A peer-reviewed version of this preprint was published in PeerJ on 28 September 2016.

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 https://doi.org/10.7717/peerj.2446

XA21-specific induction of stress-related genes following *Xanthomonas* infection of detached rice leaves

Nicholas C. Thomas[1,2*], Benjamin Schwessinger[1,2,3*], Furong Liu[1,2], Huamin Chen[1,4], Tong Wei[1,2], Yen Nguyen[1], Isaac Shaker[1], and Pamela C. Ronald[1,2]

[1]Department of Plant Pathology and the Genome Center, University of California, Davis, Davis, California, United States of America

[2]Joint BioEnergy Institute, Emeryville, CA 94608

[3]The Australian National University, Research School of Biology, Acton ACT 2601, Australia.

[4]State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China

[*] These authors contributed equally to this work and are shown in reverse alphabetical order

Corresponding author:

Pamela C. Ronald[1,2]

One Shields Avenue

Davis, CA 95616, USA

E-mail address: pcronald@ucdavis.edu

Abstract

The rice XA21 receptor kinase confers robust resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). We developed a detached leaf infection assay to quickly and reliably measure activation of the XA21-mediated immune response using genetic markers. We used RNA sequencing of elf18 treated EFR:XA21:GFP plants to identify candidate genes that could serve as markers for XA21 activation. From this analysis we identified 8 genes that

are up-regulated in both in elf18 treated EFR:XA21:GFP rice leaves and *Xoo* infected XA21 rice leaves. These results provide a rapid and reliable method to assess bacterial-rice interactions.

Introduction

Plant immunity is mediated, in part, by cell surface immune receptors that recognize molecules produced by microbes. For example, the *Arabidopsis* FLS2 (Flagellin Sensing 2) and EFR (Elongation Factor Tu Receptor) receptors recognize the flg22 peptide derived from bacterial flagellin and the elf18 peptide derived from elongation factor thermo-unstable (EF-Tu) protein, respectively (Gomez-Gomez & Boller, 2000; Zipfel et al., 2006). The rice XA21 receptor recognizes the sulfated RaxX peptide (RaxX21-sY) derived from the RaxX protein produced by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Song et al., 1995; Pruitt et al., 2015; Wei et al., 2016). XA21, EFR, and FLS2 all contain extracellular leucine rich repeat (LRR), transmembrane, and intracellular non-RD (arginine-aspartic acid) kinase domains. These receptor domains are partially interchangeable. For example, the LRR domain from EFR can be fused to the transmembrane and intracellular domain of FLS2 to form a chimeric receptor that responds to elf18 treatments when transiently expressed in *Nicotiana benthamiana* and *Arabidopsis thaliana* (Albert et al., 2010). The EFR LRR can be fused to the transmembrane and intracellular domain of XA21 to form a chimeric receptor that responds to elf18 treatment and confers partial resistance to *Xoo* in transgenic rice lines (Schwessinger et al., 2015a).

The availability of rapid and reliable assays that measure markers characteristic of immune response activation can help facilitate investigations of innate immune signaling. For example, immune signaling studies of FLS2 and EFR in *Arabidopsis* have been aided by the availability of rapid and reliable assays (Gomez-Gomez & Boller, 2000; Zipfel et al., 2006;

Chinchilla et al., 2007; Lu et al., 2010; Schulze et al., 2010; Albert et al., 2010; Schwessinger et al., 2011; Sun et al., 2013; Li et al., 2014). In contrast, studies of the XA21-mediated immune response have been limited by the lack of rapid assays and well-characterized genetic markers. Typically, disease assessments are carried out by measuring lesions on rice leaves or by assessing bacterial populations from infected leaves (Kauffman et al., 1973; Song et al., 1995; da Silva et al., 2004a; Park et al., 2008; Chen et al., 2014; Pruitt et al., 2015).

In this study we aimed to establish a rapid and efficient assay to monitor the XA21-mediated immune response after bacterial infection. For this purpose, we employed the EFR:XA21:GFP chimera composed of the EFR extracellular domain and the XA21 transmembrane and intracellular kinase domains, tagged with green fluorescent protein (EFR:XA21:GFP) (Schwessinger et al., 2015a). EFR:XA21:GFP transgenic rice plants are partially resistant to *Xoo* and detached EFR:XA21:GFP leaves respond to elf18 with stress-related gene induction, mitogen-activated protein kinase (MAPK) cascade activation, and reactive oxygen species (ROS) production (Schwessinger et al., 2015a). These results indicate that plants expressing the EFR:XA21:GFP chimeric protein are appropriate for studies to identify markers of resistance.

We used RNA sequencing (RNAseq) to identify genes differentially regulated in elf18 treated EFR:XA21:GFP rice. We then assessed if differentially regulated genes (DRGs) in elf18 treated EFR:XA21:GFP rice leaves were up-regulated in *Xoo* infected rice leaves expressing full-length XA21, which are resistant to *Xoo*. We developed a rapid and reliable assay to analyze gene expression in detached rice leaves inoculated with *Xoo*. We identified 8 DRGs from elf18 treated EFR:XA21:GFP rice that are also specifically up-regulated in detached XA21 rice leaves infected with *Xoo*.

Materials and Methods

Plant growth, peptide and bacterial treatments of detached rice leaves

For peptide treatments, wild type (WT) Kitaake and progeny from line EFR:XA21:GFP-3-8 (called EFR:XA21:GFP in this study) Kitaake rice leaves were harvested from plants grown in the greenhouse for 4.5 weeks (Schwessinger et al., 2015a). 1.5-2 cm leaf sections were collected from expanded adult leaves using surgical grade scissors. Tissue from the leaf base and leaf tip was discarded. Detached leaves were equilibrated overnight in 6-well Costar cell culture plates under constitutive light (between 5-10 μmol/(m^2*s)) (Fig. S1). Peptide treatments were performed in the morning and collected at the indicated times.

For bacterial inoculations, we used detached rice leaves harvested from 4-week old plants grown using a hydroponic growth system as described previously (Pruitt et al., 2015) under a light intensity of 280 μmol/(m^2*s). Freshly harvested leaves from Kitaake and Ubi-Myc:XA21 Kitaake rice (called Myc:XA21 rice in this study) (Park et al., 2010) were cut into 1.5-2 cm pieces and immediately (without overnight equilibration) floated on 10 mM $MgCl_2$ solution for mock treatments or 10 mM $MgCl_2$ containing fresh *Xoo* cell suspensions at $O.D._{600}$ of 0.1 (approximately 1 x 10^7 cells mL^{-1}). The samples were left overnight under constitutive light (between 5-10 μmol/(m^2*s)) and collected 24 hours post-inoculation (hpi). Leaves were floated on approximately 1.5 mL *Xoo* cell suspension media in 6-well Corning Costar cell culture plates (Fig. S1). The detached leaf infection assay allows a more uniform distribution, compared to the scissor inoculation method (Kauffman et al., 1973), of *Xoo* inoculum by floating leaves on bacterial suspensions.

RNA isolation and qPCR gene expression analysis for peptide treated and bacterial infected leaf samples

Detached leaves were frozen in liquid nitrogen and powdered using a Qiagen tissuelyser. For tissue from greenhouse grown plants, RNA was extracted from powdered tissue using TRI Reagent and precipitated with isopropanol. For tissue from hydroponically grown plants, RNA was extracted using the Spectrum Plant Total RNA Kit from Sigma-Aldrich. RNA was DNase treated using the TURBO DNase kit from Life Technologies. RNA concentrations were normalized to the lowest sample concentration in each experiment. cDNA was synthesized from 2μg of total RNA using the High Capacity cDNA Reverse Transcription Kit by Life Technologies. Gene expression changes were determined by $\Delta\Delta$Ct method (Schmittgen & Livak, 2008) normalized to *Actin* (*LOC_Os03g50885*) and compared to mock treated samples.

Identification of genes differentially regulated in elf18 treated EFR:XA21:GFP rice using RNA sequencing

Plant growth, leaf tissue isolation, and treatments were performed as described above. RNA was isolated from untreated Kitaake as well as untreated and elf18 treated EFR:XA21:GFP leaf tissue using the Spectrum Plant Total RNA Kit from Sigma-Aldrich and on-column DNase treated to remove genomic DNA contamination following the manufacturer's instructions. RNA was quantified using the Quant-IT Ribogreen RNA Assay Kit.

RNAseq libraries, sequencing, and reference alignment were performed as described previously (Schwessinger et al., 2015a). Sample correlation between Kitaake and EFR:XA21:GFP replicates and differential gene expression analysis was performed using the

Bioconductor 'edgeR' package for R (Robinson, McCarthy & Smyth, 2010; McCarthy, Chen & Smyth, 2012).

Bacterial strains and generation of mutants

We generated a PXO99AΔ*hrpA1* mutant in Philippine race 6 strain PXO99Az, a derivative of strain PXO99 (referred to as PXO99A in this study) (Salzberg et al., 2008). *Xoo* was grown in 10 g PSB (10 g Peptone (bacto-Peptone), 10 g Sucrose, 1 g sodium glutamate (glutamic acid, monosodium salt), final volume 1L, pH 7.0) or on PSA plates (PSB with 16g/L bacto-agar) at 28˚C. PXO99AΔ*hrpA1* was generated by single crossover mutagenesis using the suicide vector pJP5603 (Penfold & Pemberton, 1992). An approximately 500 base pair sequences within *hrpA1* was amplified using forward primer 5'-CGGGGTACCGTGCTGCGTGATTTGTCCG-3' and reverse primer 5'-CGCGGATCCTGACTTGGTCGATGCAGTCC-3' and cloned into the multiple cloning site of pJP5603 using the restriction enzyme sites KpnI and BamHI. PXO99A-competent cells were transformed with the suicide plasmids by electroporation and plated to PSA with kanamycin (50 µg/ml). PXO99AΔ*hrpA1* colonies with kanamycin resistance were screened by PCR for colonies with single crossover events, which contain the vector disrupting the target gene. PXO99AΔ*raxST* and PXO99AΔ*raxST(raxST)* complemented strains used in this study were described previously (Pruitt et al., 2015). PXO99AΔ*raxST* evades XA21-mediated immunity while the complemented PXO99AΔ*raxST(raxST)* strain does not.

Results

RNAseq analysis identifies 2212 genes that are differentially regulated in elf18 treated EFR:XA21:GFP rice leaves

We analyzed the transcriptomic profile of EFR:XA21:GFP rice lines treated with elf18 to identify genes differentially regulated during this response. We sequenced cDNA from EFR:XA21:GFP leaves treated with 500 nM elf18 for 0.5, 1, 3, 6, and 12 h. We also included untreated EFR:XA21:GFP and Kitaake as controls (Table S1). Multidimensional scaling of pairwise biological coefficient of variance comparisons for each sample revealed that replicate samples group together (Fig. 1A). This grouping of biological replicates demonstrates the overall transcriptional similarity between each sample (Robinson, McCarthy & Smyth, 2010).

We identified 2212 genes that were differentially regulated in EFR:XA21:GFP rice treated with elf18 compared with untreated (0 h) samples. Using a false discovery rate (FDR) cutoff of 0.05 and absolute expression log fold change (logFC) of 2 or greater, we previously reported that the transcriptomic profile of untreated Kitaake compared to untreated EFR:XA21:GFP did not differ significantly (Schwessinger et al., 2015a). Over the treatment time course, we identified 2212 DRGs (FDR < 0.05, absolute logFC > 2) using untreated EFR:XA21:GFP at 0 h as a reference. The number of DRGs that overlap between the elf18 treatment time points are summarized in Fig. 1B and File S1. Of the 2212 differentially regulated genes, there were 1420 up-regulated and 792 down-regulated genes. The highest number of DRGs (1504) was observed 6h post elf18 treatment. These results show that elf18 treated EFR:XA21:GFP rice express a substantially different set of genes over time compared to untreated (0 h) samples.

Stress response related genes are up-regulated while photosynthesis related genes are down-regulated in EFR:XA21:GFP rice treated with elf18

To examine the types of biological processes affected in elf18 treated EFR:XA21:GFP rice, we analyzed GO term enrichment of DRGs using the AgriGo analysis tool (Du et al., 2010). 1204 out of 1420 of the up-regulated DRGs and 682 of the 806 down-regulated DRGs had GO annotations. An FDR of 0.05 or less was used to define significantly enriched terms compared to the Michigan State University annotation reference as calculated by the AgriGo tool (Du et al., 2010; Kawahara et al., 2013). Fig. 1C and File S2 summarize the most enriched GO terms in each of the 3 major DRG clades. Clade 1 contains 1333 genes that are mostly up-regulated over time. Genes from clade 1 are enriched for metabolic process (GO:0008152), response to stimulus (GO:0050896) and response to stress (GO:0006950) GO terms (Fig. 1C). Clade 2 genes (122) are up-regulated across all time points and are enriched for secondary metabolic process (GO:0019748), metabolic process (GO:0008152) and response to stress (GO:0006950) GO terms (Fig. 1C). Clade 3 consists of 757 genes that are mostly down-regulated in all timepoints. Photosynthesis (GO:0015979) and response to abiotic stimulus (GO:0009628) are the most enriched GO terms associated with clade 3 genes (Fig. 1C).

qPCR validation of genes up-regulated in elf18 treated EFR:XA21:GFP plants

We chose 23 DRGs from the elf18 treated EFR:XA21:GFP rice RNAseq dataset with relatively high logFC and low FDR values after 3, 6, and 12 h for detailed analysis. We first assessed if the 23 genes up-regulated in elf18 treated EFR:XA21:GFP could be validated by qPCR analysis. Eleven out of 23 DRGs were up-regulated in EFR:XA21:GFP rice leaves after

elf18 treatment. Transcripts of the remaining 12 candidate genes were detectable by qPCR amplification but were not up-regulated in elf18 treated EFR:XA21:GFP leaves (File S3).

Establishment of bacterial infection assay of detached rice leaves

We established a detached leaf infection assay to test if genes identified in the EFR:XA21:GFP experiments are representative of genes differentially regulated in *Xoo* infected Myc:XA21 rice. We observed bacterial ooze from the detached rice leaves three days after inoculation with *Xoo* strain PXO99A (Fig. 2). To further assess if *Xoo* infects rice leaves in our detached leaf infection assay, we measured the expression level of *Os8N3* (*LOC_Os08g42350*), which was previously shown to be up-regulated in rice upon *Xoo* infection and is thus a useful marker of successful infection (Yang, Sugio & White, 2006). For these experiments, we also included a mutant PXO99A strain (PXO99AΔ*hrpA1*) that is unable to infect rice as a control. The *hrpA1* gene encodes a pilus protein that is essential for type III-secretion of effectors required for host infection (Wengelnik et al., 1996). We observed that the PXO99AΔ*hrpA1 Xoo* mutant is unable to infect Kitaake and Myc:XA21 rice plants (Fig. S2). Both WT Kitaake and Myc:XA21 detached leaves express *Os8N3* at higher levels compared to mock treatments 24 hpi with WT PXO99A, but not with PXO99AΔ*hrpA1* (Fig. 3). These results demonstrate that *Xoo* infects detached rice leaves.

We next employed the detached leaf infection assay to examine the expression of the stress-related marker *PR10b* in *Xoo* infected Myc:XA21 rice leaves. Compared with mock treated controls, *PR10b* is up-regulated in flg22 treated rice, elf18 treated EFR:XA21:GFP rice and Myc:XA21 rice treated with the RaxX21-sY (Chen et al., 2014; Schwessinger et al., 2015a; Pruitt et al., 2015). Using qPCR, we detected significant up-regulation of *PR10b* expression in Myc:XA21 rice leaves 24 hpi with PXO99A and PXO99AΔ*hrpA1*. *PR10b* up-regulation was not

observed in infected Kitaake leaves (Fig. 4). These results show that the detached leaf infection assay can be used to assess XA21-mediated marker gene expression and also indicate that RaxX expression or secretion is not affected by the *ΔhrpA1* mutation.

Eight out of 11 genes induced in efl18 treated EFR:XA21:GFP rice are up-regulated in *Xoo* infected XA21 rice

We next employed the detached leaf infection assay to identify genes up-regulated upon *Xoo* infection of Myc:XA21 rice leaves. For these assays, we examined the gene expression of the 11 genes validated by qPCR analysis of elf18 treated EFR:XA21:GFP rice described above. We inoculated Kitaake and Myc:XA21 rice with WT PXO99A, PXO99AΔ*raxST* mutants, and PXO99AΔ*raxST* complemented with *raxST* (PXO99AΔ*raxST*(*raxST*)). *Xoo* strains carrying mutations in *raxST* do not activate XA21-mediated immunity (da Silva et al., 2004a; Pruitt et al., 2015). The expression of 8 of 11 genes was specifically up-regulated in detached Myc:XA21 rice leaves 24 hpi with PXO99A and PXO99AΔ*raxST*(*raxST*) but not in Myc:XA21 rice leaves infected with PXO99AΔ*raxST* (Fig. 5 and File S3). The 8 validated marker genes encode a putative subtilisin-like protein (*LOC_Os04g03100*), a reticuline oxidase-like protein precursor (*LOC_Os06g35700*), the decarboxylase OsTDC1 (*LOC_Os08g04540*) (Kang et al., 2007), a peroxidase precursor (*LOC_Os11g02100*), RsOsPR10a (*LOC_Os12g36830*) (Hashimoto et al., 2004; Takeuchi et al., 2011), CYP71Z7 (*LOC_Os02g36190*) (Li et al., 2013), OsKO5 (*LOC_Os06g37224*) (Itoh et al., 2004), and one protein without a putative function (*LOC_Os10g28299*). The 3 remaining genes that were up-regulated in elf18 EFR:XA21:GFP rice but not in Myc:XA21 rice leaves encode a isoflavone reductase (*LOC_Os01g13610*), a

subtilisin-like protein (*LOC_Os04g03100*), and a reticuline oxidase-like protein precursor (*LOC_Os06g35700*).

Discussion

In this study we identified 8 genes that are specifically up-regulated in both elf18 treated EFR:XA21:GFP and *Xoo* infected detached Myc:XA21 rice leaves. At the time of these experiments, the activator of XA21, RaxX, had not yet been identified (Pruitt et al., 2015). We therefore treated rice plants expressing the EFR:XA21:GFP chimera with elf18 to identify candidate marker genes because EFR:XA21:GFP are partially resistant to *Xoo* and respond to elf18 treatments as described above in the introduction. Our results show that even though the EFR:XA21:GFP-mediated response does not confer robust resistance to *Xoo* (Schwessinger et al., 2015a), similar genes are up-regulated during both EFR:XA21:GFP- and Myc:XA21-mediated responses (Fig. 5). Further studies are necessary to determine why the expression of EFR:XA21:GFP in rice does not confer robust resistance to *Xoo*.

We show that stress-related gene induction of *PR10b* in Myc:XA21 rice leaves is maintained in plants inoculation with PXO99AΔ*hrpA1* mutant strains. These results suggest that RaxX expression, modification and secretion is not compromised by the Δ*hrpA1* mutation. These results indicate that RaxX function is independent of type-III secretion mediated by *hrpA1* and is consistent with the hypothesis that RaxX is a type I-secreted molecule (da Silva et al., 2004b; Pruitt et al., 2015).

The discovery of RaxX and the establishment of the detached leaf infection assay described here provide useful tools for studying XA21-mediated immunity. We can now assess XA21 activation by monitoring ROS production and marker gene expression in detached leaves

249 treated with the RaxX21-sY peptide (Pruitt et al., 2015; Schwessinger et al., 2015b). One
250 advantage of this approach is that researchers can study XA21-mediated immunity without
251 working with *Xoo*. Instead, researchers can activate XA21-mediated immunity by treating leaves
252 with RaxX21-sY peptide rather than *Xoo*. This strategy eliminates the need for select agent
253 permits, which are costly and time-consuming.

254 The detached leaf infection assay can also be used for other studies of bacterial-rice
255 interactions. For example, this system can be used to study rice immune responses conferred by
256 different resistance genes or induced by different bacterial strains. For example, the detached leaf
257 infection assay can be used to study the immune response conferred by other rice *Xa* genes
258 (Khan, Naeem & Iqbal, 2014) that confer resistance to *Xoo* such as *Xa3/Xa26*, which also
259 encodes a cell surface receptor kinase (Xiang et al., 2006; Li et al., 2012). The detached leaf
260 infection assay can also be adapted to study immune responses to other races of *Xoo* (Niño-Liu,
261 Ronald & Bogdanove, 2006) or other *Xanthomonas* pathovars such as *Xanthomonas oryzae* pv.
262 *oryzicola* (Raymundo, Perez & Co, 1992; Niño-Liu, Ronald & Bogdanove, 2006).

263

264 **Acknowledgements**

265 We would like to thank Vasanth R. Singan, Rita Kuo, Mansi Chovatia, and Christopher Daum
266 from the Joint Genome Institute for their help with RNA sequencing.

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. *The Journal of biological chemistry* 285:19035–19042.

Chen X., Zuo S., Schwessinger B., Chern M., Canlas PE., Ruan D., Zhou X., Wang J., Daudi A., Petzold CJ., Heazlewood JL., Ronald PC. 2014. An XA21-associated kinase (OsSERK2) regulates immunity mediated by the XA21 and XA3 immune receptors. *Molecular plant* 7:874–892. DOI: 10.1093/mp/ssu003.

Chinchilla D., Zipfel C., Robatzek S., Kemmerling B., Nürnberger T., Jones JDG., Felix G., Boller T. 2007. A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448:497–500. DOI: 10.1038/nature05999.

Du Z., Zhou X., Ling Y., Zhang Z., Su Z. 2010. agriGO: a GO analysis toolkit for the agricultural community. *Nucleic acids research* 38:W64–70.

Gomez-Gomez 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.

Hashimoto M., Kisseleva L., Sawa S., Furukawa T., Komatsu S., Koshiba T. 2004. A novel rice PR10 protein, RSOsPR10, specifically induced in roots by biotic and abiotic stresses, possibly via the jasmonic acid signaling pathway. *Plant & cell physiology* 45:550–559.

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* 54:533–547. DOI: 10.1023/B:PLAN.0000038261.21060.47.

290 Kang S., Kang K., Lee K., Back K. 2007. Characterization of rice tryptophan decarboxylases and
291 their direct involvement in serotonin biosynthesis in transgenic rice. *Planta* 227:263–272.
292 DOI: 10.1007/s00425-007-0614-z.
293 Kauffman HE., Reddy APK., Hsiek SPV., Marca SD. 1973. An improved technique for
294 evaluating resistance of race varieties to Xanthomonas oryzae. *Plant Disease Managment*
295 *Reports* 57:537–541.
296 Kawahara Y., de la Bastide M., Hamilton JP., Kanamori H., McCombie WR., Ouyang S.,
297 Schwartz DC., Tanaka T., Wu J., Zhou S., Childs KL., Davidson RM., Lin H., Quesada-
298 Ocampo L., Vaillancourt B., Sakai H., Lee SS., Kim J., Numa H., Itoh T., Buell CR.,
299 Matsumoto T. 2013. Improvement of the Oryza sativa Nipponbare reference genome using
300 next generation sequence and optical map data. *Rice* 6:4.
301 Khan MA., Naeem M., Iqbal M. 2014. Breeding approaches for bacterial leaf blight resistance in
302 rice (Oryza sativa L.), current status and future directions. *European journal of plant*
303 *pathology / European Foundation for Plant Pathology* 139:27–37. DOI: 10.1007/s10658-
304 014-0377-x.
305 Li H-J., Li X-H., Xiao J-H., Wing RA., Wang S-P. 2012. Ortholog alleles at Xa3/Xa26 locus
306 confer conserved race-specific resistance against Xanthomonas oryzae in rice. *Molecular*
307 *plant-microbe interactions: MPMI* 5:281–290.
308 Li W., Shao M., Yang J., Zhong W., Okada K., Yamane H., Qian G., Liu F. 2013. Oscyp71Z2
309 involves diterpenoid phytoalexin biosynthesis that contributes to bacterial blight resistance
310 in rice. *Plant science: an international journal of experimental plant biology* 207:98–107.
311 DOI: 10.1016/j.plantsci.2013.02.005.
312 Li L., Li M., Yu L., Zhou Z., Liang X., Liu Z., Cai G., Gao L., Zhang X., Wang Y., Chen S.,

313 Zhou J-M. 2014. The FLS2-associated kinase BIK1 directly phosphorylates the NADPH
314 oxidase RbohD to control plant immunity. *Cell host & microbe* 15:329–338. DOI:
315 10.1016/j.chom.2014.02.009.
316 Lu D., Wu S., Gao X., Zhang Y., Shan L., He P. 2010. A receptor-like cytoplasmic kinase,
317 BIK1, associates with a flagellin receptor complex to initiate plant innate immunity.
318 *Proceedings of the National Academy of Sciences of the United States of America* 107:496–
319 501. DOI: 10.1073/pnas.0909705107.
320 McCarthy DJ., Chen Y., Smyth GK. 2012. Differential expression analysis of multifactor RNA-
321 Seq experiments with respect to biological variation. *Nucleic acids research* 40:4288–4297.
322 Niño-Liu DO., Ronald PC., Bogdanove AJ. 2006. Xanthomonas oryzae pathovars: model
323 pathogens of a model crop. *Molecular plant pathology* 7:303–324. DOI: 10.1111/j.1364-
324 3703.2006.00344.x.
325 Park C-J., Peng Y., Chen X., Dardick C., Ruan D., Bart R., Canlas PE., Ronald PC. 2008. Rice
326 XB15, a protein phosphatase 2C, negatively regulates cell death and XA21-mediated innate
327 immunity. *PLoS biology* 6:e231.
328 Park C-J., Lee S-W., Chern M., Sharma R., Canlas PE., Song M-Y., Jeon J-S., Ronald PC. 2010.
329 Ectopic expression of rice Xa21 overcomes developmentally controlled resistance to
330 Xanthomonas oryzae pv. oryzae. *Plant science: an international journal of experimental*
331 *plant biology* 179:466–471. DOI: 10.1016/j.plantsci.2010.07.008.
332 Penfold RJ., Pemberton JM. 1992. An improved suicide vector for construction of chromosomal
333 insertion mutations in bacteria. *Gene* 118:145–146.
334 Pruitt RN., Schwessinger B., Joe A., Thomas N., Liu F., Albert M., Robinson MR., Chan LJG.,
335 Luu DD., Chen H., Bahar O., Daudi A., De Vleesschauwer 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:e1500245. DOI: 10.1126/sciadv.1500245.

Raymundo AK., Perez MT., Co S. 1992. Xanthomonas oryzae pv. oryzicola. In: *2. Asia-Pacific Biotechnology Congress, College, Laguna (Philippines), 22-24 May 1991*. agris.fao.org,.

Robinson MD., McCarthy DJ., Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26:139–140.

Salzberg SL., Sommer DD., Schatz MC., Phillippy AM., Rabinowicz PD., Tsuge S., Furutani A., Ochiai H., Delcher AL., Kelley D., Madupu R., Puiu D., Radune D., Shumway M., Trapnell C., Aparna G., Jha G., Pandey A., Patil PB., Ishihara H., Meyer DF., Szurek B., Verdier V., Koebnik R., Dow JM., Ryan RP., Hirata H., Tsuyumu S., Won Lee S., Seo Y-S., Sriariyanum M., Ronald PC., Sonti RV., Van Sluys M-A., Leach JE., White FF., Bogdanove AJ. 2008. Genome sequence and rapid evolution of the rice pathogen Xanthomonas oryzae pv. oryzae PXO99A. *BMC genomics* 9:204. DOI: 10.1186/1471-2164-9-204.

Schmittgen TD., Livak KJ. 2008. Analyzing real-time PCR data by the comparative CT method. *Nature protocols* 3:1101–1108. DOI: 10.1038/nprot.2008.73.

Schulze B., Mentzel T., Jehle AK., Mueller K., Beeler S., Boller T., Felix G., Chinchilla D. 2010. Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. *The Journal of biological chemistry* 285:9444–9451.

Schwessinger B., Roux M., Kadota Y., Ntoukakis V., Sklenar J., Jones A., Zipfel C. 2011.

359 Phosphorylation-Dependent Differential Regulation of Plant Growth, Cell Death, and Innate
360 Immunity by the Regulatory Receptor-Like Kinase BAK1. *PLoS genetics* 7:e1002046. DOI:
361 10.1371/journal.pgen.1002046.
362 Schwessinger B., Bahar O., Thomas N., Holton N., Nekrasov V., Ruan D., Canlas PE., Daudi A.,
363 Petzold CJ., Singan VR., Kuo R., Chovatia M., Daum C., Heazlewood JL., Zipfel C.,
364 Ronald PC. 2015a. Transgenic Expression of the Dicotyledonous Pattern Recognition
365 Receptor EFR in Rice Leads to Ligand-Dependent Activation of Defense Responses. *PLoS*
366 *pathogens* 11:e1004809. DOI: 10.1371/journal.ppat.1004809.
367 Schwessinger B., Li X., Ellinghaus TL., Chan LJG., Wei T., Joe A., Thomas N., Pruitt R.,
368 Adams PD., Chern MS., Petzold CJ., Liu CC., Ronald PC. 2015b. A second-generation
369 expression system for tyrosine-sulfated proteins and its application in crop protection.
370 *Integrative Biology*. DOI: 10.1039/C5IB00232J.
371 da Silva FG., Shen Y., Dardick C., Burdman S., Yadav RC., de Leon AL., Pc R. 2004a. Bacterial
372 genes involved in type I secretion and sulfation are required to elicit the rice Xa21-mediated
373 innate immune response. *Molecular plant-microbe interactions: MPMI* 17:593–601.
374 da Silva FG., Shen Y., Dardick C., Burdman S., Yadav RC., de Leon AL., Ronald PC. 2004b.
375 Bacterial genes involved in type I secretion and sulfation are required to elicit the rice
376 Xa21-mediated innate immune response. *Molecular plant-microbe interactions: MPMI*
377 17:593–601. DOI: 10.1094/MPMI.2004.17.6.593.
378 Song WY., Wang GL., Chen LL., Kim HS., Pi LY., Holsten T., Gardner J., Wang B., Zhai WX.,
379 Zhu LH., Fauquet C., Ronald P. 1995. A receptor kinase-like protein encoded by the rice
380 disease resistance gene, Xa21. *Science* 270:1804–1806.
381 Sun Y., Li L., Macho AP., Han Z., Hu Z., Zipfel C., Zhou J-M., Chai J. 2013. Structural basis for

382 flg22-induced activation of the Arabidopsis FLS2-BAK1 immune complex. *Science*
383 342:624–628. DOI: 10.1126/science.1243825.
384 Takeuchi K., Gyohda A., Tominaga M., Kawakatsu M., Hatakeyama A., Ishii N., Shimaya K.,
385 Nishimura T., Riemann M., Nick P., Hashimoto M., Komano T., Endo A., Okamoto T.,
386 Jikumaru Y., Kamiya Y., Terakawa T., Koshiba T. 2011. RSOsPR10 expression in response
387 to environmental stresses is regulated antagonistically by jasmonate/ethylene and salicylic
388 acid signaling pathways in rice roots. *Plant & cell physiology* 52:1686–1696. DOI:
389 10.1093/pcp/pcr105.
390 Wei T., Chern M., Liu F., Ronald PC. 2016. Suppression of bacterial infection in rice by
391 treatment with a sulfated peptide. *Molecular plant pathology*. DOI: 10.1111/mpp.12368.
392 Wengelnik K., Marie C., Russel M., Bonas U. 1996. Expression and localization of HrpA1, a
393 protein of Xanthomonas campestris pv. vesicatoria essential for pathogenicity and induction
394 of the hypersensitive reaction. *Journal of bacteriology* 178:1061–1069.
395 Xiang Y., Cao Y., Xu C., Li X., Wang S. 2006. Xa3, conferring resistance for rice bacterial
396 blight and encoding a receptor kinase-like protein, is the same as Xa26. *TAG. Theoretical*
397 *and applied genetics. Theoretische und angewandte Genetik* 113:1347–1355.
398 Yang B., Sugio A., White FF. 2006. Os8N3 is a host disease-susceptibility gene for bacterial
399 blight of rice. *Proceedings of the National Academy of Sciences of the United States of*
400 *America* 103:10503–10508. DOI: 10.1073/pnas.0604088103.
401 Zipfel C., Kunze G., Chinchilla D., Caniard A., Jones JDG., Boller T., Felix G. 2006. Perception
402 of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated
403 transformation. *Cell* 125:749–760.

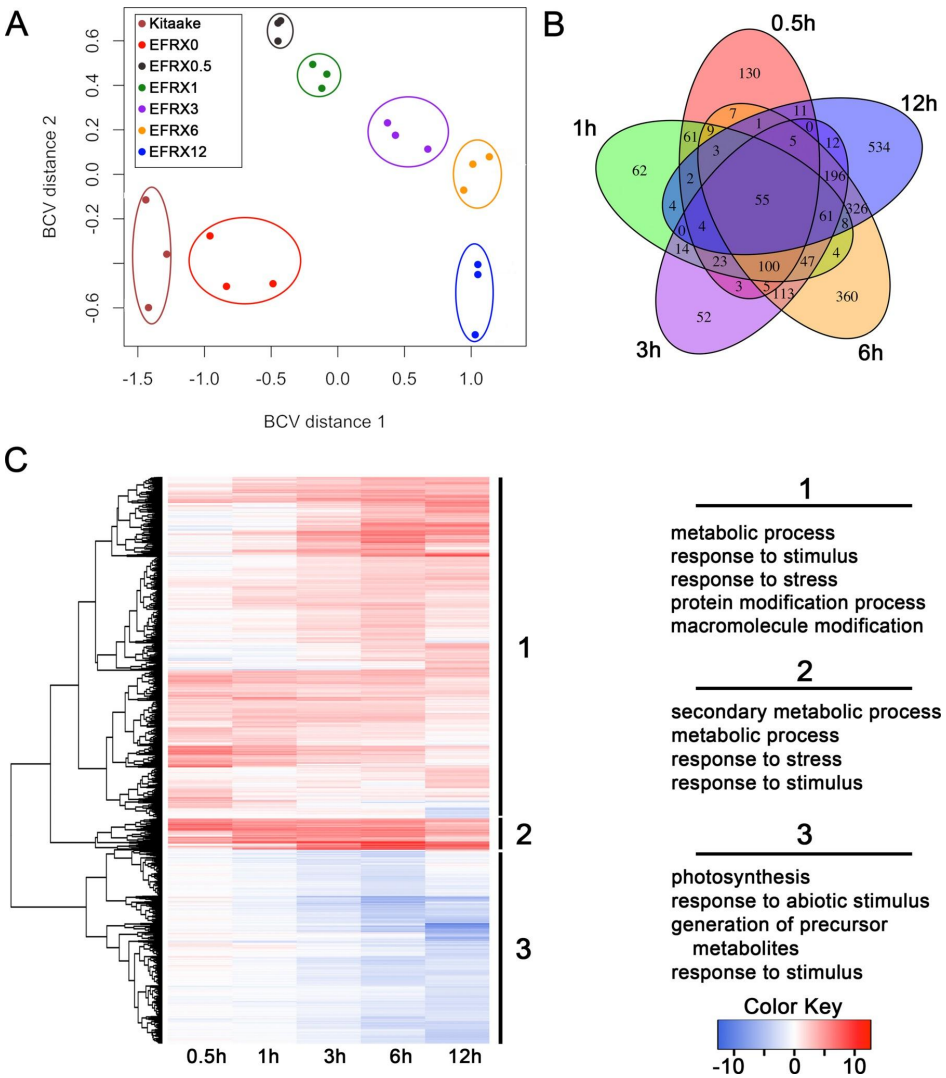


Figure 1. The transcriptomic profile of elf18 treated EFR:XA21:GFP rice is enriched for stress response related and photosynthesis-related genes. (A) Multi-dimensional scaling comparing biological coefficients of variance between each sample. Samples labeled Kit0 are Kitaake rice leaf samples without treatment. Samples labeled Kitaake represent untreated Kitaake samples at 0 h, EFRX represent EFR:XA21:GFP untreated samples (EFRX0) and samples treated with 500 nM elf18 at 0.5h (EFRX0.5), 1 h (EFRX1), 3 h (EFRX3), 6 h (EFRX6), and 12 h (EFRX12). Groups of technical replicates are circled and sample color codes are indicated in upper left legend. (B) A five-way Venn diagram indicating number of unique and overlapping differentially regulated genes between time points . (C) Heatmap representing expression levels of differentially regulated genes (DRGs) for EFR:XA21:GFP samples treated with elf18 for indicated durations. The three major DRG clades, determined by expression profile, are labeled 1, 2 and 3 and are indicated to the right of the heatmap. Significantly enriched gene ontology terms with a false discovery rate less than 0.5, compared to the reference, for each clade are shown on the right under the respective clade number. The heatmap color key indicates $\log_2$ fold change values compared with untreated, EFR:XA21:GFP 0h samples.

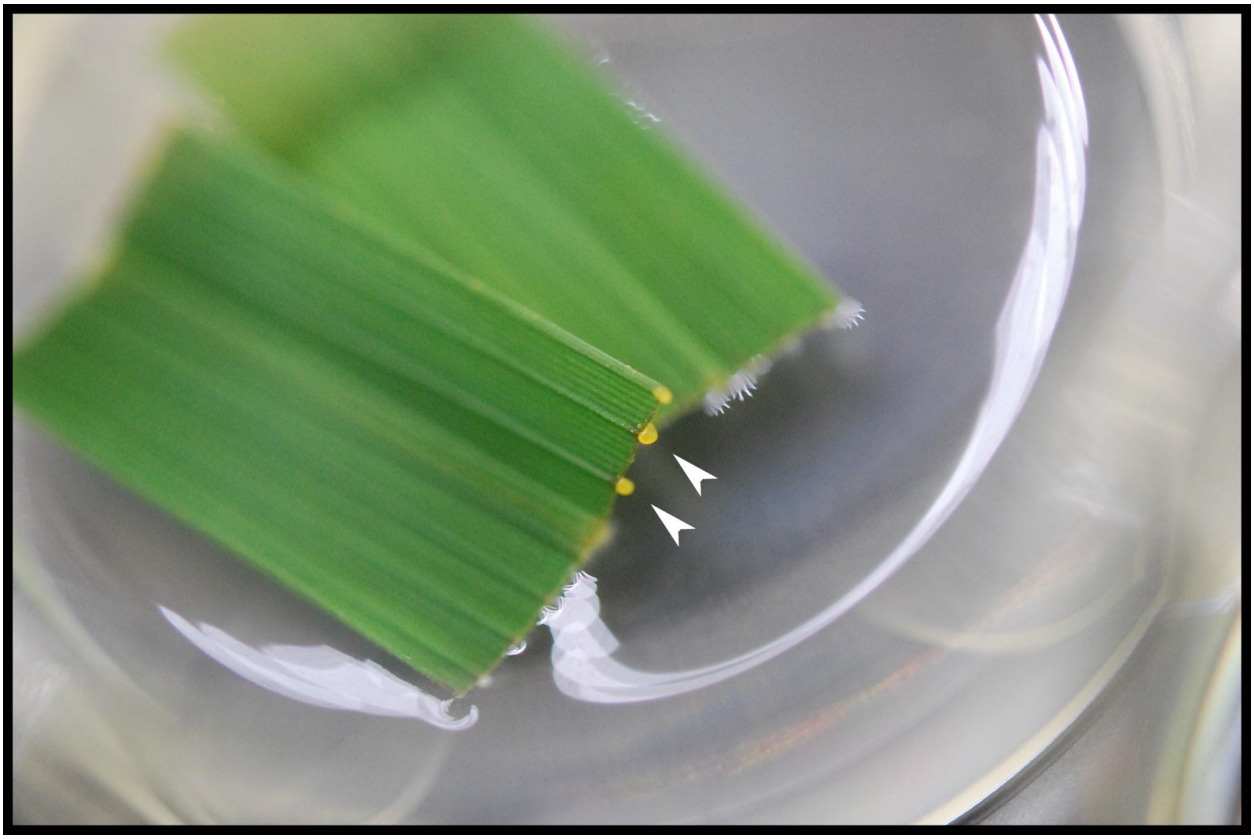


Figure 2. Bacterial oozes from an infected rice leaf. Bacterial oozing (white arrowheads) was observed from rice leaf xylem vessels three days post infection. This image shows detached Kitaake rice leaves infected with PXO99A in a 6-well cell culture plate. Bacterial oozing was consistently observed in Kitaake and Myc:XA21 detached leaves infected with PXO99A. Rice leaves were collected from 4-week old, hydroponically grown plants and floated on *Xoo* cell suspension media.

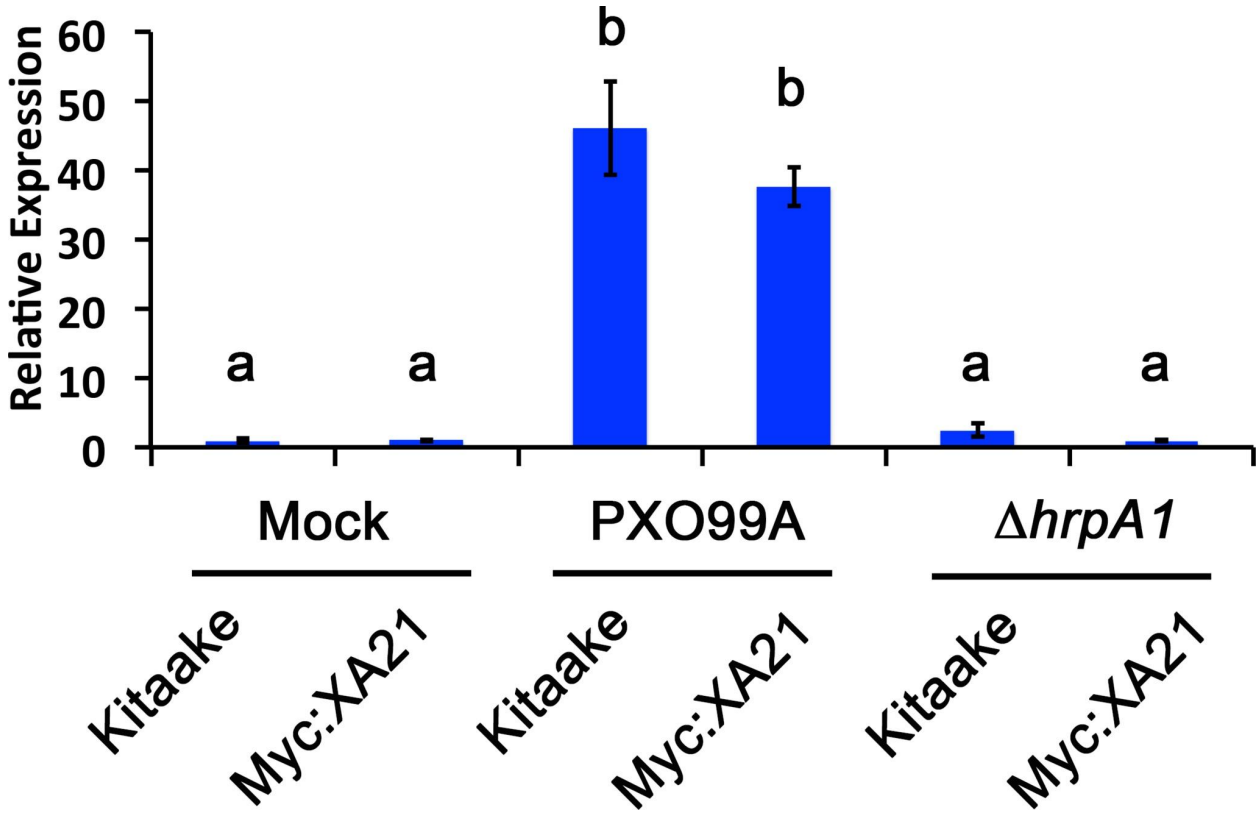


Figure 3. A marker gene of *Xanthomonas* infection, *Os8N3*, is up-regulated in PXO99A infected leaves. *Os8N3* expression in detached Kitaake and Myc:XA21 rice leaves with 10 mM $MgCl_2$ mock treatment or infected with PXO99A or PXO99AΔ*hrpA1* (Δ*hrpA1*) at an $O.D._{600}$ of 0.1. Letters represent statistically significant differences between mean expression values ($p < 0.05$) determined by using a Tukey-Kramer HSD test. This experiment was repeated three times with similar results.

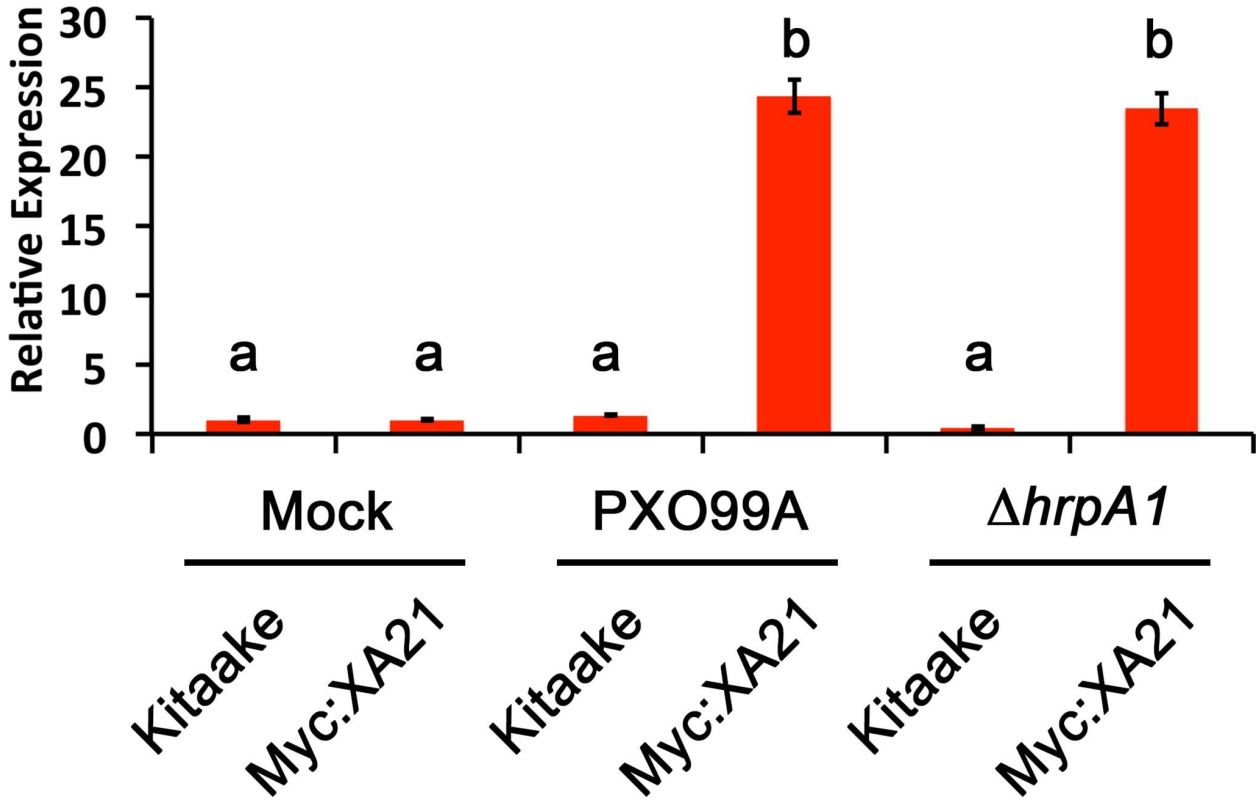


Figure 4. The stress-related marker gene *PR10b* is up-regulated in *Xanthomonas* infected XA21 rice. *PR10b* expression in detached Kitaake and Myc:XA21 rice leaves with 10 mM $MgCl_2$ mock treatment or infected with PXO99A or PXO99AΔ*hrpA1* (Δ*hrpA1*) at an $O.D._{600}$ of 0.1. Letters represent statistically significant differences between mean expression values ($p < 0.05$) determined by using a Tukey-Kramer HSD test. This experiment was repeated three times with similar results.

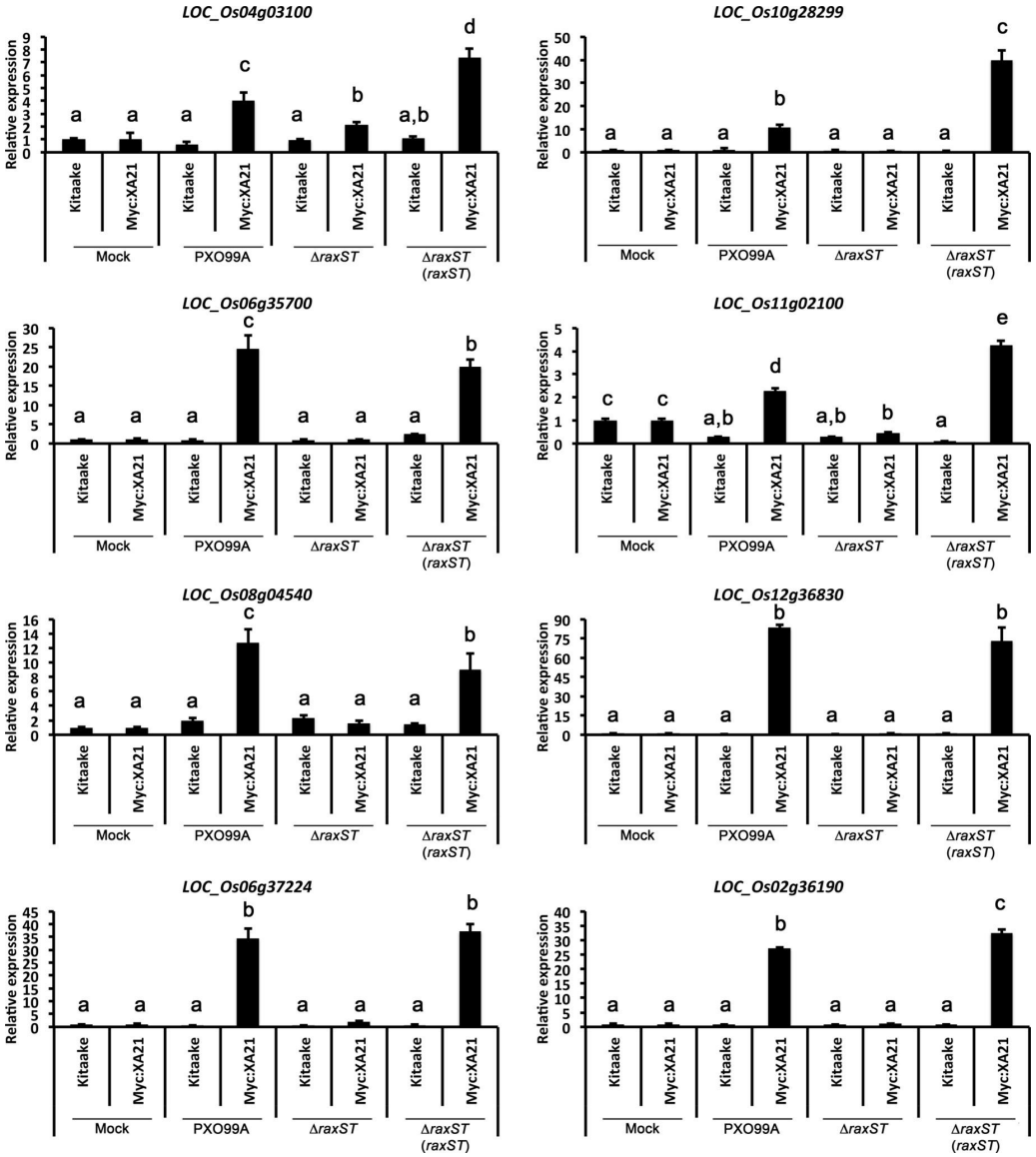
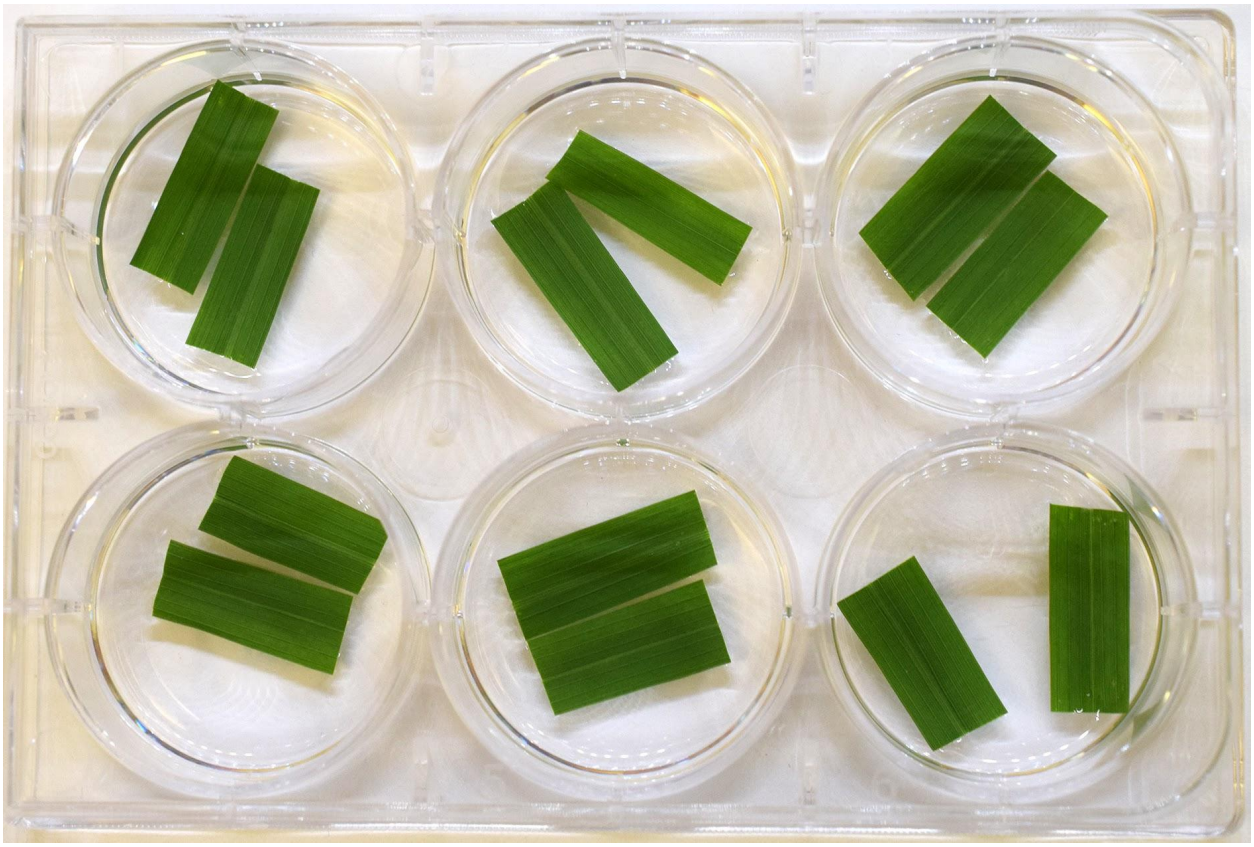
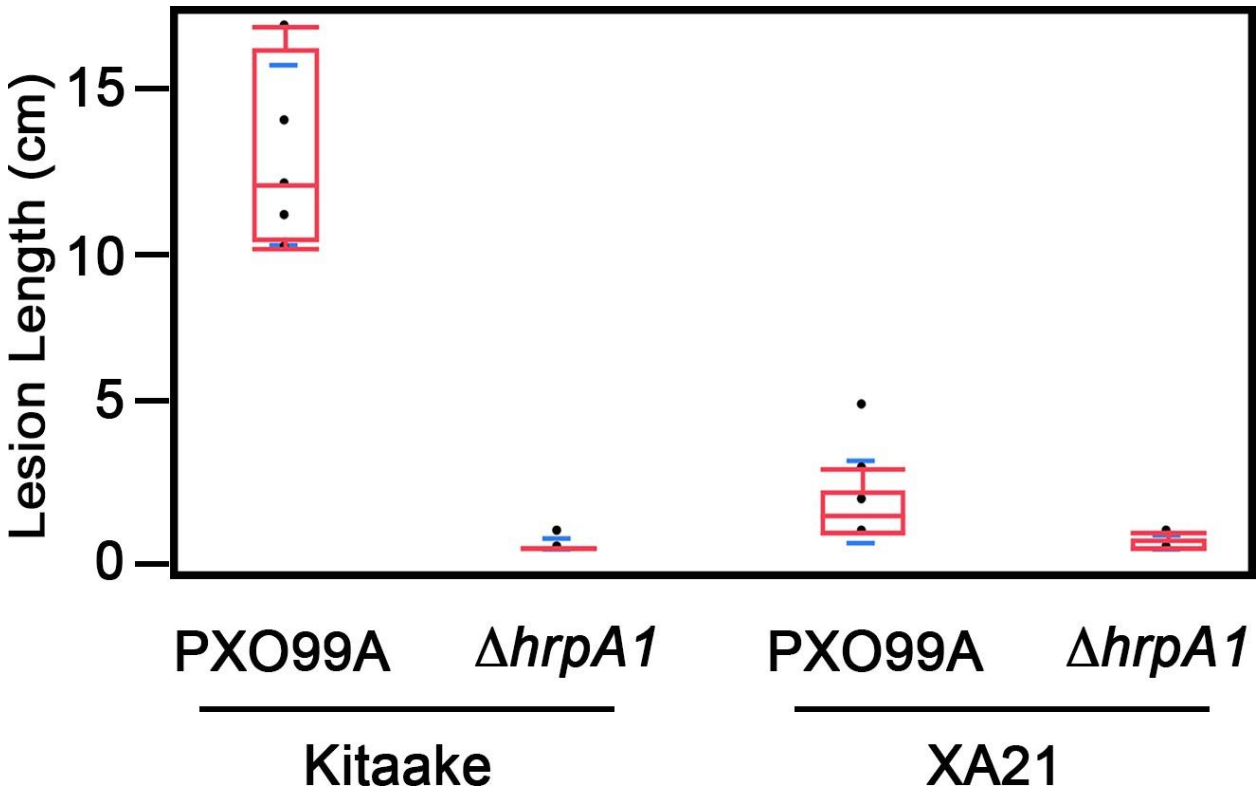


Figure 5. Eight marker genes are specifically up-regulated in detached rice leaves undergoing the XA21-mediated immune response. Expression of 8 genes was measured in detached Kitaake and Myc:XA21 leaves infected with different *Xoo* strains. Mock samples were treated with 10 mM $MgCl_2$. *Xoo* strains used for infection were WT PXO99A, a PXO99AΔ*raxST* mutant strain that evades XA21-mediated immunity (Δ*raxST*), and the PXO99AΔ*raxST* mutant strain complemented with *raxST* (Δ*raxST* (*raxST*)). or mock treated with. Expression levels are normalized to *Actin* then compared to mock treated samples. Shown is one of three biological replicates. Bars indicate mean expression levels ± standard deviation of three technical replicates. Letters represent statistically significant differences between mean expression values ($p < 0.05$) determined by using a Tukey-Kramer HSD test. This experiment was repeated twice with similar results.



Supplemental Figure 1. Image of *Xoo* infection of detached rice leaves. Image of detached rice leaf assay setup. 1.5-2cm detached leaves are floated on ~1.5mL of bacterial suspension in 6-well flat bottom cell culture plates (approximately 12.5 x 8.5 x 2 cm).

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2289v1 | CC BY 4.0 Open Access | rec: 13 Jul 2016, publ: 13 Jul 2016



Supplemental Figure 2. Infection with PXO99AΔ*hrpA1* mutants does not form lesions on Kitaake or XA21 rice leaves. Kitaake or Myc:XA21 rice were inoculated with scissors dipped in PXO99A or PXO99AΔ*hrpA1* (Δ*hrpA1*) at an approximate cell density of $8x10^8$ cells mL^{-1}. Boxplots (red) represent distribution of lesion measurements from three different plants taken 14 days after infection with at least three measurements from each plant ($n \geq 9$). Blue lines indicate standard deviation of the mean.