related symptoms as BRI1:XA21 (He et al., 2000). These results suggested that the XA21 endodomain was activated upon BRI1 recognition of brassinosteroid and that the specific type of response was most consistent with the response mediated by the XA21 endodomain and not the BRI1 ectodomain.

Another chimera study compared the responses of receptors consisting of ectodomain and endodomain exchanges between EFR and WAK1. WAK1 recognizes oligogalacturonides (OGs) released from damaged plant cell walls (Decreux & Messiaen, 2005; Decreux et al., 2006; Cabrera et al., 2008). Elf18 treated wild-type plants and OG treated plants expressing a WAK1:EFR (WEG) chimeric RLK both produced ROS, ethylene and expressed the EFR-induced genes (At3g22270 and At4g37640) while EFR:WAK1 (EWAK) expressing plants did not. Instead, EWAK plants retained WAK1-like responses by producing ROS but not ethylene in response to OGs (Ferrari et al., 2008; Brutus et al., 2010). WEG and EWAK responses were therefore most consistent with the response conferred by the respective endodomain portion of each fusion protein. Another study showed that fusing the XA21 endodomain to the fungal chitin RLP CEBiP (Kaku et al., 2006) (CRXA-1, and CRXA-3 in the original publication) conferred a more robust immune response to fungal infection by *Magnaporthe oryzae* than when expressing or overexpressing CEBiP alone (Kishimoto et al., 2010). These results suggested

that the XA21 endodomain was responsible for conferring the enhanced immune response to *M. oryzae*. Together, these studies indicate that the endodomain of several immune receptors dictate the specific signaling events that lead to disease resistance in whole plants or defense responses in plant cells. These studies also suggest chimeras carrying the XA21 endodomain, when treated with the appropriate ligand, can initiate an immune response similar to that mediated by the full-length receptor XA21.

To further explore the function and specificity of the XA21 endodomain and ectodomain, we previously generated transgenic rice lines expressing EFR, tagged with green fluorescent protein (EFR:GFP), or a chimeric EFR:XA21 protein, consisting of the EFR ectodomain and the XA21 transmembrane and intracellular domain, tagged with GFP (EFR:XA21:GFP). The maize ubiquitin promoter drove expression for both *EFR:GFP* and *EFR:XA21:GFP* transgenes (Schwessinger et al., 2015a). Both *EFR:GFP* and *EFR:XA21:GFP* rice plants were susceptible to *Xoo* strain PXO99A and conferred partial resistance to weakly virulent strains, which suggested EF-Tu from *Xoo* was still recognized by *EFR:GFP* rice (Schwessinger et al., 2015a). These studies suggested that although both receptors were capable of recognizing EF-Tu, they were still unable to initiate a robust immune response to PXO99A. As noted in the paper's discussion, these results were counterintuitive based on earlier domain swap studies that indicated that the endodomain dictates immune signaling and disease resistance (He et al., 2000; Brutus et al., 2010; Albert et al., 2010; Kishimoto et al., 2010).

Although it is unclear why the EFR and EFR:XA21 study conflicted with findings from previous chimeric receptor studies, there are several possibilities to explain these discrepancies. In the case of the EFR:WAK1 and WAK1:EFR study, it could be that the type of kinase domain dictated the distinct signaling mediated by each chimeric receptor because the WAK1 and EFR kinase domains belong to different kinase classes. The WAK1 kinase domain contains an arginine (R) aspartate (D) motif while the EFR kinase domain is non-RD, as described above. The non-RD kinases are almost always associated with immune responses in plants and animals and are likely regulated differently than RD kinases (Dardick & Ronald, 2006; Ronald & Beutler, 2010; Dardick, Schwessinger & Ronald, 2012). Thus, the presence of the non-RD domain may dictate an immune response when appropriately activated and the presence of the RD domain may specify a WAK1-like response.

For both BRI1:XA21 and CEBiP:XA21 studies, it is possible that the origin of the kinase domain from XA21 was less important than the fact that the kinase belonged to the non-RD class. For example, it is unclear if fusing BRI1 or CEBiP to other non-RD kinases, such as the kinases from EFR or OsFLS2, would have produced similar results (Takai et al., 2008).

Previous studies have shown that the XA21 ectodomain plays a critical role in the immune response. For example, the *Xa21D* paralog, which lacks a transmembrane and intracellular domain, confer partial resistance to *Xoo* (Wang et al., 1998). Unlike *Xa21*, *Xa21D* only encodes an ectodomain that is nearly identical to the XA21 ectodomain, differing only in 15 amino acid residues compared to the XA21 ectodomain. Similarly, expression of a catalytically inactive variant of XA21, carrying a mutation in the catalytic domain of the kinase (K736E), in rice maintained partial resistance to *Xoo* (Andaya & Ronald, 2003).

Together, these studies indicate that the XA21 ectodomain is sufficient to confer partial resistance to *Xoo*, even in the absence of a functional kinase domain.

To further explore the function and importance of the XA21 ectodomain, we generated transgenic rice lines expressing a chimeric protein containing the XA21 ectodomain fused to the EFR transmembrane and intracellular domain, tagged with GFP (XA21:EFR:GFP) (Holton *et al.*, 2015). We found that XA21:EFR:GFP rice display robust resistance to *Xoo* strain PXO99A. We also show that XA21:EFR:GFP was specifically activated by RaxX as measured by bacterial infection, defense response gene expression and ROS production (Pruitt *et al.*, 2015; Schwessinger *et al.*, 2015b; Wei *et al.*, 2016). These results indicate that the XA21 ectodomain and its recognition of RaxX specify robust resistance to *Xoo* even in the absence of the XA21 endodomain.

## MATERIALS AND METHODS

### Plant material and methods

Rice seeds were germinated on water-soaked filter paper for 5–7 days at 28 °C and then transplanted into 2.6-l pots. Plants were grown in an approximately 80/20 (sand/peat) soil mixture in an environmentally-controlled greenhouse with temperature set between 28 and 30 °C with 75–80% humidity.

### Transgenic rice production

The *Xa21:EFR:GFP* (XA21 aa residues 1–650 fused to EFR aa residues 650–1,031) binary vector used in rice transformation was described previously (Holton *et al.*, 2015). Transgenic Kitaake plants expressing the *Xa21:EFR:GFP* transgene, under control of the maize ubiquitin promoter, were generated by the UC Davis Plant Transformation Facility as described previously (Hiei *et al.*, 1994). pCAMBIA1300 binary vectors carrying the *Xa21:EFR:GFP* construct were transformed into Kitaake calli by *Agrobacterium*-mediated transformation. Regenerated plants were selected on hygromycin. The presence of the transgene was confirmed in each generation by PCR using transgene-specific primers (Table S1).

### Bacterial infection of rice plants

*Xoo* isolates PXO99A, PXO99A $\Delta$ *raxX*, and PXO99A $\Delta$ *raxX*(*raxX*) (Pruitt *et al.*, 2015) were plated on peptone sucrose agar plates for three days. *Xoo* was suspended in water to approximately  $5 \times 10^8$  colony forming units (CFU)/mL for inoculation. Greenhouse-grown plants were transported into environmentally-controlled growth chambers at the four-week-old stage. Chamber conditions were set to 26 °C, 85% humidity with 12 h light/dark cycles. Plants were acclimated to the chamber conditions for two to three days before scissor inoculation (Kauffman *et al.*, 1973).

### Segregation analysis

The presence of each transgene was identified using PCR genotyping using genomic DNA templates and transgene-specific primers (Table S1). Chi-square tests were used to determine possible multiple transgene insertions.

### Gene expression analysis by qRT-PCR

Total RNA was extracted from detached leaves frozen in liquid nitrogen and powdered using a Qiagen tissuelyser. RNA was extracted from powdered tissue using TRI Reagent and precipitated with isopropanol. RNA was DNase treated using the TURBO DNase kit from Life Technologies, Carlsbad, CA USA. RNA concentrations were normalized to the lowest sample concentration in each experiment. cDNA was synthesized from 2  $\mu$ g of total RNA using the High Capacity cDNA Reverse Transcription Kit by Life Technologies, Carlsbad, CA USA. Gene expression changes were determined by  $\Delta\Delta$ Ct method ([Livak & Schmittgen, 2001](#)) normalizing gene expression to Actin (*LOC\_Os03g50885*) and using mock treated samples as the reference for stress gene expression. Quantitative real time PCR (qRT-PCR) was performed using a Bio-Rad CFX96 Real-Time System coupled to a C1000 Thermal Cycler (Bio-Rad, Hercules, CA USA) using the Bio-Rad SsoFast EvaGreen Supermix. qRT-PCR primer pairs used are described in [Table S1](#). qRT-PCR reactions were run for 40 cycles with annealing and amplification at 62 °C for 5 s and denaturation at 95 °C for 5 s. Single melting curves were observed for all primer pairs used indicating negligible off-target amplification.

### Western blot analysis for protein expression

Anti-GFP (Santa Cruz Biotech, Santa Cruz, CA USA) was used to detect EFR:GFP, EFR:XA21:GFP, XA21:GFP, and XA21:EFR:GFP. Secondary anti-mouse antibodies (Santa Cruz Biotech) conjugated to horseradish peroxidase were used in combination with chemiluminescence substrates (Thermo, Waltham, MA, USA) to detect proteins on a Biorad ChemiDoc.

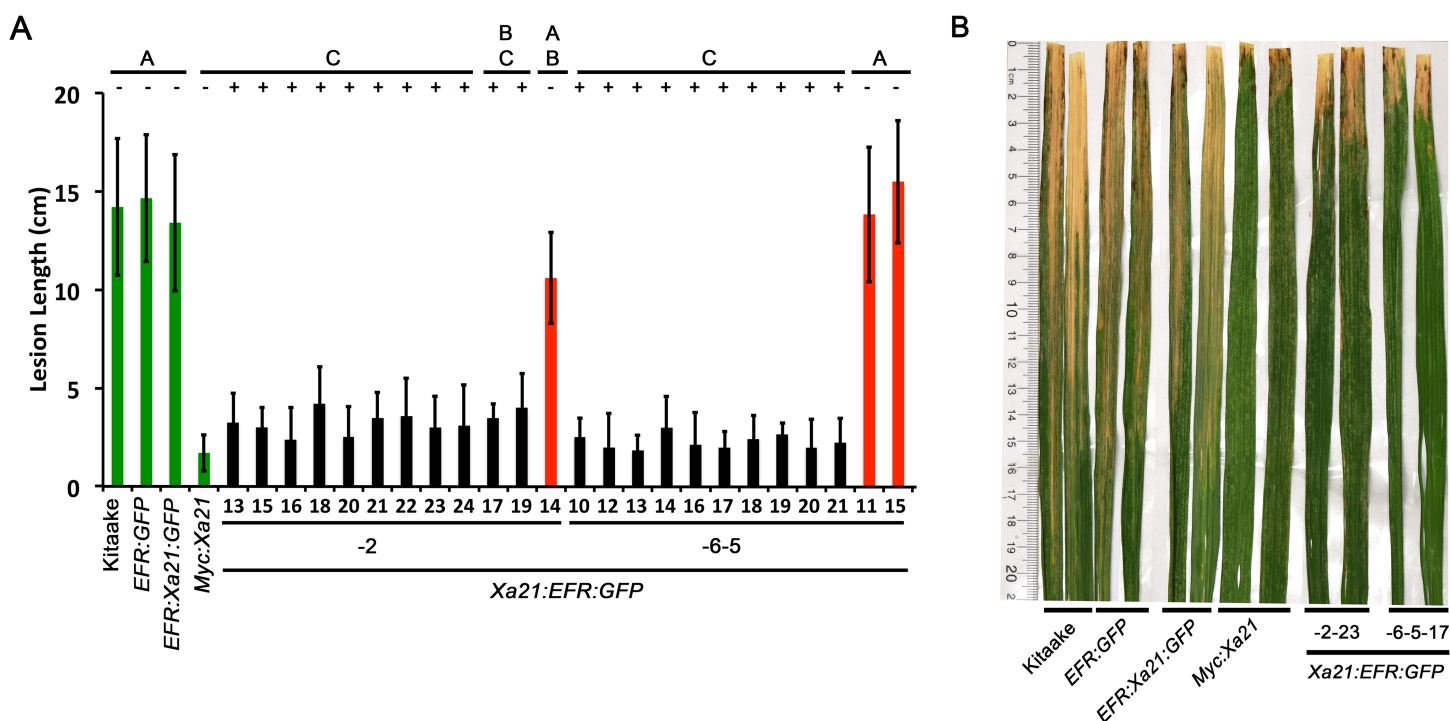
### Reactive oxygen species production

Leaves of three- to four-week-old rice plants were cut longitudinally along the mid vein and then into 1–1.5 mm thick pieces. Leaf pieces were floated on sterile water overnight. The following morning, two leaf pieces were transferred into one well of a 96-well white plate containing 100  $\mu$ l elicitation solution (20  $\mu$ M L-012 (Wako Chemicals, Neuss, Germany), 2  $\mu$ g/mL HRP (Sigma, St. Louis, MO, USA)). A total of 500 nM of elf18 (*Escherichia coli*) or RaxX21-sY peptides were used for treatments. ROS production was measured for 0.5 s per reading with a high sensitivity plate reader (TriStar; Berthold, Bad Wildbad, Germany).

## RESULTS

### Transgenic rice expressing the XA21:EFR chimeric receptor display robust resistance to *Xoo*

We produced transgenic rice lines expressing an *Xa21:EFR:GFP* chimeric construct to test whether the XA21 ectodomain confers resistance to *Xoo* when fused to the EFR cytoplasmic domain. This construct encodes the XA21 ectodomain (XA21 residues 1–650) fused to the EFR transmembrane, juxtamembrane, and cytoplasmic domain (EFR residues 651–1,031) with a carboxyl-terminal GFP fusion ([Holton et al., 2015](#)) expressed under the maize ubiquitin promoter. We generated 10 independent transgenic  $T_0$  lines and inoculated the



**Figure 1** Rice expressing *Xa21:EFR:GFP* are resistant to *Xoo* infection. (A) The bar graph represents the average lesion length observed on rice plants infected with *Xoo*. Control lines used were Kitaake, *EFR:GFP*, *EFR:XA21:GFP*, and *Myc:XA21* rice (green bars). Experimental samples include individuals PCR positive for the *Xa21:EFR:GFP* transgene (black bars) from line 2 and 6 and PCR negative individuals (red bars). Five-week-old greenhouse-grown plants were scissor inoculated with PXO99A ( $5 \times 10^8$  CFU/mL) and disease lesions were scored approximately two weeks post inoculation. Error bars represent standard deviation from the mean lesion length. Mean lesion lengths are the average of lesion measurements from individual leaves from the same plant ( $n \geq 3$ ). Black lines and letters above the graph represent statistical groupings using the Tukey–Kramer HSD test. Different letters indicate significant differences ( $p < 0.05$ ). This experiment was repeated at least three times with similar results. (B) Photograph of select leaves from the same experiment in A. The photograph shows Kitaake, *EFR:GFP*, *EFR:XA21:GFP*, *Myc:XA21*, *Xa21:EFR:GFP* individual -2-23, and -6-5-17 leaves infected with *Xoo* and was taken approximately two weeks after inoculation. Nicholas Thomas provided the photograph. [Full-size DOI: 10.7717/peerj.4456/fig-1](https://doi.org/10.7717/peerj.4456/fig-1)

plants with *Xoo* using a leaf clipping method followed by lesion length measurements, which allows a comparable but more rapid assessment of resistance than bacterial population counting. We found that eight of these lines (lines 2, 3, 4, 5, 6, 7, 9, and 10) displayed enhanced resistance to *Xoo* compared with the Kitaake parent line (Fig. S1).

To assess if the resistance phenotype was transmitted to the next generation, we self-pollinated five of the eight  $T_0$  lines (lines 2, 4, 5, 6, 7) and collected  $T_1$  seed. These  $T_1$  plants, as well as rice plants expressing and lacking *Xa21* as controls, were inoculated with *Xoo* and assessed for resistance by measuring the lengths of disease-induced lesions. We observed that  $T_1$  individuals that were PCR positive for the transgene in lines 2, 4, 5, and 6 co-segregated with resistance to *Xoo* (PCR positive to negative ratios 8:4, 21:0, 8:7, and 16:5, respectively). Lesion length averages were approximately 5 cm in resistant individuals compared to approximately 13 cm for susceptible controls (Fig. 1 and Fig. S2). All  $T_1$  individuals from line 4 were PCR positive for *Xa21:EFR:GFP* (21:0) which could have been from multiple transgene insertions ( $X^2(1) = 1.4$ ,  $p = 0.24$ ) and were resistant to

*Xoo*. All  $T_1$  individuals from line 7 were also PCR positive for the *Xa21:EFR:GFP* transgene. However, these plants showed varying degrees of resistance (Fig. S2).

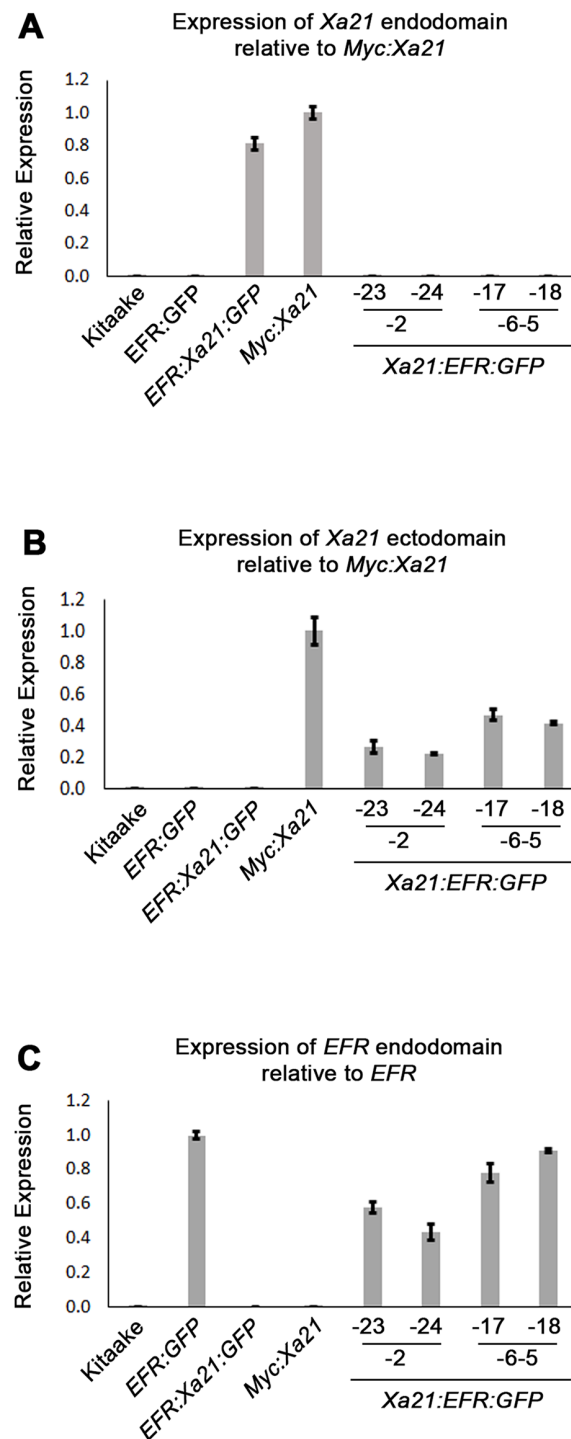
To determine if *Xoo* resistance in *Xa21:EFR:GFP* plants is mediated through XA21 perception of RaxX, we infected two different  $T_1$  progeny from two different -2 lines (-2-13 and -2-19) and  $T_2$  progeny from line -6-5-1 with wild-type PXO99A, PXO99A  $\Delta$ *raxX* mutants ( $\Delta$ *raxX*) that evade full-length XA21-mediated immunity, and  $\Delta$ *raxX* strains complemented with *raxX* ( $\Delta$ *raxX(raxX)*) that do not evade XA21-mediated immunity (Pruitt et al., 2015). We found that Kitaake plants were susceptible to all strains used (average lesion lengths approximately 20 cm), whereas *Xa21:GFP* control plants were susceptible to  $\Delta$ *raxX* infections with approximately 19 cm average lesion lengths and resistant to WT PXO99A and complemented  $\Delta$ *raxX(raxX)* strains with approximately 7 cm average lesion lengths consistent with previous findings (Pruitt et al., 2015). For segregants carrying the *Xa21:EFR:GFP* transgene, plants were significantly more resistant to WT PXO99A, with approximately 5 cm average lesion lengths, compared to  $\Delta$ *raxX* infections that developed lesions approximately 17 cm long. *Xa21:EFR:GFP* segregants carrying the transgene were significantly more resistant to  $\Delta$ *raxX(raxX)* with approximately 8 cm average lesion lengths compared to null segregants that developed significantly longer average lesion lengths of approximately 17 cm (Fig. S3). These results indicate that the XA21:EFR:GFP protein perceives RaxX.

For subsequent experiments, we focused on two *Xa21:EFR:GFP* lines (-2 and -6) for further molecular characterization. For these experiments,  $T_1$  plants were used for line 2 and  $T_2$  plants were used for line 6 (we self-pollinated  $T_1$  individuals and collected  $T_2$  seed for line 6) to test if similar phenotypes are observable in different lines and in subsequent generations. We found that  $T_2$  individuals from line 6 maintained *Xoo* resistance that segregated with the *Xa21:EFR:GFP* transgene (Fig. 1). Because  $T_1$  and  $T_2$  individuals from lines 2 and 6, respectively, were still segregating for the transgene, we performed experiments on individual plants that carried the *Xa21:EFR:GFP* transgene, selected by PCR genotyping. We used null segregant individuals as controls.

### **The *Xa21:EFR:GFP* chimeric transgene is expressed and XA21:EFR:GFP protein accumulates in stable transgenic lines**

We used qRT-PCR to assess if plants containing the *Xa21:EFR:GFP* transgene express the *Xa21* ectodomain and *EFR* cytoplasmic domain. We assessed transcript levels using domain-specific primers for regions that encode the XA21 ectodomain, XA21 cytoplasmic domain, and the EFR cytoplasmic domain (Table S1) (Figs. 2A–2C). Our results show *Xa21:EFR:GFP*-2-23, -2-24, -6-5-17, and -6-5-18 that carry the transgene specifically express regions encoding the XA21 ectodomain and the EFR cytoplasmic domain. Additionally, these plants do not express regions encoding the XA21 endodomain. Because these plants are not expressing the full-length *Xa21* transcript or endodomain, any immune responses observed in these plants are not mediated by full-length XA21 or the XA21 endodomain.





**Figure 2** *Xa21*:EFR:GFP transcripts are expressed in stable transgenic lines. Bar graphs represent the relative expression of transgenic transcripts. (A) Relative amplification of the *Xa21* endodomain with *Myc:Xa21* rice as the expression reference. (B) Amplification of the *Xa21* endodomain with *Myc:Xa21* rice as the expression reference. (C) Amplification of the *EFR* cytoplasmic domain with *EFR:GFP* rice as the expression reference. Gene expression was measured by quantitative real-time PCR using cDNA amplified from total RNA as a template. Each gene expression measurement is the average of two technical replicates and error bars represent the standard deviation between the two measurements.

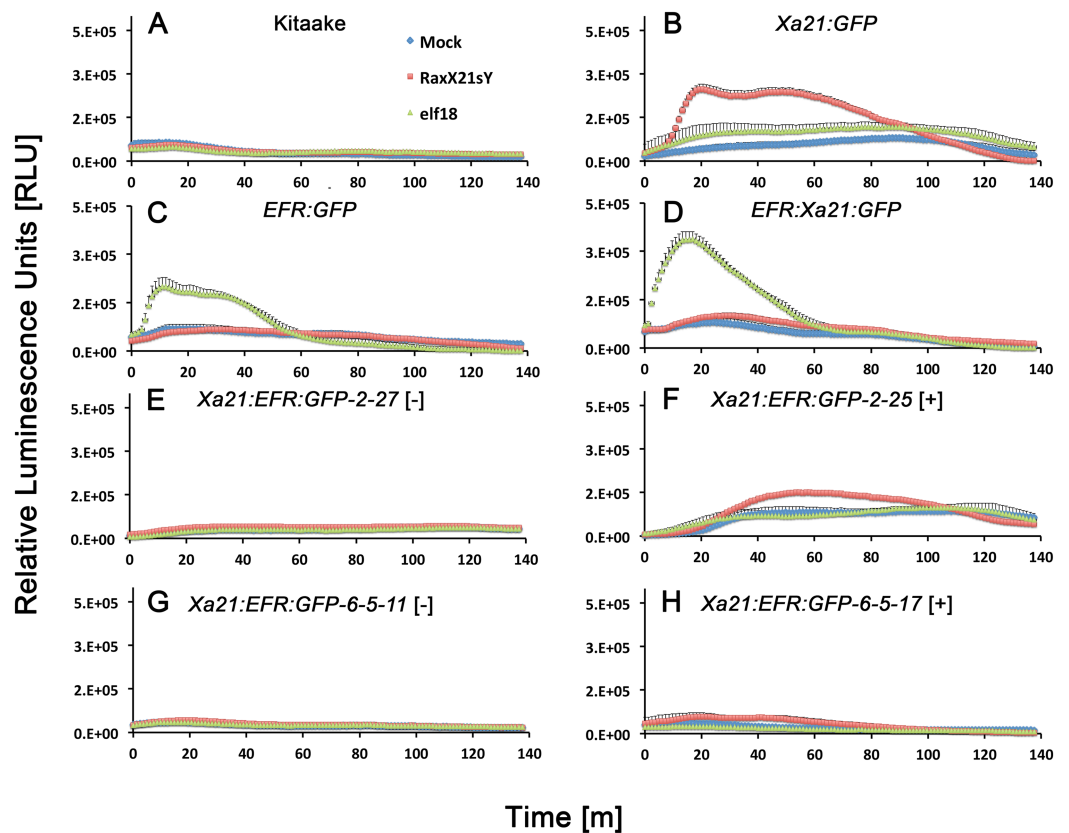
Full-size DOI: 10.7717/peerj.4456/fig-2

In addition to the specific *Xa21:EFR:GFP* transcript, we show that XA21:EFR:GFP protein accumulates in transgenic rice. We performed Western Blot analysis to determine if XA21:EFR:GFP protein accumulates in *Xa21:EFR:GFP* transgenic rice using primary anti-GFP antibodies. Our results show that XA21:EFR:GFP protein is detectable in *Xa21:EFR:GFP-2-28*, *-2-29*, *-6-5-4*, and *-6-5-7* that carry the *Xa21:EFR:GFP* transgene. Wild-type Kitaake and null segregants *Xa21:EFR:GFP-2-32* and *Xa21:EFR:GFP-6-5-6* do not express any GFP tagged protein (Fig. S3). Together, RNA and protein expression indicate that two independent *Xa21:EFR:GFP* transgenic lines express *Xa21:EFR:GFP* transcript and accumulate XA21:EFR:GFP protein.

### RaxX21-sY treated *Xa21:EFR:GFP* rice leaves produce reactive oxygen species and highly express stress-related genes

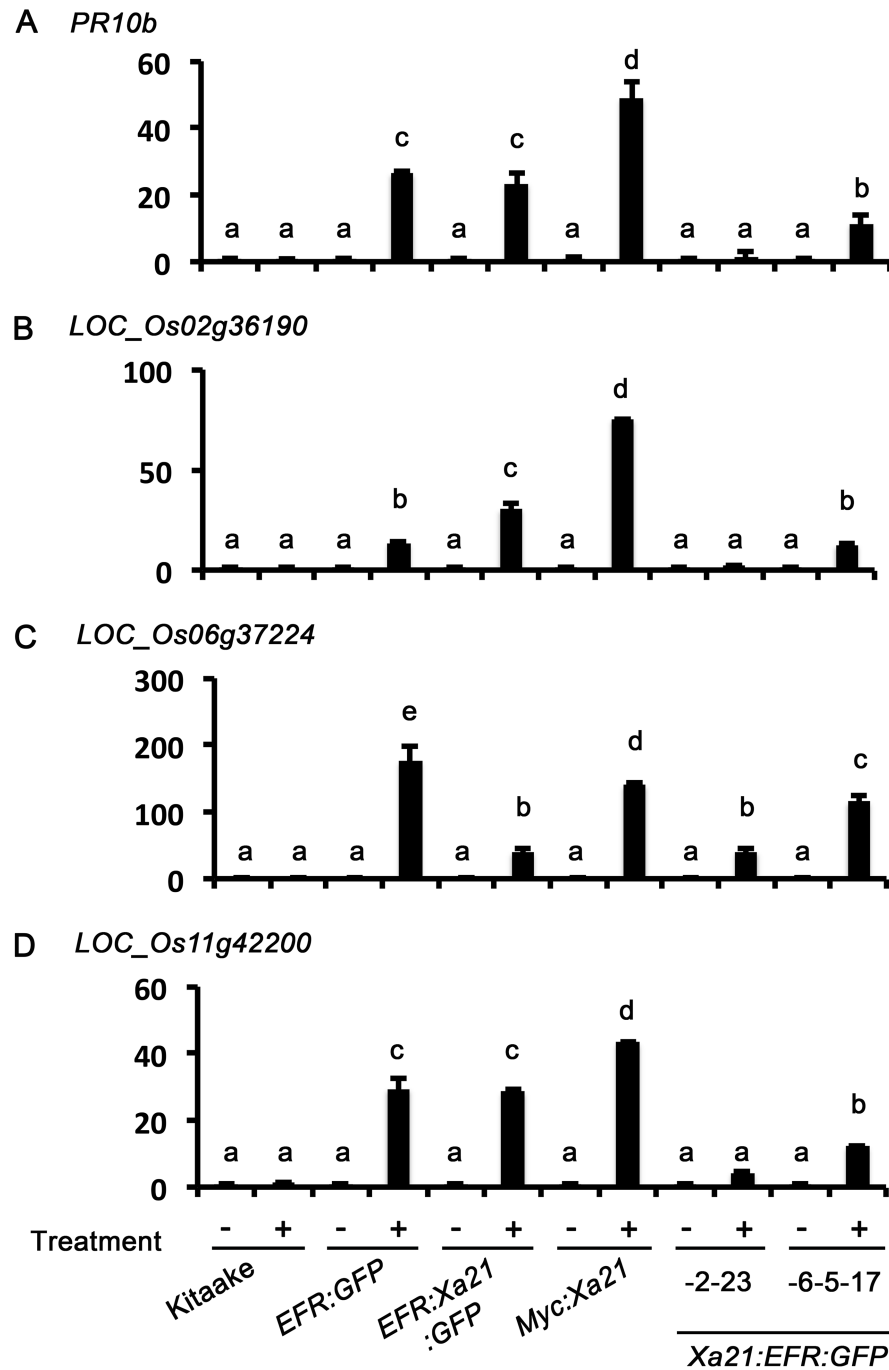
We next assessed if *Xa21:EFR:GFP* rice are able to activate immune responses after RaxX treatments. We used a commercially synthesized, sulfated RaxX peptide, composed of 21 amino acids from the *Xoo* RaxX protein sequence in PXO99A (RaxX21-sY) previously shown to activate XA21-mediated immunity (Pruitt et al., 2015; Wei et al., 2016). Bursts of ROS are commonly measured to assess immune responses because ROS are rapidly produced as a defense response to pathogen attack (Wojtaszek, 1997; Jones & Dangl, 2006; Macho & Zipfel, 2014). We therefore measured ROS production in *Xa21:EFR:GFP* rice after RaxX21-sY treatment to determine if plants carrying the chimeric protein respond similarly to RaxX21-sY treated plants carrying full-length XA21 (Pruitt et al., 2015). *Xa21:EFR:GFP* rice accumulate ROS in response to RaxX21-sY treatments, but not to mock or elf18 treatments (Fig. 3). In addition, we confirmed that RaxX21-sY treated XA21:GFP rice, expressing the full-length XA21 protein tagged with GFP (Fig. S4), accumulate ROS (Fig. 3B). Null segregants did not produce ROS bursts in response to RaxX21-sY treatments (Fig. 3). *EFR:GFP* and *EFR:XA21:GFP* rice responded to elf18, but not to RaxX21-sY, showing that the XA21 ectodomain in full-length XA21 and XA21:EFR:GFP proteins is necessary for RaxX-triggered immune responses (Figs. 3C and 3D).

We next measured stress-related marker gene expression in RaxX21-sY treated *Xa21:EFR:GFP* rice to further characterize the XA21:EFR:GFP-mediated response. We measured the expression of rice defense marker genes *PR10b*, *LOC\_Os02g36190*, *LOC\_Os06g37224*, and *LOC\_Os11g42200*. *PR10b* encodes a putative ribonuclease and is up-regulated upon fungal infection in rice (McGee, Hamer & Hodges, 2001), *LOC\_Os02g36190* is involved in phytoalexin biosynthesis and bacterial blight resistance (Li et al., 2013), *LOC\_Os06g37224* encodes an ent-Kaurene Oxidase up-regulated after ultraviolet light stress (Itoh et al., 2004), and *LOC\_Os11g42200* encodes a laccase precursor protein and is up-regulated after Atrazine herbicide treatment (Huang et al., 2016). The up-regulation of these genes were previously established as markers of the rice stress-response including the XA21-mediated immune response to sulfated RaxX peptides and to *Xoo* infection (Chen et al., 2014; Pruitt et al., 2015; Thomas et al., 2016) using a detached leaf treatment assay. RNA was extracted from detached leaves of four-week-old plants mock treated with water or with 500 nM of RaxX21-sY for 6 h. Gene expression



**Figure 3** Reactive oxygen species accumulate after peptide treatments. Reactive oxygen species (ROS) production after water (mock, blue diamonds), 500 nM RaxX21-sY (red squares), or 500 nM elf18 peptide treatments (green triangles). (A) ROS production in wild-type Kitaaake rice and (B) Xa21:GFP rice, (C) EFR:GFP rice and (D) EFR:Xa21:GFP rice. (E) and (G) ROS production in T1 and T2 null-segregant individuals from Xa21:EFR:GFP line -2 and line -6-5, respectively. (F) and (H) ROS production in T1 and T2 individuals from line -2 and -6-5, respectively, that segregate for the Xa21:EFR:GFP transgene. Each datapoint represents an average of four technical replicate measurements and error bars represent the standard error of the averages. These experiments have been repeated three times with similar results. [Full-size !\[\]\(ba1b80118482ccef74a5d718ca4d7242\_img.jpg\) DOI: 10.7717/peerj.4456/fig-3](https://doi.org/10.7717/peerj.4456/fig-3)

was measured in individuals *Xa21:EFR:GFP-2-23* and *-6-5-17* by quantitative real-time PCR. Higher expression was observed in each of the stress-related genetic markers in RaxX21-sY treated *Myc:Xa21* and *Xa21:EFR:GFP* rice (Figs. 4A–4D). Gene expression was not induced in any mock treated samples or Kitaaake samples treated with RaxX21-sY. Only Individual 2–23 showed higher induction of *LOC\_Os11g42200* and *LOC\_Os06g37224*, and only induction of *LOC\_Os06g37224* was significant (Fig. 4C). Although the magnitude of ROS and stress gene induction in response to RaxX21-sY is relatively lower in *Xa21:EFR:GFP* rice compared to *XA21:GFP* rice (Figs. 3 and 4), both show robust resistance to *Xoo* infection as measured by lesion length progression (Fig. 1) Together, the results from ROS and gene expression experiments suggest that the XA21 ectodomain in XA21:EFR:GFP is sufficient to recognize RaxX and that the EFR endodomain can be substituted for the XA21 endodomain to transduce immune responses after RaxX treatment.



**Figure 4** *Xa21:EFR:GFP* rice express stress-related genes after RaxX21-sY treatment. Gene expression profiles of four stress-related genes, (A) *PR10b*, (B) *LOC\_Os02g36190*, (C) *LOC\_06g37224*, and (D) *LOC\_Os11g42200*. Samples are rice leaves from wild-type Kitaake, *Myc:XA21* rice, and individuals -23 from *Xa21:EFR:GFP* line -2 and individual -17 from line -6-5. Leaves were mock treated with water (-) or with 500 nM RaxX21-sY for *Myc:XA21* and *XA21:EFR* rice and 500 nM elf18 for *EFR:GFP* and *EFR:XA21:GFP* rice (+). Kitaake was treated with both RaxX21-sY and elf18 in these experiments. Letters indicate significant difference in gene expression compared to mock using the Tukey-Kramer HSD test ( $\alpha = 0.05$ ). Expression levels are normalized to mock treatment of the same line. Bars depict average expression level relative to actin expression  $\pm$  standard error of three technical replicates. This experiment was repeated twice with similar results. [Full-size !\[\]\(5f471a71b78d7676bc356df190b88ab4\_img.jpg\) DOI: 10.7717/peerj.4456/fig-4](https://doi.org/10.7717/peerj.4456/fig-4)

## DISCUSSION

Here we show that the ectodomain of XA21 is sufficient to confer full resistance to *Xoo* strain PXO99A when fused to the intracellular domain of the *Arabidopsis* immune receptor EFR (Fig. 1 and Figs. S1–S3). We previously demonstrated that a functional EFR:XA21:GFP is not able to confer resistance to *Xoo* when expressed in rice. Together these results suggest that the XA21 extracellular domain and the recognition of RaxX are the key properties that dictate the robust immune response of XA21. Both the native XA21 endodomain as well as the EFR endodomain fused to the XA21 ectodomain appear to be interchangeable as both XA21 and EFR kinases can confer robust resistance when fused with the XA21 ectodomain. This result slightly contrasts with previous domain swap studies that indicated that the endodomains of immune receptors were the defining properties of the immune receptor responses (He et al., 2000; Brutus et al., 2010; Albert et al., 2010; Kishimoto et al., 2010). Although XA21 mutants and XA21 derivatives that lack a functional kinase domain maintain partial resistance, it appears that a functional kinase domain is required for robust resistance (Wang et al., 1998; Andaya & Ronald, 2003).

Despite the evidence that rice expressing XA21:EFR:GFP are resistant to *Xoo*, it is unclear why plants expressing the reciprocal EFR:XA21:GFP protein are susceptible to *Xoo* (Schwessinger et al., 2015a). Previous results indicate that elf18 and RaxX21-sY have similar EC<sub>50</sub> values for immune activation (Schwessinger et al., 2015a; Pruitt et al., 2015), but it is unclear if RaxX and EF-Tu are available at similar levels during infection, and it is also possible that *Xoo* masks EF-Tu, preventing optimal EFR ectodomain recognition. We hypothesize that the XA21 ectodomain is critical for conferring robust resistance because it interacts with additional rice-specific signaling components that the EFR ectodomain is unable to bind. In partial support of this hypothesis, we previously showed that the EFR kinase domain does not interact with some of the previously identified XA21 kinase domain signaling components, including the negative regulator XB15 and positive regulator XB3 (Schwessinger et al., 2015a). It is therefore possible that unidentified positive regulators of XA21-mediated immunity that interact with the XA21 ectodomain do not associate with the EFR ectodomain. Future studies might be aimed at identifying these elusive ectodomain-specific signaling partners to better understand XA21-mediated immunity.

## ACKNOWLEDGEMENTS

We would like to thank Dr. Nicholas Holton and Prof. Dr. Cyril Zipfel from the Sainsbury Laboratory for providing the *Xa21:EFR:GFP* construct.

## ADDITIONAL INFORMATION AND DECLARATIONS

### Funding

This project was funded through NIH grant GM59962 and the NSF PGRP grant IOS-1237975. Benjamin Schwessinger was supported by a Human Frontiers Science Program long-term postdoctoral fellowship (LT000674/2012) and a Discovery Early Career Research Award (DE150101897). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Grant Disclosures

The following grant information was disclosed by the authors:

NIH grant: GM59962.

NSF PGRP grant: IOS-1237975.

Human Frontiers Science Program long-term postdoctoral fellowship: LT000674/2012.

Discovery Early Career Research Award: DE150101897.

### Competing Interests

Pamela C. Ronald is an Academic Editor for PeerJ.

### Author Contributions

- Nicholas C. Thomas conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Nir Oksenberg conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Furong Liu performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Daniel Caddell performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Alina Nalyvayko performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Yen Nguyen performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Benjamin Schwessinger conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Pamela C. Ronald conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

### Data Availability

The following information was supplied regarding data availability:

The data are represented in the Figures and [Supplemental Files](#).

### Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.4456#supplemental-information>.

## REFERENCES

- Albert M, Jehle AK, Mueller K, Eisele C, Lipschis M, Felix G. 2010. Arabidopsis thaliana pattern recognition receptors for bacterial elongation factor Tu and flagellin can be combined to form functional chimeric receptors. *Journal of Biological Chemistry* **285**(25):19035–19042  
DOI [10.1074/jbc.m110.124800](https://doi.org/10.1074/jbc.m110.124800).

- Andaya CB, Ronald PC. 2003.** A catalytically impaired mutant of rice Xa21 receptor kinase confers partial resistance to *Xanthomonas oryzae* pv. *oryzae*. *Physiological and Molecular Plant Pathology* **62**(4):203–208 DOI [10.1016/s0885-5765\(03\)00060-2](https://doi.org/10.1016/s0885-5765(03)00060-2).
- Brutus A, Sicilia F, Macone A, Cervone F, De Lorenzo G. 2010.** A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proceedings of the National Academy of Sciences of the United States of America* **107**(20):9452–9457 DOI [10.1073/pnas.1000675107](https://doi.org/10.1073/pnas.1000675107).
- Cabrera JC, Boland A, Messiaen J, Cambier P, Van Cutsem P. 2008.** Egg box conformation of oligogalacturonides: the time-dependent stabilization of the elicitor-active conformation increases its biological activity. *Glycobiology* **18**(6):473–482.
- Chen X, Zuo S, Schwessinger B, Chern M, Daudi A, Canlas PE, Ruan DL, Zhou X, Wang J, Petzold C, Heazlewood J, Ronald PC. 2014.** An XA21-associated kinase (OsSERK2) regulates immunity by the XA21 and XA3 immune receptors. *Molecular Plant* **7**(5):874–892 DOI [10.1093/mp/ssu003](https://doi.org/10.1093/mp/ssu003).
- Chinchilla D, Bauer Z, Regenass M, Boller T, Felix G. 2006.** The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell* **18**(2):465–476 DOI [10.1105/tpc.105.036574](https://doi.org/10.1105/tpc.105.036574).
- Dardick C, Ronald P. 2006.** Plant and animal pathogen recognition receptors signal through non-RD kinases. *PLoS Pathogens* **2**(1):e2 DOI [10.1371/journal.ppat.0020002](https://doi.org/10.1371/journal.ppat.0020002).
- Dardick C, Schwessinger B, Ronald P. 2012.** Non-arginine-aspartate (non-RD) kinases are associated with innate immune receptors that recognize conserved microbial signatures. *Current Opinion in Plant Biology* **15**(4):358–366 DOI [10.1016/j.pbi.2012.05.002](https://doi.org/10.1016/j.pbi.2012.05.002).
- Decreux A, Messiaen J. 2005.** Wall-associated kinase WAK1 interacts with cell wall pectins in a calcium-induced conformation. *Plant & Cell Physiology* **46**(2):268–278 DOI [10.1093/pcp/pci026](https://doi.org/10.1093/pcp/pci026).
- Decreux A, Thomas A, Spies B, Brasseur R, Van Cutsem P, Messiaen J. 2006.** In vitro characterization of the homogalacturonan-binding domain of the wall-associated kinase WAK1 using site-directed mutagenesis. *Phytochemistry* **67**(11):1068–1079 DOI [10.1016/j.phytochem.2006.03.009](https://doi.org/10.1016/j.phytochem.2006.03.009).
- Felix G, Duran JD, Volko S, Boller T. 1999.** Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant Journal* **18**(3):265–276 DOI [10.1046/j.1365-313x.1999.00265.x](https://doi.org/10.1046/j.1365-313x.1999.00265.x).
- Ferrari S, Galletti R, Pontiggia D, Manfredini C, Lionetti V, Bellincampi D, Cervone F, De Lorenzo G. 2008.** Transgenic expression of a fungal endo-polygalacturonase increases plant resistance to pathogens and reduces auxin sensitivity. *Plant Physiology* **146**(2):669–681 DOI [10.1104/pp.107.109686](https://doi.org/10.1104/pp.107.109686).
- Gómez-Gómez L, Boller T. 2000.** FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular Cell* **5**:1003–1011.
- He Z, Wang ZY, Li J, Zhu Q, Lamb C, Ronald PC, Chory J. 2000.** Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. *Science* **288**(5475):2360–2363 DOI [10.1126/science.288.5475.2360](https://doi.org/10.1126/science.288.5475.2360).
- Hiei Y, Ohta S, Komari T, Kumashiro T. 1994.** Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *Plant Journal* **6**(2):271–282 DOI [10.1046/j.1365-313x.1994.6020271.x](https://doi.org/10.1046/j.1365-313x.1994.6020271.x).
- Holton N, Nekrasov V, Ronald PC, Zipfel C. 2015.** The phylogenetically-related pattern recognition receptors EFR and XA21 recruit similar immune signaling components in monocots and dicots. *PLoS Pathogens* **11**(1):e1004602 DOI [10.1371/journal.ppat.1004602](https://doi.org/10.1371/journal.ppat.1004602).

- Huang MT, Lu YC, Zhang S, Luo F, Yang H. 2016. Rice (*Oryza sativa*) laccases involved in modification and detoxification of herbicides atrazine and isoproturon residues in plants. *Journal of Agricultural and Food Chemistry* **64**(33):6397–6406 DOI [10.1021/acs.jafc.6b02187](https://doi.org/10.1021/acs.jafc.6b02187).
- Itoh H, Tatsumi T, Sakamoto T, Otomo K, Toyomasu T, Kitano H, Ashikari M, Ichihara S, Matsuoka M. 2004. A rice semi-dwarf gene, Tan-Ginbozu (D35), encodes the gibberellin biosynthesis enzyme, ent-kaurene oxidase. *Plant Molecular Biology* **54**(4):533–547 DOI [10.1023/b:plan.0000038261.21060.47](https://doi.org/10.1023/b:plan.0000038261.21060.47).
- Jones JDG, Dangl JL. 2006. The plant immune system. *Nature* **444**(7117):323–329 DOI [10.1038/nature05286](https://doi.org/10.1038/nature05286).
- Kaku H, Nishizawa Y, Ishii-Minami N, Akimoto-Tomiya C, Dohmae N, Takio K, Minami E, Shibuya N. 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proceedings of the National Academy of Sciences of the United States of America* **103**(29):11086–11091 DOI [10.1073/pnas.0508882103](https://doi.org/10.1073/pnas.0508882103).
- Kauffman HE, Reddy APK, Hsiek SPV, Marca SD. 1973. An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. *Plant Disease Management Reports* **57**:537–541.
- Kishimoto K, Kouzai Y, Kaku H, Shibuya N, Minami E, Nishizawa Y. 2010. Perception of the chitin oligosaccharides contributes to disease resistance to blast fungus *Magnaporthe oryzae* in rice. *Plant Journal* **64**(2):343–354 DOI [10.1111/j.1365-3113.2010.04328.x](https://doi.org/10.1111/j.1365-3113.2010.04328.x).
- Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, Felix G. 2004. The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. *Plant Cell* **16**(12):3496–3507 DOI [10.1105/tpc.104.026765](https://doi.org/10.1105/tpc.104.026765).
- Li J, Chory J. 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* **90**(5):929–938 DOI [10.1016/s0092-8674\(00\)80357-8](https://doi.org/10.1016/s0092-8674(00)80357-8).
- Li W, Shao M, Yang J, Zhong W, Okada K, Yamane H, Qian G, Liu F. 2013. OsCYP71Z2 involves diterpenoid phytoalexin biosynthesis that contributes to bacterial blight resistance in rice. *Plant Science* **207**:98–107 DOI [10.1016/j.plantsci.2013.02.005](https://doi.org/10.1016/j.plantsci.2013.02.005).
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta C_T}$  method. *Methods* **25**(4):402–408 DOI [10.1006/meth.2001.1262](https://doi.org/10.1006/meth.2001.1262).
- Macho AP, Zipfel C. 2014. Plant PRRs and the activation of innate immune signaling. *Molecular Cell* **54**(2):263–272 DOI [10.1016/j.molcel.2014.03.028](https://doi.org/10.1016/j.molcel.2014.03.028).
- McGee JD, Hamer JE, Hodges TK. 2001. Characterization of a PR-10 pathogenesis-related gene family induced in rice during infection with *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions* **14**(7):877–896 DOI [10.1094/mpmi.2001.14.7.877](https://doi.org/10.1094/mpmi.2001.14.7.877).
- Pruitt RN, Schwessinger B, Joe A, Thomas N, Liu F, Albert M, Robinson MR, Chan LJG, Luu DD, Chen H, Bahar O, Daudi A, De Vleeschauwer D, Caddell D, Zhang W, Zhao X, Li X, Heazlewood JL, Ruan D, Majumder D, Chern M, Kalbacher H, Midha S, Patil PB, Sonti RV, Petzold CJ, Liu CC, Brodbelt JS, Felix G, Ronald PC. 2015. The rice immune receptor XA21 recognizes a tyrosine-sulfated protein from a gram-negative bacterium. *Science Advances* **1**(6):e1500245 DOI [10.1126/sciadv.1500245](https://doi.org/10.1126/sciadv.1500245).
- Ronald PC, Beutler B. 2010. Plant and animal sensors of conserved microbial signatures. *Science* **330**(6007):1061–1064 DOI [10.1126/science.1189468](https://doi.org/10.1126/science.1189468).
- Schwessinger B, Bahar O, Thomas N, Holton N, Nekrasov V, Ruan D, Canlas PE, Daudi A, Petzold CJ, Singan VR, Kuo R, Chovatia M, Daum C, Heazlewood JL, Zipfel C, Ronald PC. 2015a. Transgenic expression of the dicotyledonous pattern recognition receptor EFR in rice leads to ligand-dependent activation of defense responses. *PLoS Pathogens* **11**(3):e1004809 DOI [10.1371/journal.ppat.1004809](https://doi.org/10.1371/journal.ppat.1004809).



- Schwessinger B, Li X, Ellinghaus TL, Chan LJG, Wei T, Joe A, Thomas N, Pruitt R, Adams PD, Chern MS, Petzold CJ, Liu CC, Ronald PC. 2015b. A second-generation expression system for tyrosine-sulfated proteins and its application in crop protection. *Integrative Biology* 8(4):542–545 DOI [10.1039/c5ib00232j](https://doi.org/10.1039/c5ib00232j).
- Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang B, Zhai WX, Zhu LH, Fauquet C, Ronald P. 1995. A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science* 270(5243):1804–1806 DOI [10.1126/science.270.5243.1804](https://doi.org/10.1126/science.270.5243.1804).
- Takai R, Isogai A, Takayama S, Che F-S. 2008. Analysis of flagellin perception mediated by flg22 receptor OsFLS2 in rice. *Molecular Plant-Microbe Interactions* 21(12):1635–1642 DOI [10.1094/mpmi-21-12-1635](https://doi.org/10.1094/mpmi-21-12-1635).
- Thomas NC, Schwessinger B, Liu F, Chen H, Wei T, Nguyen YP, Shaker IWF, Ronald PC. 2016. XA21-specific induction of stress-related genes following *Xanthomonas* infection of detached rice leaves. *PeerJ* 4:e2446.
- Wang GL, Ruan DL, Song WY, Sideris S, Chen L, Pi LY, Zhang S, Zhang Z, Fauquet C, Gaut BS, Whalen MC, Ronald PC. 1998. Xa21D encodes a receptor-like molecule with a leucine-rich repeat domain that determines race-specific recognition and is subject to adaptive evolution. *Plant Cell* 10:765–779 DOI [10.2307/3870663](https://doi.org/10.2307/3870663).
- Wei T, Chern M, Liu F, Ronald PC. 2016. Suppression of bacterial infection in rice by treatment with a sulfated peptide. *Molecular Plant Pathology* 17(9):1493–1498 DOI [10.1111/mpp.12368](https://doi.org/10.1111/mpp.12368).
- Wojtaszek P. 1997. Oxidative burst: an early plant response to pathogen infection. *Biochemical Journal* 322(3):681–692 DOI [10.1042/bj3220681](https://doi.org/10.1042/bj3220681).
- Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T, Felix G. 2006. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* 125(4):749–760 DOI [10.1016/j.cell.2006.03.037](https://doi.org/10.1016/j.cell.2006.03.037).