

1 **The Rice XA21 Ectodomain Fused to the Arabidopsis EFR Cytoplasmic Domain**
2 **Confers Resistance to *Xanthomonas oryzae* pv. *oryzae***

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14

15 **Summary**

16 • Rice (*Oryza sativa*) plants expressing the XA21 cell surface receptor kinase are
17 resistant to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) infection. We previously
18 demonstrated that expressing a chimeric protein containing the EFR
19 (ELONGATION FACTOR Tu RECEPTOR) ectodomain and the XA21
20 endodomain (EFR:XA21) in rice does not confer robust resistance to *Xoo*.

21 • To test if the XA21 ectodomain is required for *Xoo* resistance, we produced

22 transgenic rice lines expressing a chimeric protein consisting of the XA21
23 ectodomain and EFR endodomain (XA21:EFR) and inoculated these lines with
24 *Xoo*. We also tested if the XA21:EFR rice plants respond to a synthetic sulfated
25 21 amino acid derivative (RaxX21-sY) derived from the activator of XA21-
26 mediated immunity, RaxX.

27 ● We found that five independently transformed XA21:EFR rice lines displayed
28 resistance to *Xoo* as measured by lesion length analysis, and showed that five
29 lines express markers of the XA21 defense response (generation of reactive
30 oxygen species and defense response gene expression) after treatment with
31 RaxX21-sY.

32 ● Our results indicate that expression of the XA21:EFR chimeric receptor in rice
33 confers resistance to *Xoo*. These results suggest that the endodomain of the
34 EFR and XA21 immune receptors are interchangeable and the XA21 ectodomain
35 is the key determinant conferring robust resistance to *Xoo*.

36

37 **Introduction**

38 Plant cell surface immune receptors confer defense against pathogen infection.
39 Cell surface mediated immunity in plants is mainly conferred by receptor like proteins
40 (RLPs) and receptor like kinases (RLKs) that recognize pathogen associated molecular
41 patterns (PAMPs) (Jones and Dangl 2006; Macho and Zipfel 2014). Three well-studied
42 cell-surface RLKs that confer resistance to bacterial pathogens include FLAGELLIN
43 SENSING2 (FLS2; At5G6330) (Gómez-Gómez and Boller 2000), EF-TU RECEPTOR

44 (EFR; At5g20480) (Zipfel et al. 2006) from *Arabidopsis* and XA21 (U37133) from *Oryza*
45 *longistaminata* (Song et al. 1995). The identification of the microbial molecules
46 recognized by these three receptors have enhanced in depth characterization of their
47 functional properties. The FLS2 receptor binds the flg22 peptide derived from bacterial
48 Flagellin (Felix et al. 1999; Gómez-Gómez and Boller 2000; Chinchilla et al. 2006). EFR
49 recognizes the elf18 peptide derived from the bacterial Elongation Factor Thermo-
50 unstable protein (EF-Tu) (Kunze et al. 2004; Zipfel et al. 2006). XA21 recognizes the
51 sulfated RaxX (required for activation of Xa21-mediated immunity X) protein produced
52 by *Xoo* (Pruitt et al. 2015). Although these receptors specifically recognize different
53 molecules, they share similar domain structures including ectodomains containing
54 leucine rich repeats and endodomains containing intracellular kinases of the non-
55 arginine aspartate (non RD) class (Dardick and Ronald, 2006).

56 Domain swap studies between cell-surface receptors have led to the hypothesis
57 that the nature of the endodomains is the primary determinant dictating the specific
58 disease resistance outcome. For example, studies of a chimeric receptor generated by
59 fusion of the *Arabidopsis* BRASSINOSTEROID-INSENSITIVE1 (BRI1) receptor, which
60 recognizes brassinosteroid hormones, to the XA21 endodomain (BRI1:XA21) (Li and
61 Chory 1997) indicated that the chimeric receptor could be activated by brassinosteroid
62 treatment. Rice cells expressing BRI1:XA21 (NRG-1 in the original publication) and
63 treated with brassinosteroid initiated cell death, produced ROS, and expressed stress-
64 related genes. The stress-related symptoms were attributed to the activation of the
65 XA21 endodomain because the full-length BRI1 receptor does not induce the same

66 stress-related symptoms as BRI1:XA21 (He et al. 2000). These results suggested that
67 the XA21 endodomain was activated upon BRI1 recognition of brassinosteroid and that
68 the specific type of response was most consistent with the response mediated by the
69 XA21 endodomain and not the BRI1 ectodomain.

70 Another chimera study compared the responses of receptors consisting of
71 ectodomain and endodomain exchanges between EFR and WAK1. WAK1 recognizes
72 oligogalacturonides (OGs) released from damaged plant cell walls (A. Decreux and
73 Messiaen 2005; Annabelle Decreux et al. 2006; Cabrera et al. 2008). Elf18 treated wild-
74 type plants and OG treated plants expressing a WAK1:EFR (WEG) chimeric RLK both
75 produced ROS, ethylene and expressed the EFR-induced genes (At3g22270 and
76 At4g37640) while EFR:WAK1 (EWAK) expressing plants did not. Instead, EWAK plants
77 retained WAK1-like responses by producing ROS but not ethylene in response to OGs
78 (Brutus et al. 2010; Ferrari et al. 2008). WEG and EWAK responses were therefore
79 most consistent with the response conferred by the respective endodomain portion of
80 each fusion protein. Another study showed that fusing the XA21 endodomain to the
81 fungal chitin receptor like protein CEBiP (Kaku et al. 2006) (CRXA-1, and CRXA-3 in the
82 original publication) conferred a more robust immune response to fungal infection by
83 *Magnaporthe oryzae* than when expressing or overexpressing CEBiP alone (Kishimoto
84 et al. 2010). These results suggested that the XA21 endodomain was responsible for
85 conferring the enhanced immune response to *M. oryzae*. Together, these studies
86 indicate that the endodomain of several immune receptors dictate the specific signaling
87 events that lead to disease resistance in whole plants or defense responses in plant

88 cells. These studies also suggest chimeras carrying the XA21 endodomain, when
89 treated with the appropriate ligand, can initiate an immune response similar to that
90 mediated by the full-length receptor XA21.

91 To further explore the function and specificity of the XA21 endodomain and
92 ectodomain, we previously generated transgenic rice lines expressing EFR, tagged with
93 green fluorescent protein (EFR:GFP), or a chimeric EFR:XA21 protein, consisting of the
94 EFR ectodomain and the XA21 transmembrane and intracellular domain, tagged with
95 GFP (EFR:XA21:GFP) (Schwessinger, Bahar, et al. 2015). Both *EFR:GFP* and
96 *EFR:XA21:GFP* rice plants were susceptible to *Xoo* strain PXO99A and conferred
97 partial resistance to weakly virulent strains (Schwessinger, Bahar, et al. 2015). These
98 studies suggested that although both receptors were capable of recognizing EF-Tu,
99 they were still unable to initiate a robust immune response to PXO99A. As noted in the
100 paper's discussion, these results were counterintuitive based on earlier domain swap
101 studies that indicated that the endodomain dictates immune signaling and disease
102 resistance (He et al. 2000; Brutus et al. 2010; Kishimoto et al. 2010; Albert et al. 2010).

103 Although it is unclear why the EFR and EFR:XA21 study conflicted with findings
104 from previous chimeric receptor studies, there are several possibilities to explain these
105 discrepancies. In the case of the EFR:WAK1 and WAK1:EFR study, it could be that the
106 type of kinase domain dictated the distinct signaling mediated by each chimeric receptor
107 because the WAK1 and EFR kinase domains belong to different kinase classes. The
108 WAK1 kinase domain contains an arginine (R) aspartate (D) motif while the EFR kinase
109 domain is non-RD, as described above. The non-RD kinases are almost always

110 associated with immune responses in plants and animals and are likely regulated
111 differently than RD kinases (Christopher Dardick and Ronald 2006; Ronald and Beutler
112 2010; Chris Dardick, Schwessinger, and Ronald 2012). Thus, the presence of the non-
113 RD domain may dictate an immune response when appropriately activated and the
114 presence of the RD domain may specify a WAK1-like response.

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

120 Previous studies have shown that the XA21 ectodomain plays a critical role in the
121 immune response. For example, the *Xa21D* paralog, which lacks a transmembrane and
122 intracellular domain, confer partial resistance to *Xoo* (Wang et al. 1998). Unlike *Xa21*,
123 *Xa21D* only encodes an ectodomain that is nearly identical to the XA21 ectodomain,
124 differing only in 15 amino acid residues compared to the XA21 ectodomain. Similarly,
125 expression of a catalytically inactive variant of XA21, carrying a mutation in the catalytic
126 domain of the kinase (K736E), in rice maintained partial resistance to *Xoo* (Cynthia B.
127 Andaya and Ronald 2003). Together, these studies indicate that the XA21 ectodomain
128 is sufficient to confer partial resistance to *Xoo*, even in the absence of a functional
129 kinase domain.

130 To further explore the function and importance of the XA21 ectodomain, we
131 generated transgenic rice lines expressing a chimeric protein containing the XA21

132 ectodomain fused to the EFR transmembrane and intracellular domain, tagged with
133 GFP (XA21:EFR:GFP) (Holton et al. 2015). We found that *XA21:EFR:GFP* rice display
134 robust resistance to *Xoo* strain PXO99A. We also show that *XA21:EFR:GFP* was
135 specifically activated by RaxX as measured by defense response gene expression and
136 ROS production (Pruitt et al. 2015; Schwessinger, Li, et al. 2015; Wei et al. 2016).
137 These results indicate that the XA21 ectodomain and its recognition of RaxX specify
138 robust resistance to *Xoo* even in the absence of the XA21 endodomain.

139

140 **Materials and Methods**

141 *Plant material and methods*

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

146

147 *Transgenic rice production*

148 The *Xa21:EFR:GFP* (XA21 aa residues 1-650 fused to EFR aa residues 650-1031)
149 binary vector used in rice transformation was described previously (Holton et al. 2015).
150 Transgenic Kitaake plants expressing the *Xa21:EFR:GFP* transgene were generated by
151 the UC Davis Plant Transformation Facility as described previously (Hiei et al. 1994).
152 pCAMBIA1300 binary vectors carrying the *Xa21:EFR:GFP* construct were transformed
153 into Kitaake calli by *Agrobacterium*-mediated transformation. Regenerated plants were

154 selected on hygromycin. The presence of the transgene was confirmed in each
155 generation by PCR using transgene specific primers (Table S1).

156

157 *Segregation analysis (genotyping, infection, plant conditions)*

158 *Xoo* isolates (PXO99A strain) were plated on peptone sucrose agar plates for 3 days.

159 *Xoo* was suspended in water to approximately 5×10^8 colony forming units (CFU)/ mL.

160 Greenhouse-grown plants were transported into environmentally controlled growth

161 chambers at the 4 week-old stage. Chamber conditions were set to 26°C, 85% humidity

162 with 12h light/dark cycles. Plants were acclimated to the chamber conditions for 2–3

163 days before scissor inoculation (Kauffman et al. 1973). The presence of the transgene

164 was identified using PCR genotyping with transgene-specific primers (Table S1).

165

166 *Gene expression analysis by qRT-PCR*

167 Total RNA was extracted from detached leaves frozen in liquid nitrogen and powdered

168 using a Qiagen tissuelyser. RNA was extracted from powdered tissue using TRI

169 Reagent and precipitated with isopropanol. RNA was DNase treated using the TURBO

170 DNase kit from Life Technologies. RNA concentrations were normalized to the lowest

171 sample concentration in each experiment. cDNA was synthesized from 2µg of total RNA

172 using the High Capacity cDNA Reverse Transcription Kit by Life Technologies. Gene

173 expression changes were determined by $\Delta\Delta\text{Ct}$ method (Livak and Schmittgen 2001)
174 normalizing gene expression to Actin (*LOC_Os03g50885*) and using mock treated
175 samples as the reference for stress gene expression. Quantitative real time PCR (qRT-
176 PCR) was performed using a Bio-Rad CFX96 Real-Time System coupled to a C1000
177 Thermal Cycler (Bio-Rad) using the Bio-Rad SsoFast EvaGreen Supermix. qRT-PCR
178 primer pairs used are described in Table S1. qRT-PCR reactions were run for 40 cycles
179 with annealing and amplification at 62°C for 5 sec and denaturation at 95°C for 5 sec.
180 Single melting curves were observed for all primer pairs used indicating negligible off-
181 target amplification.

182

183 *Western Blot Analysis for Protein expression*

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

188

189 *Reactive oxygen species production*

190 Leaves of 3- to 4-week-old rice plants were cut longitudinally along the mid vein and
191 then into 1 to 1.5 mm thick pieces. Leaf pieces were floated on sterile water overnight.
192 The following morning, two leaf pieces were transferred into one well of a 96-well white
193 plate containing 100 μl elicitation solution (20 μM LO-12 [Wako, Japan], 2 $\mu\text{g/ml}$ HRP

194 [Sigma]). 500 nM of elf18, RaxX21 or RaxX21-sY peptides were used for treatments.
195 ROS production was measured for 0.5s per reading with a high sensitivity plate reader
196 (TriStar, Berthold, Germany).

197

198 **Results**

199 **Transgenic rice expressing the XA21:EFR chimeric receptor display robust** 200 **resistance to *Xoo*.**

201 We produced transgenic rice lines expressing an *Xa21:EFR:GFP* chimeric
202 construct to test whether the XA21 ectodomain confers resistance to *Xoo* when fused to
203 the EFR cytoplasmic domain. This construct encodes the XA21 ectodomain (XA21
204 residues 1-650) fused to the EFR transmembrane, juxtamembrane and cytoplasmic
205 domain (EFR residues 651-1031) with a carboxyl-terminal GFP fusion (Holton et al.
206 2015) expressed under the maize ubiquitin promoter. We generated 10 independent
207 transgenic T₀ lines and inoculated the plants with *Xoo* using a leaf clipping method. We
208 found that 8 of these lines (lines 2, 3, 4, 5, 6, 7, 9, and 10) displayed enhanced
209 resistance to *Xoo* compared with the Kitaake parent line (Fig. S1).

210 To assess if the resistance phenotype was transmitted to the next generation, we
211 self-pollinated 5 of the 8 T₀ lines (lines 2,4,5,6,7) and collected T₁ seed. These T₁
212 plants, as well as rice plants expressing and lacking Xa21 as controls, were inoculated
213 with *Xoo* and assessed for resistance by measuring the lengths of disease-induced
214 lesions. We observed that T₁ individuals that were PCR positive for the transgene in
215 lines 2,4,5, and 6 co-segregated with resistance to *Xoo* (PCR positive to negative ratios

216 8:4, 21:0, 8:7, and 16:5, respectively). Lesion length averages were approximately 5 cm
217 in resistant individuals compared to approximately 13 cm for susceptible controls (Fig.
218 1, Fig S2). All T₁ individuals from line 4 were PCR positive for *Xa21:EFR:GFP* (21:0)
219 which could have been from multiple transgene insertions (χ^2 (1) = 1.4, p = 0.24) and
220 were resistant to *Xoo*. All T₁ individuals from line 7 were also PCR positive for the
221 *Xa21:EFR:GFP* transgene. However, these plants showed varying degrees of
222 resistance (Fig. S2).

223 For subsequent experiments, we focused on two *Xa21:EFR:GFP* lines (-2 and -
224 6) for further molecular characterization experiments (Figs 3, 4, S4). For these
225 experiments, T₁ plants were used for line 2 and T₂ plants were used for line 6 (we self-
226 pollinated T₁ individuals and collected T₂ seed for line 6) to test if similar phenotypes are
227 observable in different lines and in subsequent generations. We found that T₂
228 individuals from line 6 maintained *Xoo* resistance that segregated with the
229 *Xa21:EFR:GFP* transgene (Fig. 1). Because T₁ and T₂ individuals from lines 2 and 6,
230 respectively, were still segregating for the transgene, we performed experiments on
231 individual plants that carried the *Xa21:EFR:GFP* transgene, selected by PCR
232 genotyping. We used null segregant individuals as controls.

233

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

236 We used qRT-PCR to assess if plants containing the *Xa21:EFR:GFP* transgene
237 express the *Xa21* ectodomain and *EFR* cytoplasmic domain. We assessed transcript

238 levels using domain specific primers for regions that encode the XA21 ectodomain,
239 XA21 cytoplasmic domain, and the EFR cytoplasmic domain (Table S1) (Fig. 2A-C).
240 Our results show *Xa21:EFR:GFP-2-23*, *-2-24*, *-6-5-17* and *-6-5-18* that carry the
241 transgene specifically express regions encoding the XA21 ectodomain and the EFR
242 cytoplasmic domain. Additionally, these plants do not express regions encoding the
243 XA21 endodomain. Because these plants are not expressing the full-length *Xa21*
244 transcript or endodomain, any immune responses observed in these plants are not
245 mediated by full-length XA21 or the XA21 endodomain.

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

256

257 **RaxX21-sY treated *Xa21:EFR:GFP* rice leaves produce reactive oxygen species** 258 **and highly express stress-related genes**

259 We next assessed if *Xa21:EFR:GFP* rice are able to activate immune responses

260 after RaxX treatments. We used a commercially synthesized, sulfated RaxX peptide,
261 composed of 21 amino acids from the Xoo RaxX protein sequence in PXO99A
262 (RaxX21-sY) previously shown to activate XA21-mediated immunity (Pruitt et al. 2015;
263 Wei et al. 2016). Bursts of reactive oxygen species (ROS) are commonly measured to
264 assess immune responses because ROS are rapidly produced as a defense response
265 to pathogen attack (Wojtaszek 1997; Jones and Dangl 2006; Macho and Zipfel 2014).
266 We therefore measured ROS production in *Xa21:EFR:GFP* rice after RaxX21-sY
267 treatment to determine if plants carrying the chimeric protein respond similarly to
268 RaxX21-sY treated plants carrying full-length XA21 (Pruitt et al. 2015). *Xa21:EFR:GFP*
269 rice accumulate ROS in response to RaxX21-sY treatments, but not to mock or elf18
270 treatments (Figs 3f, S4). In addition, we confirmed that RaxX21-sY treated XA21:GFP
271 rice, expressing the full length XA21 protein tagged with GFP (Fig. S3), accumulate
272 ROS (Fig. 3b). Null segregants did not produce ROS bursts in response to RaxX21-sY
273 treatments (Figs 3e, S4). *EFR:GFP* and *EFR:XA21:GFP* rice responded to elf18, but not
274 to RaxX21-sY, showing that the XA21 ectodomain in full-length XA21 and
275 XA21:EFR:GFP proteins is necessary for RaxX-triggered immune responses (Figs 3c
276 and 3d).

277 We next measured stress-related marker gene expression in RaxX21-sY treated
278 *Xa21:EFR:GFP* rice to further characterize the XA21:EFR:GFP-mediated response. We
279 measured the expression of rice defense marker genes *PR10b*, *LOC_Os02g36190*,
280 *LOC_Os06g37224*, and *LOC_Os11g42200* (Chen et al. 2014; Pruitt et al. 2015;
281 Thomas et al. 2016) using a detached leaf treatment assay. RNA was extracted from

282 detached leaves of 4 week old plants mock treated with water or with 500 nM of
283 RaxX21-sY for 6 hours. Gene expression was measured in individuals XA21:EFR:GFP-
284 2-23 and -6-5-17 by quantitative real-time PCR. Higher expression was observed in
285 each of the stress-related genetic markers in RaxX21-sY treated *Myc:XA21* and
286 *Xa21:EFR:GFP* rice (Fig. 4a-d). Gene expression was not induced in any mock treated
287 samples or Kitaake samples treated with RaxX21-sY. Individual 2-23 only showed
288 higher induction of *LOC_Os11g42200* and *LOC_Os06g37224*, and only induction of
289 *LOC_Os06g37224* was significant (Fig. 4c). Together, the results from ROS and gene
290 expression experiments suggest that the XA21 ectodomain in XA21:EFR:GFP is
291 sufficient to recognize RaxX and that the EFR endodomain can be substituted for the
292 XA21 endodomain to transduce immune responses after RaxX treatment.

293

294 **Discussion**

295 Here we show that the ectodomain of XA21 is sufficient to confer full resistance
296 to *Xoo* strain PXO99A when fused to the intracellular domain of the Arabidopsis
297 immune receptor EFR (Figs 1, S1, S2). We previously demonstrated that a functional
298 EFR:XA21:GFP is not able to confer resistance to *Xoo* when expressed in rice.
299 Together these results suggest that the XA21 extracellular domain and the recognition
300 of RaxX are the key properties that dictate the robust immune response of XA21. Both
301 the native XA21 endodomain as well as the EFR endodomain fused to the XA21
302 ectodomain appear to be interchangeable as both XA21 and EFR kinases can confer
303 robust resistance when fused with the XA21 ectodomain. This result slightly contrasts

304 with previous domain swap studies that indicated that the endodomains of immune
305 receptors were the defining properties of the immune receptor responses (He et al.
306 2000; Brutus et al. 2010; Kishimoto et al. 2010; Albert et al. 2010). Although XA21
307 mutants and XA21 derivatives that lack a functional kinase domain maintain partial
308 resistance, it appears that a functional kinase domain is required for robust resistance
309 (C. B. Andaya and Ronald 2003) (Wang et al. 1996).

310 Despite the evidence that rice expressing XA21:EFR:GFP are resistant to *Xoo*, it
311 is unclear why plants expressing the reciprocal EFR:XA21:GFP protein are susceptible
312 to *Xoo* (Schwessinger, Bahar, et al. 2015). We hypothesize that the XA21 ectodomain is
313 critical for conferring robust resistance because it interacts with additional rice specific
314 signaling components that the EFR ectodomain is unable to bind. In partial support of
315 this hypothesis, we previously showed that the EFR kinase domain does not interact
316 with some of the previously identified XA21 kinase domain signaling components,
317 including the negative regulator XB15 and positive regulator XB3 (Schwessinger, Bahar,
318 et al. 2015). Future studies might be aimed at identifying these elusive ectodomain
319 specific signaling partners to better understand XA21-mediated immunity.

320

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324

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330

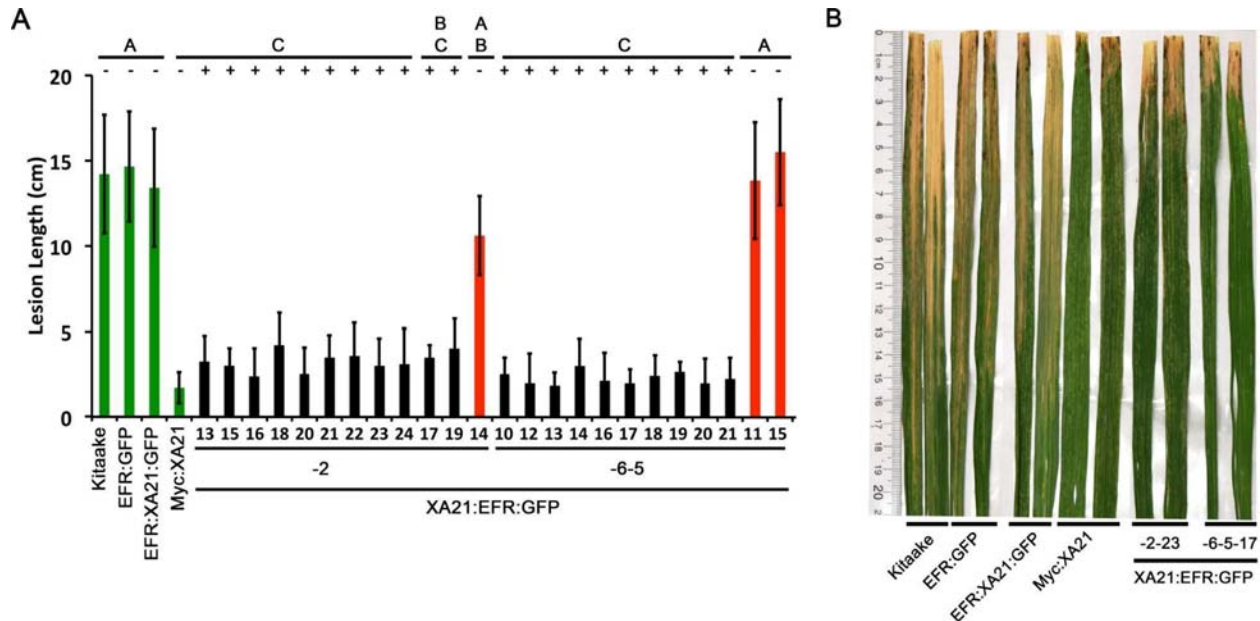
331 References

- 332 Albert, M., A. K. Jehle, K. Mueller, C. Eisele, M. Lipschis, and G. Felix. 2010.
333 "Arabidopsis Thaliana Pattern Recognition Receptors for Bacterial Elongation
334 Factor Tu and Flagellin Can Be Combined to Form Functional Chimeric Receptors."
335 *The Journal of Biological Chemistry* 285 (25): 19035–42.
- 336 Andaya, C. B., and P. C. Ronald. 2003. "A Catalytically Impaired Mutant of Rice Xa21
337 Receptor Kinase Confers Partial Resistance to Xanthomonas Oryzae Pv. Oryzae."
338 *Physiological and Molecular Plant Pathology* 62: 203–8.
- 339 Andaya, Cynthia B., and Pamela C. Ronald. 2003. "A Catalytically Impaired Mutant of
340 the Rice Xa21 Receptor Kinase Confers Partial Resistance to Xanthomonas
341 Oryzae Pv Oryzae." *Physiological and Molecular Plant Pathology* 62 (4): 203–8.
- 342 Brutus, Alexandre, Francesca Sicilia, Alberto Macone, Felice Cervone, and Giulia De
343 Lorenzo. 2010. "A Domain Swap Approach Reveals a Role of the Plant Wall-
344 Associated Kinase 1 (WAK1) as a Receptor of Oligogalacturonides." *Proceedings*
345 *of the National Academy of Sciences of the United States of America* 107 (20):
346 9452–57.
- 347 Cabrera, Juan Carlos, Aurelien Boland, Johan Messiaen, Pierre Cambier, and Pierre
348 Van Cutsem. 2008. "Egg Box Conformation of Oligogalacturonides: The Time-
349 Dependent Stabilization of the Elicitor-Active Conformation Increases Its Biological
350 Activity." *Glycobiology* 18 (6): 473–82.
- 351 Chen, X., S. Zuo, B. Schwessinger, M. Chern, A. Daudi, P. E. Canlas, D. L. Ruan, et al.
352 2014. "An XA21-Associated Kinase (OsSERK2) Regulates Immunity by the XA21
353 and XA3 Immune Receptors." *Molecular Plant-Microbe Interactions: MPMI* doi:
354 10.1093/mp/ssu003.
- 355 Chinchilla, D., Z. Bauer, M. Regenass, T. Boller, and G. Felix. 2006. "The Arabidopsis
356 Receptor Kinase FLS2 Binds flg22 and Determines the Specificity of Flagellin
357 Perception." *The Plant Cell* 18 (2): 465–76.
- 358 Dardick, Chris, Benjamin Schwessinger, and Pamela Ronald. 2012. "Non-Arginine-
359 Aspartate (non-RD) Kinases Are Associated with Innate Immune Receptors That
360 Recognize Conserved Microbial Signatures." *Current Opinion in Plant Biology* 15
361 (4): 358–66.
- 362 Dardick, Christopher, and Pamela Ronald. 2006. "Plant and Animal Pathogen
363 Recognition Receptors Signal through Non-RD Kinases." *PLoS Pathogens* 2 (1):
364 e2.
- 365 Decreux, A., and J. Messiaen. 2005. "Wall-Associated Kinase WAK1 Interacts with Cell
366 Wall Pectins in a Calcium-Induced Conformation." *Plant & Cell Physiology* 46 (2):
367 268–78.
- 368 Decreux, Annabelle, Annick Thomas, Benoît Spies, Robert Brasseur, Pierre Van
369 Cutsem, and Johan Messiaen. 2006. "In Vitro Characterization of the
370 Homogalacturonan-Binding Domain of the Wall-Associated Kinase WAK1 Using
371 Site-Directed Mutagenesis." *Phytochemistry* 67 (11): 1068–79.

- 372 Felix, G., J. D. Duran, S. Volko, and T. Boller. 1999. "Plants Have a Sensitive
373 Perception System for the Most Conserved Domain of Bacterial Flagellin." *The*
374 *Plant Journal: For Cell and Molecular Biology* 18 (3): 265–76.
- 375 Ferrari, Simone, Roberta Galletti, Daniela Pontiggia, Cinzia Manfredini, Vincenzo
376 Lionetti, Daniela Bellincampi, Felice Cervone, and Giulia De Lorenzo. 2008.
377 "Transgenic Expression of a Fungal Endo-Polygalacturonase Increases Plant
378 Resistance to Pathogens and Reduces Auxin Sensitivity." *Plant Physiology* 146 (2):
379 669–81.
- 380 Gómez-Gómez, Lourdes, and Thomas Boller. 2000. "FLS2: An LRR Receptor-like
381 Kinase Involved in the Perception of the Bacterial Elicitor Flagellin in Arabidopsis."
382 *Molecular Cell* 5 (6): 1003–11.
- 383 He, Z., Z. Y. Wang, J. Li, Q. Zhu, C. Lamb, P. C. Ronald, and J. Chory. 2000.
384 "Perception of Brassinosteroids by the Extracellular Domain of the Receptor Kinase
385 BRI1." *Science* 288 (5475): 2360–63.
- 386 Hiei, Y., S. Ohta, T. Komari, and T. Kumashiro. 1994. "Efficient Transformation of Rice
387 (*Oryza Sativa* L.) Mediated by *Agrobacterium* and Sequence Analysis of the
388 Boundaries of the T-DNA." *The Plant Journal: For Cell and Molecular Biology* 6 (2):
389 271–82.
- 390 Holton, Nicholas, Vladimir Nekrasov, Pamela C. Ronald, and Cyril Zipfel. 2015. "The
391 Phylogenetically-Related Pattern Recognition Receptors EFR and XA21 Recruit
392 Similar Immune Signaling Components in Monocots and Dicots." *PLoS Pathogens*
393 11 (1): e1004602.
- 394 Jones, Jonathan D. G., and Jeffery L. Dangl. 2006. "The Plant Immune System." *Nature*
395 444 (7117): 323–29.
- 396 Kaku, H., Y. Nishizawa, N. Ishii-Minami, C. Akimoto-Tomiyama, N. Dohmae, K. Takio,
397 E. Minami, and N. Shibuya. 2006. "Plant Cells Recognize Chitin Fragments for
398 Defense Signaling through a Plasma Membrane Receptor." *Proceedings of the*
399 *National Academy of Sciences of the United States of America* 103 (29): 11086–91.
- 400 Kauffman, H. E., A. P. K. Reddy, S. P. V. Hsieh, and S. D. Marca. 1973. "An Improved
401 Technique for Evaluating Resistance of Rice Varieties to *Xanthomonas Oryzae*."
402 *Plant Disease Management Reports* 57 (January): 537–41.
- 403 Kishimoto, K., Y. Kouzai, H. Kaku, N. Shibuya, E. Minami, and Y. Nishizawa. 2010.
404 "Perception of the Chitin Oligosaccharides Contributes to Disease Resistance to
405 Blast Fungus *Magnaporthe Oryzae* in Rice." *The Plant Journal: For Cell and*
406 *Molecular Biology* 64 (2): 343–54.
- 407 Kunze, Gernot, Cyril Zipfel, Silke Robatzek, Karsten Niehaus, Thomas Boller, and
408 Georg Felix. 2004. "The N Terminus of Bacterial Elongation Factor Tu Elicits Innate
409 Immunity in Arabidopsis Plants." *The Plant Cell*.
- 410 Li, J., and J. Chory. 1997. "A Putative Leucine-Rich Repeat Receptor Kinase Involved in
411 Brassinosteroid Signal Transduction." *Cell* 90 (5): 929–38.
- 412 Livak, K. J., and T. D. Schmittgen. 2001. "Analysis of Relative Gene Expression Data
413 Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method." *Methods*
414 25 (4): 402–8.

- 415 Macho, Alberto P., and Cyril Zipfel. 2014. "Plant PRRs and the Activation of Innate
416 Immune Signaling." *Molecular Cell* 54 (2): 263–72.
- 417 Pruitt, Rory N., Benjamin Schwessinger, Anna Joe, Nicholas Thomas, Furong Liu,
418 Markus Albert, Michelle R. Robinson, et al. 2015. "The Rice Immune Receptor
419 XA21 Recognizes a Tyrosine-Sulfated Protein from a Gram-Negative Bacterium."
420 *Science Advances* 1 (6). American Association for the Advancement of Science:
421 e1500245.
- 422 Ronald, P. C., and B. Beutler. 2010. "Plant and Animal Sensors of Conserved Microbial
423 Signatures." *Science* 330 (6007): 1061–64.
- 424 Schwessinger, Benjamin, Ofir Bahar, Nicolas Thomas, Nicolas Holton, Vladimir
425 Nekrasov, Deling Ruan, Patrick E. Canlas, et al. 2015. "Transgenic Expression of
426 the Dicotyledonous Pattern Recognition Receptor EFR in Rice Leads to Ligand-
427 Dependent Activation of Defense Responses." *PLoS Pathogens* 11 (3): e1004809.
- 428 Schwessinger, Benjamin, Xiang Li, Thomas L. Ellinghaus, Leanne Jade G. Chan, Tong
429 Wei, Anna Joe, Nicholas Thomas, et al. 2015. "A Second-Generation Expression
430 System for Tyrosine-Sulfated Proteins and Its Application in Crop Protection."
431 *Integrative Biology* 8 (4). Royal Society of Chemistry: 542–45.
- 432 Song, W. Y., G. L. Wang, L. L. Chen, H. S. Kim, L. Y. Pi, T. Holsten, J. Gardner, et al.
433 1995. "A Receptor Kinase-like Protein Encoded by the Rice Disease Resistance
434 Gene, Xa21." *Science* 270 (5243): 1804–6.
- 435 Takai, Ryota, Akira Isogai, Seiji Takayama, and Fang-Sik Che. 2008. "Analysis of
436 Flagellin Perception Mediated by flg22 Receptor OsFLS2 in Rice." *Molecular Plant-
437 Microbe Interactions: MPMI* 21 (12): 1635–42.
- 438 Thomas, Nicholas C., Benjamin Schwessinger, Furong Liu, Huamin Chen, Tong Wei,
439 Yen P. Nguyen, Isaac W. F. Shaker, and Pamela C. Ronald. 2016. "XA21-Specific
440 Induction of Stress-Related Genes Following Xanthomonas Infection of Detached
441 Rice Leaves." *PeerJ* 4 (September). PeerJ Inc.: e2446.
- 442 Wang, G. L., D. L. Ruan, W. Y. Song, S. Sideris, L. Chen, L. Y. Pi, S. Zhang, et al.
443 1998. "Xa21D Encodes a Receptor-like Molecule with a Leucine-Rich Repeat
444 Domain That Determines Race-Specific Recognition and Is Subject to Adaptive
445 Evolution." *The Plant Cell* 10 (5): 765–79.
- 446 Wei, Tong, Mawsheng Chern, Furong Liu, and Pamela C. Ronald. 2016. "Suppression
447 of Bacterial Infection in Rice by Treatment with a Sulfated Peptide." *Molecular Plant
448 Pathology*, January. doi:10.1111/mpp.12368.
- 449 Wojtaszek, P. 1997. "Oxidative Burst: An Early Plant Response to Pathogen Infection."
450 *Biochemical Journal* 322 (Pt 3) (March): 681–92.
- 451 Zipfel, Cyril, Gernot Kunze, Delphine Chinchilla, Anne Caniard, Jonathan D. G. Jones,
452 Thomas Boller, and Georg Felix. 2006. "Perception of the Bacterial PAMP EF-Tu
453 by the Receptor EFR Restricts Agrobacterium-Mediated Transformation." *Cell* 125
454 (4): 749–60.

455 **Figures**



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457 **Figure 1. Rice expressing *Xa21:EFR:GFP* are resistant to *Xoo* infection**

458 A, The bar graph represents the average lesion length observed on rice plants infected
 459 with *Xoo*. Control lines used were Kitaake, *EFR:GFP*, *EFR:XA21:GFP*, and *Myc:XA21*
 460 rice (green bars). Experimental samples include individuals PCR positive for the
 461 *Xa21:EFR:GFP* transgene (black bars) from line 2 and 6 and PCR negative individuals
 462 (red bars). Five week old greenhouse grown plants were scissor inoculated with
 463 PXO99A (5×10^8 colony forming units (CFU)/ mL) and disease lesions were scored
 464 approximately 2 weeks post inoculation. Error bars represent standard deviation from
 465 the mean lesion length. Mean lesion lengths are the average of lesion measurements
 466 from individual leaves from the same plant ($n \geq 3$). Black lines and letters above the
 467 graph represent statistical groupings using the Tukey-Kramer HSD test. Different letters
 468 indicate significant differences ($p < 0.05$). This experiment was repeated at least three
 469 times with similar results. B, Photograph of select leaves from the same experiment in
 470 A. The photograph shows Kitaake, *EFR:GFP*, *EFR:XA21:GFP*, *Myc:XA21*,
 471 *Xa21:EFR:GFP* individual -2-23, and -6-5-17 leaves infected with *Xoo* and was taken
 472 approximately 2 weeks after inoculation.

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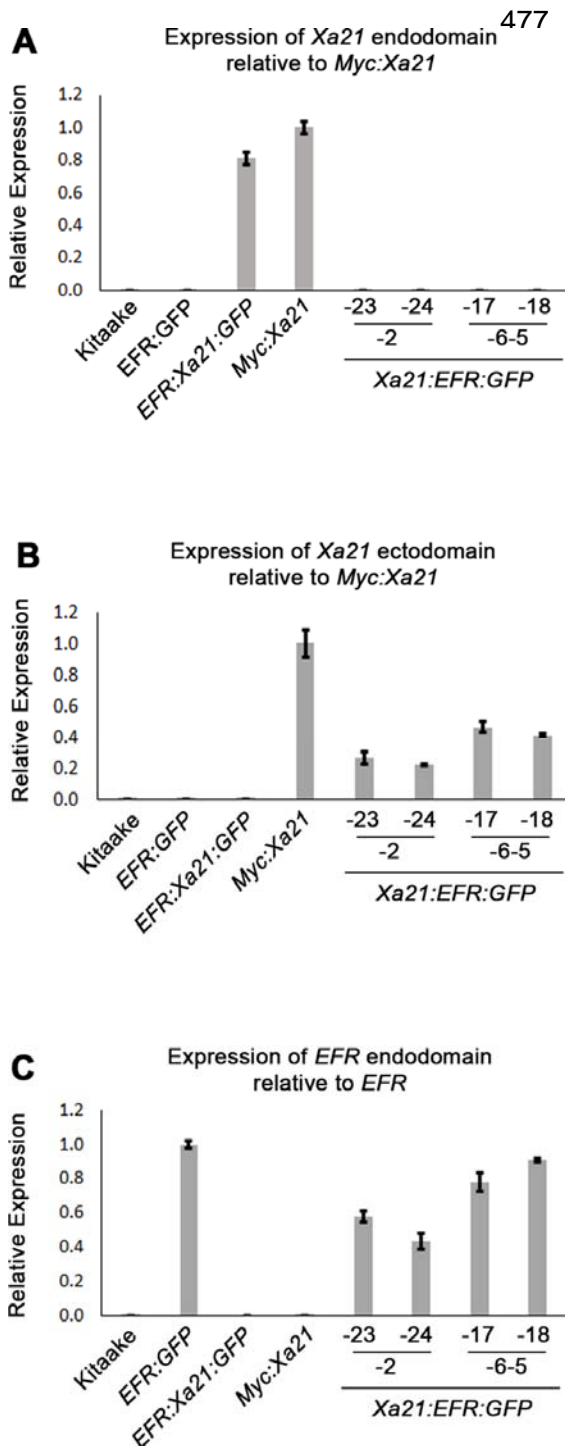
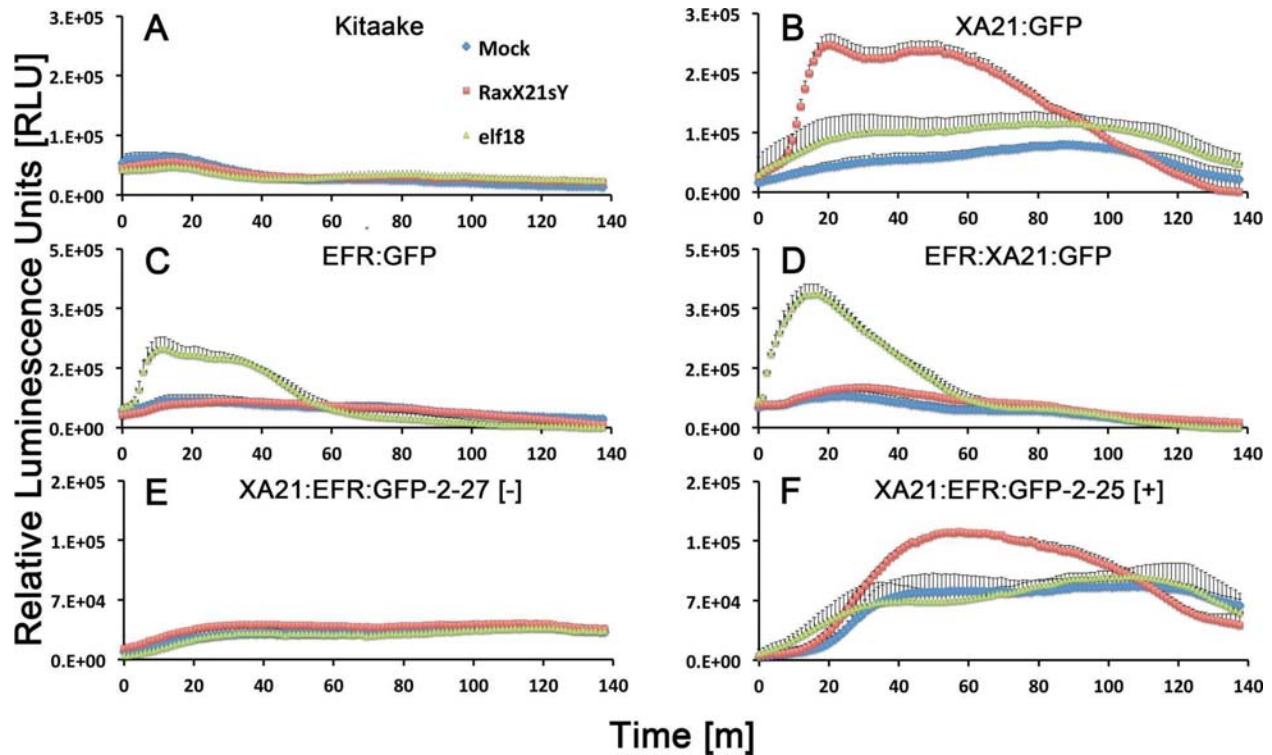


Figure 2. *Xa21:EFR:GFP* transcripts are expressed in stable transgenic lines

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* ectodomain 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 2 technical replicates and error bars represent the standard deviation between the two measurements.

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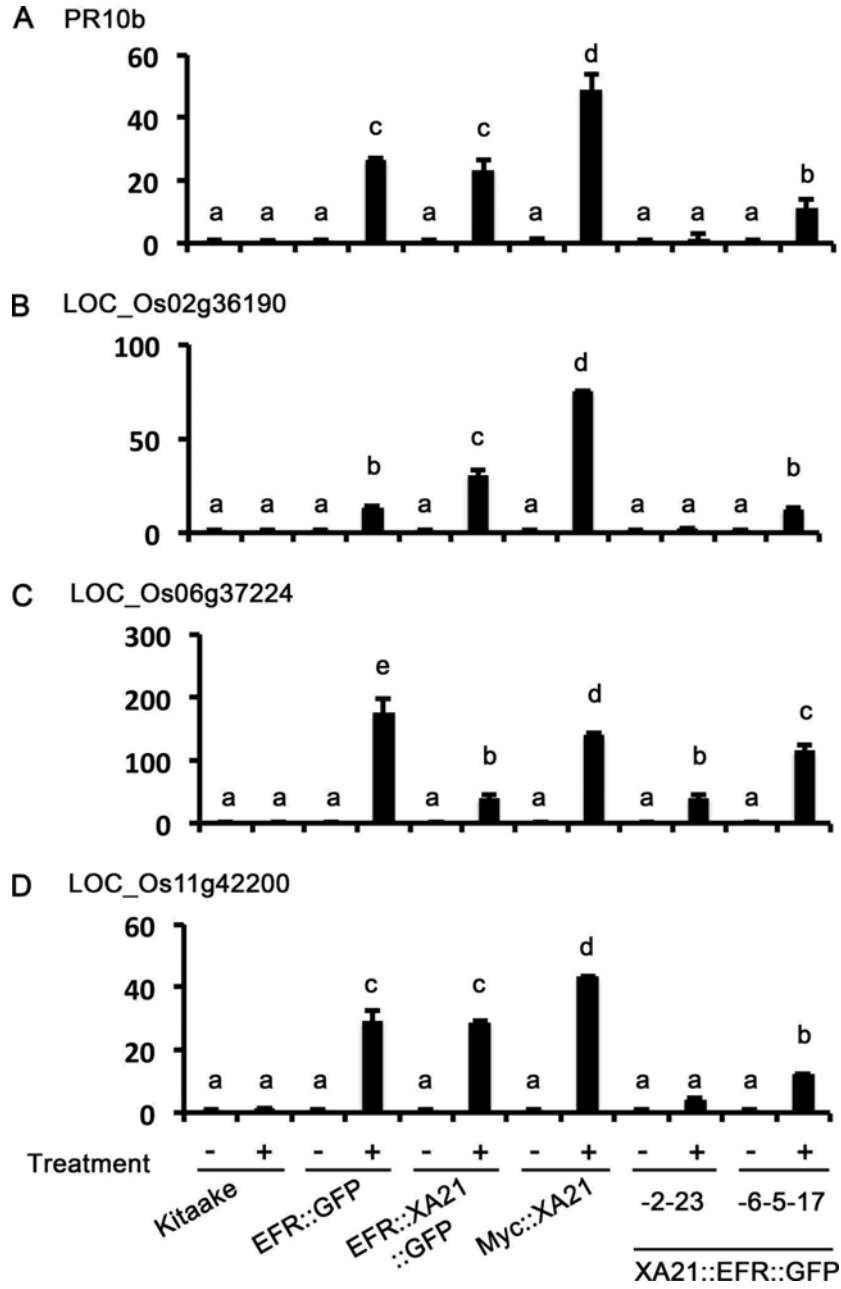


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Figure 3. Reactive oxygen species accumulate after peptide treatments

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 Kitaake rice and B, *Xa21:GFP* rice C, *EFR:GFP* rice and D, *EFR:XA21:GFP* rice. E, ROS production in a T_1 null-segregant individual from *Xa21:EFR:GFP* line -2. F, ROS production in a T_1 individual from line -2 that segregates 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.

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Figure 4. *Xa21::EFR::GFP* rice express stress related genes after RaxX21-sY treatment

519 Gene expression profiles of four stress-related genes, *PR10b*, *LOC_Os2g36190*,
520 *LOC_06g37224*, and *LOC_Os11g42200*. Samples are rice leaves from wild-type
521 Kitaake, Myc:XA21 rice, and individuals -23 from *Xa21:EFR:GFP* line -2 and individual -
522 17 from line -6-5. Leaves were mock treated with water (-) or with 500 nM RaxX21-sY
523 (+). Letters indicate significant difference in gene expression compared to mock using
524 the Tukey-Kramer HSD test ($\alpha = 0.05$). Expression levels are normalized to mock
525 treatment of the same line. Bars depict average expression level relative to actin
526 expression \pm standard error of three technical replicates. This experiment was repeated
527 twice with similar results.