Cloning of the rice *Xo1* resistance gene and interaction of the Xo1 protein with the defense-suppressing *Xanthomonas* effector Tal2h

Andrew C. Read,[1†] Mathilde Hutin,[1,2†] Matthew J. Moscou,[3] Fabio C. Rinaldi,[1] and Adam J. Bogdanove[1]*

[1] Plant Pathology and Plant-Microbe Biology Section, School of Integrative Plant Science, Cornell University, Ithaca, NY 14853

[2] IRD, CIRAD, Université Montpellier, IPME, 34000 Montpellier, France

[3] The Sainsbury Laboratory, University of East Anglia, Norwich Research Park, Norwich, NR4 7UK, United Kingdom

[†] These authors contributed equally.

Current address for F. C. Rinaldi: Vertex Pharmaceuticals, 50 Northern Avenue, Boston, MA 02210

* Corresponding author: A. J. Bogdanove; ajb7@cornell.edu

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Abstract

The *Xo1* locus in the heirloom rice variety Carolina Gold Select confers resistance to bacterial leaf streak and bacterial blight, caused by *Xanthomonas oryzae* pvs. oryzicola and oryzae, respectively. Resistance is triggered by pathogen-delivered transcription activator-like effectors (TALEs) independent of their ability to activate transcription, and is suppressed by variants called truncTALEs common among Asian strains. By transformation of the susceptible variety Nipponbare, we show that one of 14 nucleotide-binding, leucine-rich repeat (NLR) protein genes at the locus, with a zfBED domain, is the *Xo1* gene. Analyses of published transcriptomes revealed that the *Xo1*-mediated response is similar to those of NLR resistance genes *Pia* and *Rxo1* and distinct from that associated with induction of the executor resistance gene *Xa23*, and that a truncTALE dampens or abolishes activation of defense-associated genes by *Xo1*. In *Nicotiana benthamiana* leaves, fluorescently-tagged Xo1 protein, like TALEs and truncTALEs, localized to the nucleus. And, endogenous Xo1 specifically co-immunoprecipitated from rice leaves with a pathogen-delivered, epitope-tagged truncTALE. These observations suggest that suppression of Xo1-function by truncTALEs occurs through direct or indirect physical interaction. They further suggest

that effector co-immunoprecipitation may be effective for identifying or characterizing other resistance genes.

Bacterial leaf streak of rice, caused by *Xanthomonas oryzae* pv. oryzicola (Xoc), is an increasing threat to production in many parts of the world, especially in Africa. Bacterial blight of rice, caused by *X. oryzae* pv. oryzae (Xoo) has long been a major constraint in Asia and is becoming prevalent in Africa. The purified American heirloom rice variety Carolina Gold Select (hereafter Carolina Gold; McClung and Fjellstrom, 2010) is resistant to all tested African strains of Xoc and some tested strains of Xoo (Read et al., 2016). Using an African strain of Xoc, the resistance was mapped to chromosome 4 and designated as *Xo1* (Triplett et al., 2016). Both Xoc and Xoo deploy multiple type III-secreted transcription activator-like effectors (TALEs) during infection. TALEs enter the plant nucleus and bind to promoters, each with different sequence specificity, to transcriptionally activate effector-specific target genes (Perez-Quintero and Szurek, 2019). Some of these genes, called susceptibility genes, contribute to disease development (Hutin et al., 2015). In some host genotypes, a TALE may activate a so-called executor resistance gene, leading to host cell death that stops the infection (Bogdanove et al., 2010). Most of the cloned resistance genes for bacterial blight are in fact executor genes (Zhang et al., 2015). *Xo1* is different. It mediates resistance in response to TALEs non-specifically, independent of their ability to activate transcription (Triplett et al., 2016). Also, unlike executor genes, *Xo1* function is suppressed by a

variant class of these effectors known as truncTALEs (also called iTALEs), which nuclear localize (Ji et al., 2016) but do not bind DNA (Read et al., 2016).

Xo1 maps to a region that in the reference rice genome (cv. Nipponbare) contains seven nucleotide-binding, leucine-rich repeat protein genes ("NLR" genes) (Triplett et al., 2016). NLR genes are the largest class of plant disease resistance genes. NLR proteins recognize specific, corresponding pathogen effector proteins typically through direct or indirect protein-protein interactions, and mediate downstream defense signaling that leads to expression of defense genes and a programmed localized cell death, the hypersensitive reaction (HR) (Lolle et al., 2020). Recently, by whole genome sequencing, we determined that the *Xo1* locus in Carolina Gold comprises 14 NLR genes. We identified one of these, $Xo1_{11}$, as a strong candidate based on its structural similarity to the previously cloned and only known NLR resistance gene for bacterial blight, *Xa1* (Read et al., 2020). *Xa1*, originally identified in the rice variety Kogyoku, maps to the same location (Yoshimura et al., 1998) and behaves similarly to *Xo1*: it responds to TALEs non-specifically (and thus confers resistance also to bacterial leaf streak), and its activity is suppressed by truncTALEs (Ji et al., 2016). $Xo1_{11}$ and *Xa1* are members of a small subfamily of NLR genes that encode an unusual N-terminal domain comprising a zinc finger BED motif (Read et al., 2020).

To ascertain whether $Xo1_{11}$ is the gene responsible for *Xo1* resistance, we generated transgenic Nipponbare plants expressing it. We amplified the genomic $Xo1_{11}$ coding sequence (5,882 bp) as well as the 993 bp promoter region upstream of the start codon and cloned them together into a binary vector with a 35S terminator to generate plasmid pAR902. *Agrobacterium tumefaciens* strain EHA101 carrying pAR902 was

used for the transformation, which was performed by the Cornell University Plant Transformation Facility. After rooting, regenerants from two independent events were moved to soil and maintained in a growth chamber. These T0 plants were inoculated by syringe infiltration with African Xoc strain CFBP7331, which has no truncTALE of its own, carrying either an empty vector (EV) or the plasmid-borne truncTALE gene *tal2h* (p2h) from the Asian Xoc strain BLS256 (Read et al., 2016). Inoculum was confirmed on untransformed Nipponbare and Carolina Gold plants (**Fig. S1**). Plants from both *Xo1* events displayed resistance to the strain with the EV, manifesting as HR (necrosis) and lack of water-soaking, and this was suppressed by Tal2h (**Fig. 1**), demonstrating that $Xo1_{11}$ is the *Xo1* gene.

NLR protein activation is characteristically followed by a suite of responses that includes massive transcriptional reprogramming leading both to HR and to activation of a large number of defense-associated genes (Cui et al., 2015). To gain insight into the nature of *Xo1*-mediated resistance, we compared the global profile of differentially expressed genes during *Xo1*-mediated defense to those of two other NLR genes in rice, and to the profile associated with an executor gene. We used our previously reported RNAseq data from Carolina Gold plants inoculated with CFBP7331(EV) or mock inoculum (Read et al., 2020), data for the NLR gene *Pia* for resistance to the rice blast pathogen *Magnaporthe oryzae* (Tanabe et al., 2014), data from rice resistant to bacterial leaf streak due to transgenic expression of the maize NLR gene *Rxo1* (Xie et al., 2007; Zhou et al., 2010), and data for the transcriptomic response associated with induction of the executor resistance gene *Xa23* by an Xoo strain with the corresponding TALE (Tariq et al., 2018). Differentially expressed genes (log_2-fold change >1 or <-1; *p*-

value >0.05) in the comparison between pathogen-inoculated and mock-inoculated plants were compared across the four datasets. The total number of DEGs ranged from 10,050 for *Xo1* to 628 for *Xa23* (**Fig. 2A**, **Table S1**). For each resistance gene, there were a number of DEGs found only in the pathogen to mock comparison for that dataset, and this was highest for *Xo1* (7,121 genes) (**Fig. 2A, Table S1**). These DEGs may be specific to the rice-genotype and pathogen combinations assayed, or they may be due to differences in the expression assay (RNAseq vs. microarray), annotation, or timepoints used. Overall, the DEG profile for *Xo1* was more similar to those of *Pia* and *Rxo1* than to the profile for *Xa23* (**Fig. 2A**). This was even more apparent when the expression of 340 rice genes associated with plant defense response (gene ontology group 0006952) was examined (**Fig. 2B**). The *Xo1* profile comprised the largest number of plant defense DEGs (99), and these included 16 of 26 total defense DEGs for *Rxo1*, 26 of 46 for *Pia*, and 8 of only 14 for *Xa23* (**Fig. 2B** and **Table S2**).

We also compared DEGs relative to mock in Carolina Gold plants inoculated with CFBP7331(EV) and Carolina Gold plants inoculated with CFBP7331(p2h) (Read et al., 2020), to gain insight into how *Xo1*-mediated resistance is overcome by a pathogen delivering a truncTALE. In contrast to the 99 defense response genes differentially expressed in response to CFBP7331(EV), only 18 defense genes were differentially expressed in response to CFBP7331(p2h) (**Fig. 2C**). Of these 18 genes, 7 were differentially expressed only in the response to the strain with *tal2h*, 4 up and 3 down. Of the remaining 11, 3 were up and 2 were down in both responses, but each less so in the response to the strain with *tal2h*. The other 6 moved in opposite directions entirely, up in the absence but repressed in the presence of *tal2h*, relative to mock. This

136 expression profile during suppression of *Xo1*-mediated resistance is consistent with
137 Tal2h functioning early in the defense cascade. Interestingly, the Xoc susceptibility gene
138 *OsSULTR3;6* is strongly induced by both CFBP7331(EV) and CFBP7331(p2h),
139 indicating that TALE function is not compromised by Xo1 or by Tal2h.

140 The observation that Xo1 reprograms transcription in a manner consistent with
141 other rice NLR proteins upon recognition of the cognate pathogen effector and that
142 reprogramming by Xo1 is essentially blocked by Tal2h led us to explore whether Xo1
143 localizes to the same subcellular location as TALEs and truncTALEs. Some, but not all,
144 NLR proteins nuclear localize (Shen et al., 2007; Wirthmueller et al., 2007; Caplan et al.,
145 2008; Cheng et al., 2009), and we previously identified putative nuclear localization
146 signals (NLSs) in $Xo1_{11}$ (Read et al., 2020). We generated expression constructs for a
147 green fluorescent protein (GFP) fusion to the N-terminus of Xo1 as well as an N-
148 terminal monomeric red fluorescent protein (mRFP) fusion both to a TALE (Tal1c of Xoc
149 BLS256) and to Tal2h. These constructs were delivered into *Nicotiana benthamiana*
150 leaves using *A. tumefaciens* strain GV3101, and the leaves imaged with a Zeiss 710
151 confocal microscope (**Fig. 3**). GFP-Xo1 in the absence of either effector but with free
152 mRFP localized to foci that appeared to be nuclei. Co-expression with mRFP-Tal1c or
153 with mRFP-Tal2h confirmed that these foci were nuclei.

154 The localization of Xo1, the TALE, and the truncTALE to the nucleus when
155 transiently expressed in *N. benthamiana* led us to pursue the hypothesis that Xo1
156 physically interacts with one or both of these proteins in the native context. We
157 generated plasmid constructs that add a 3x FLAG tag to the C-terminus of Tal1c or
158 Tal2h (Tal1c-FLAG and Tal2h-FLAG) and introduced them individually into the TALE-

deficient *X. oryzae* strain X11-5A (Triplett et al., 2011) for co-immunoprecipitation from inoculated Carolina Gold leaves (**Fig. 4**). We included also a plasmid for expression of a second, untagged TALE (Tal3c from BLS256) and a plasmid for untagged Tal2h. By pairing the X11-5A transformants with each other or with the untransformed control strain, we were able to probe for Carolina Gold proteins interacting with the tagged TALE or truncTALE, and for interactions of these proteins with each other or with the second TALE. Select combinations were inoculated to Nipponbare leaves for comparison. Inoculation was done by syringe infiltration, in 30-40 contiguous spots on each side of the leaf midrib. For each co-inoculation, tissue was harvested at 48 hours and ground in liquid N_2, then soluble extract was incubated with anti-FLAG agarose beads and washed to immunopurify the tagged and interacting proteins. Immunoprecipitates were eluted, and an aliquot of each was subjected to western blotting with anti-TALE antibody (**Fig. S2**). The remainders were then resolved on a 4-20% SDS-PAGE and eluates from gel slices containing proteins between approximately 60 and 300 kDa (**Fig. S3**) were digested and the peptides analyzed by mass spectrometry. Proteins were considered present in a sample if at least three peptides mapped uniquely to any of the pertinent annotated genomes searched: the *X. oryzae* strain X11-5A genome (Triplett et al., 2011) plus the TALE(s) or TruncTALE being expressed, the Nipponbare genome (MSU 7; Kawahara et al., 2013), and the Carolina Gold genome (Read et al., 2020). For the Carolina Gold genome, we re-annotated using the RNAseq data from CFBP7331(EV), CFBP7331(p2h), and mock-inoculated plants cited earlier. We carried out the experiment twice.

In the western blot for each experiment (**Fig. S2**), we detected the tagged TALE or truncTALE in each corresponding sample, with the exception of a Tal1c-FLAG/Tal3c/Nipponbare sample in the first experiment. No Tal3c or untagged Tal2h was detected in any sample. The mass spectrometry confirmed these observations, suggesting that neither TALEs with truncTALEs nor TALEs with other TALEs interact appreciably (**Fig. 4**). Xo1 was consistently detected in the Carolina Gold/Tal2h-FLAG samples, irrespective of any co-delivered Tal1c or Tal3c, and not in any other sample (**Fig. 4**). No other protein consistently co-purified with Tal2h-FLAG or Tal1c-FLAG in either Carolina Gold or Nipponbare samples (**Dataset S1**).

In summary, we have shown that 1) an NLR protein gene at the *Xo1* locus, harboring an integrated zfBED domain, is *Xo1*; 2) the *Xo1*-mediated response is similar to those mediated by two other NLR resistance genes and distinct from that associated with TALE-specific transcriptional activation of an executor resistance gene; 3) a truncTALE abolishes or dampens activation of defense-associated genes by *Xo1*; 4) the Xo1 protein, like TALEs and truncTALEs, localizes to the nucleus, and 5) Xo1 specifically co-immunoprecipitates from rice leaves with a pathogen-delivered, epitope-tagged truncTALE. Thus, *Xo1* is an allele or paralog of *Xa1*, and suppression of Xo1 function by a truncTALE is likely the result of physical interaction between the resistance protein and the effector.

Whether the interaction is direct or indirect is not certain, but that fact that no other protein was detected consistently that co-immunoprecipitated with Tal2h and Xo1 suggests the interaction is direct. It is tempting to speculate that TALEs trigger Xo1-mediated resistance also by direct interaction with the protein and that truncTALEs

function by disrupting the association. This is consistent with the results of our comparative analysis of the Xo1 DEG profile during TALE-triggered HR, which showed it to be a typical NLR protein-mediated response and thus plausibly the result of direct interaction with the TALE. And it is consistent with the Xo1 DEG profile during suppression by Tal2h, which suggested that Tal2h functions early in the defense cascade, perhaps by blocking TALE recognition. While tagged Tal1c did not detectably pull down Xo1, it is possible that the interaction might be weak, or transient, or that any complex of the proteins in the plant cells had begun to degrade with the developing HR at the 48 hour time point sampled. An alternative hypothesis is that Tal2h interacts with TALEs and masks them from the resistance protein, but both our co-immunoprecipitation results and the fact that Tal2h did not impact TALE activation of the *OsSULTR3;6* susceptibility suggest that this is not the case.

The results presented constitute an important step toward understanding how Xo1 works, and how its function can be suppressed by the pathogen. An immediate next step might be to determine the portion(s) of Xo1 involved in its interaction with Tal2h. Our previous comparison of the motifs present in $Xo1_{11}$, Xa1, and the closest Nipponbare homolog (Nb-$xo1_5$, which is expressed) revealed that the zfBED and CC domains are identical and the NB-ARC domains nearly so (Read et al., 2020). In contrast, the leucine rich repeat domain of Nb-$xo1_5$ differs markedly from those of Xo1 and Xa1, which, with the exception of an additional repeat in Xa1, are very similar. Thus, the LRR may be the determinative interacting domain. Supporting this hypothesis, differences in the LRR determine the pathogen race specificities of some flax rust resistance genes (Ellis et al., 1999). More broadly, the ability of tagged Tal2h to pull

227 down Xo1 suggests that effector co-immunoprecipitation may be an effective approach
228 to characterizing pathogen recognition mechanisms of other resistance proteins, or for
229 identifying a resistance gene *de novo*.
230
231
232 **Figure legends**
233
234 **Fig. 1.** Transgenic Nipponbare plants expressing $Xo1_{11}$ are resistant to African Xoc
235 strain CFBP7331 and the resistance suppressed by a truncTALE. Susceptible cultivar
236 Nipponbare was transformed with pAR902, and leaves of T0 plants from two events
237 were syringe-infiltrated with African Xoc strain CFBP7311 carrying either empty vector
238 (EV) or *tal2h* (p2h) adjusted to OD_{600} 0.4. Leaves were photographed on a light box at 4
239 days after inoculation. Resistance is apparent as HR (necrosis) at the site of inoculation
240 and disease as expanded, translucent watersoaking.
241
242 **Fig. 2.** The Xo1-mediated transcriptomic response is similar to those of other NLR
243 genes and is essentially eliminated by Tal2h. **A,** Expression heatmaps (columns)
244 showing all differentially expressed genes (DEGs) in plants undergoing the resistant
245 response compared to mock inoculated plants for *Xo1*, the NLR genes *Pia* and *Rxo1*,
246 and the executor resistance gene *Xa23*. White numbers for each on the heatmap
247 indicate the number of DEGs specific to each response (see **Table S1**). Total numbers
248 of DEGs are indicated below. **B,** Heatmaps for the subset of DEGs from (A) that belong
249 to gene ontology group 0006952, defense response, with totals displayed at bottom. **C,**

Heatmaps for the 18 defense response DEGs identified in the comparison of Carolina Gold plants inoculated with CFBP7331(p2h) to mock inoculated plants. The “EV” heatmap shows their expression relative to mock in Carolina Gold plants inoculated with CFBP7331(EV) (resistance), and the “p2h” column shows their expression relative to mock in the presence of Tal2h (disease). The DEGs have been divided into five categories: **I**, induced in both; **II**, down-regulated in both; **III**, down-regulated in resistance and induced in disease; **IV**, not differentially expressed in resistance and down-regulated in disease; and **V**, not differentially expressed in resistance and induced in disease.

Fig. 3. Xo1 localizes to the nucleus. Using *Agrobacterium* co-infiltrations, an expression construct for Xo1 with GFP at the N-terminus (GFP-Xo1) together with a p19 silencing suppressor construct were introduced into *Nicotiana benthamiana* leaves alone or with a construct for mRFP, mRFP fused to TALE Tal1c (mRFP-Tal1c), or mRFP fused to the truncTALE Tal2h (mRFP-Tal2h). Confocal image stacks were taken at 3 days after inoculation and are presented as maximum intensity projections. Insets are magnifications of individual nuclei. The scale bars represent 50 µm.

Fig. 4. Xo1 co-immunoprecipitates with Tal2h. **Top,** strategy used for co-immunoprecipitation (Co-IP) of truncTALE Tal2h or TALE Tal1c and any interactors. Plasmid borne expression constructs for Tal2h or Tal1c with a C-terminal 3x FLAG tag, as well as untagged Tal2h and a second TALE,Tal3c were introduced into *Xanthomonas oryzae* strain X11-5. Paired combinations of the transformants with each

other or with the untransformed control strain, or the control strain alone, were co-infiltrated into leaves of rice varieties Carolina Gold and Nipponbare at a final OD_{600} 0.5 for each transformant. Samples were collected 48 hours after inoculation, ground, and sonicated before Co-IP using anti-FLAG agarose beads. After elution and SDS-PAGE separation, proteins between approximately 60 and 300 kDa were eluted, digested and analyzed by mass spectrometry. The experiment was conducted twice. **Bottom**, co-IP results. For each immunoprecipitate, the numbers of unique peptides detected that matched Tal2h, Tal3c, Tal1c, or Xo1 in each experiment are shown. "-" indicates that ≤ 2 unique peptides were detected.

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Author contributions

AR, MH, FR, and AB conceived and designed the study; AR, MH, and FR carried out the experiments; AR, MH, FR, MM, and AB analyzed data; AR, MH, and AB wrote the manuscript.

Supplemental files

1. Supplemental text and figures

Materials and methods

Fig. S1. Confirmation of CFBP7331(EV) and CFBP(p2h) inoculum on Nipponbare and Carolina Gold plants

Fig. S2. Western blot of immunoprecipitates using anti-TALE antibody

Fig. S3. SDS-PAGE of immunoprecipitates and size range excised for mass spectrometry

Supplemental references

2. Supplemental tables

Table S1. DEGs in Fig. 2A (all DEGS)

Table S2. DEGs in Fig. 2B (GO:0006952 DEGs)

Table S3. DEGs in Fig. 2C (GO:0006952 in disease)

3. Dataset S1. Mass spectrometry data

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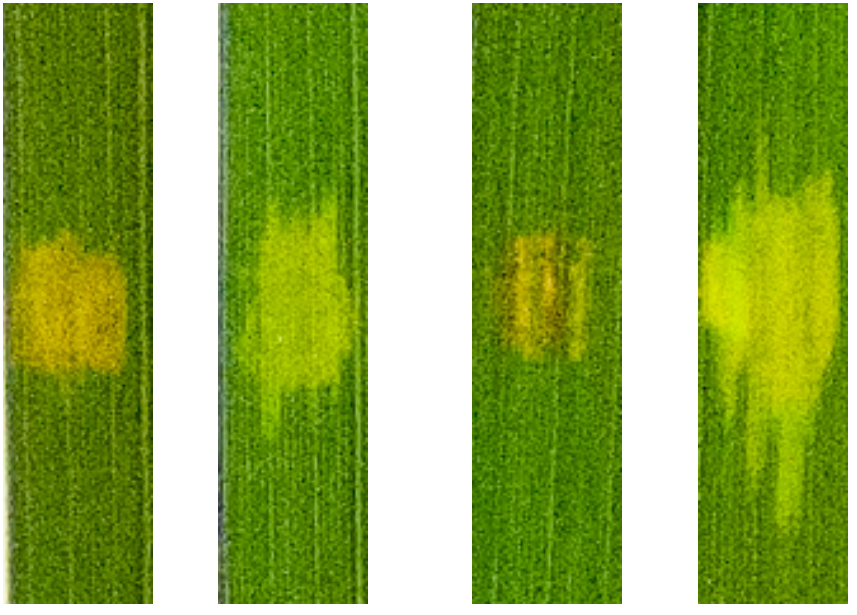
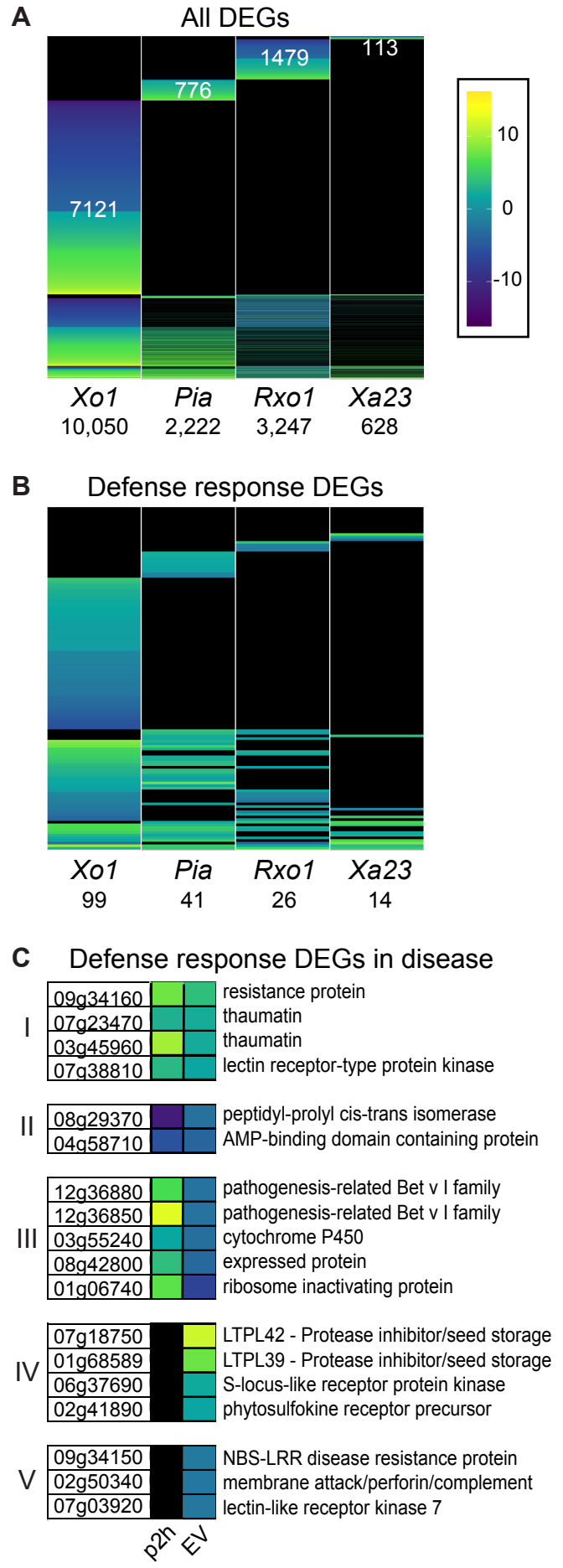
Xoc CFBP7331

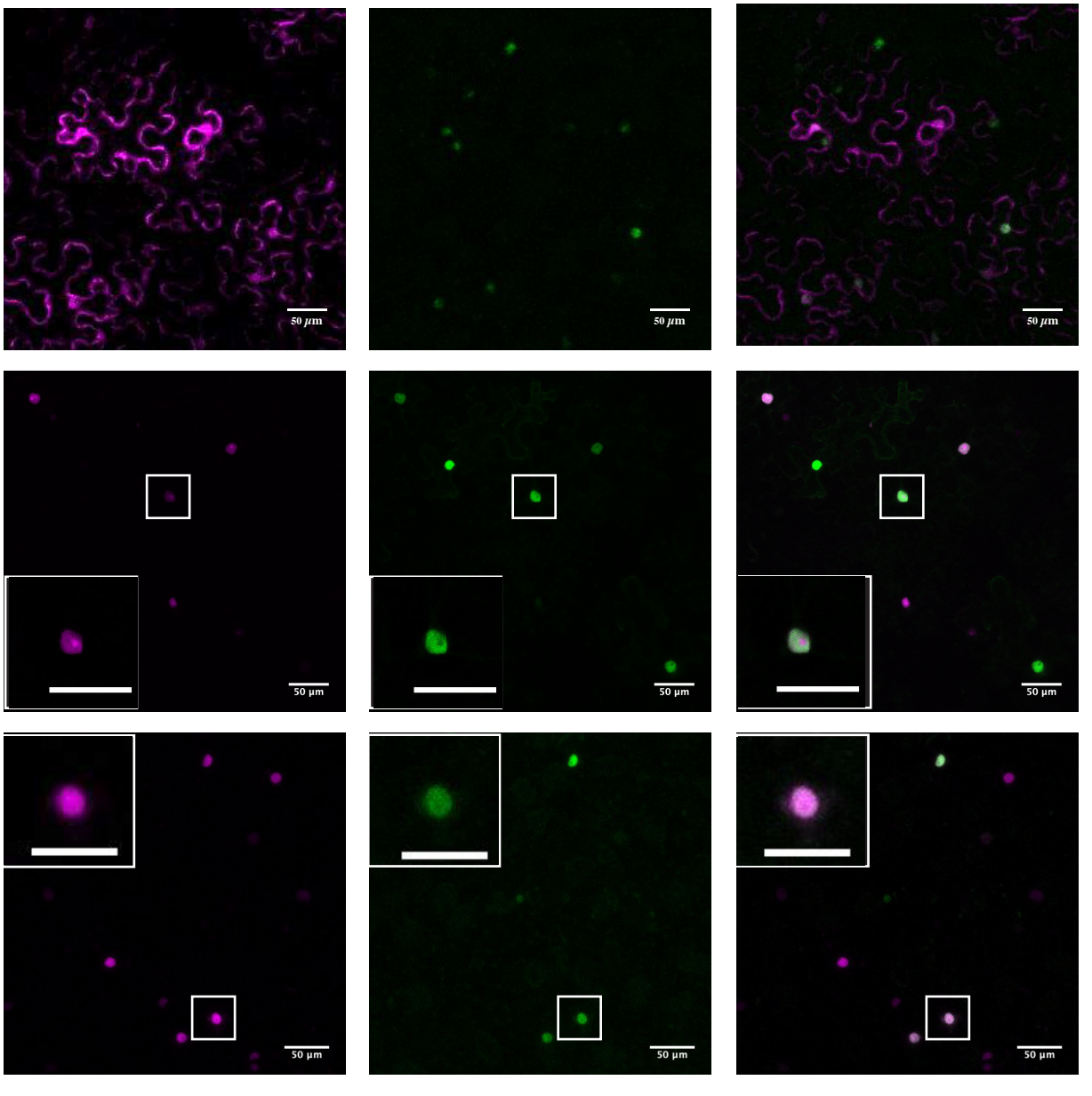
Event 1 Event 2

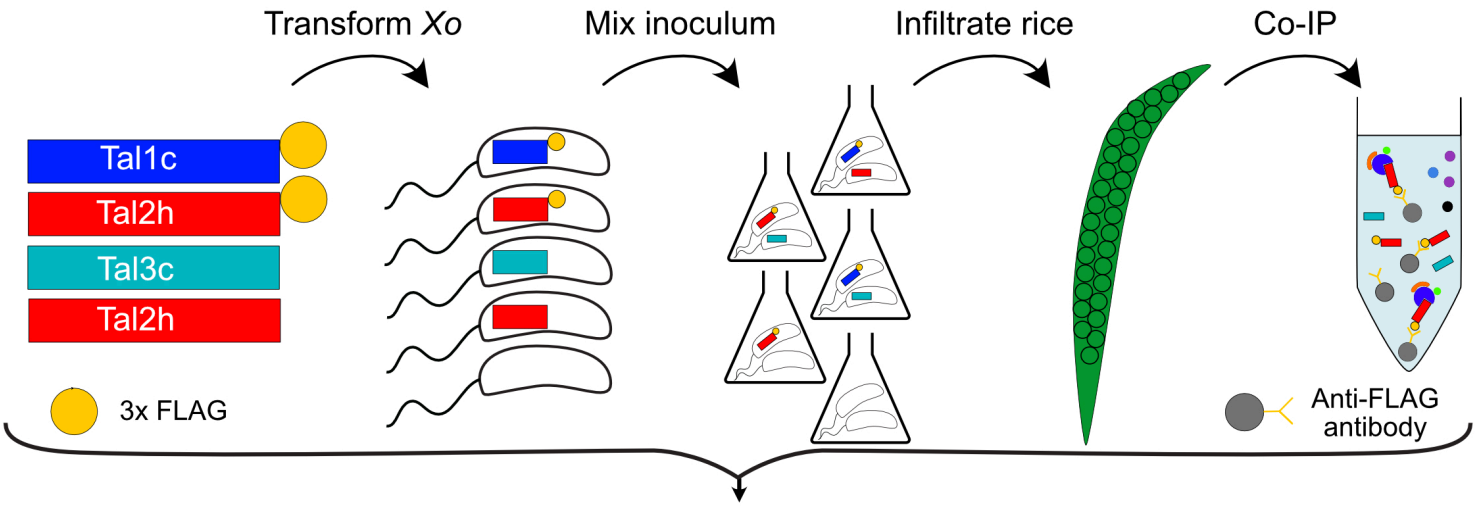
EV p2h EV p2h



R D R D


A
All DEGs
1479
113
776
7121
10
0
-10
Xo1
Pia
Rxo1
Xa23
10,050
2,222
3,247
628
B
Defense response DEGs
Xo1
Pia
Rxo1
Xa23
99
41
26
14
C
Defense response DEGs in disease
I
09g34160 resistance protein
07g23470 thaumatin
03g45960 thaumatin
07g38810 lectin receptor-type protein kinase
II
08g29370 peptidyl-prolyl cis-trans isomerase
04g58710 AMP-binding domain containing protein
III
12g36880 pathogenesis-related Bet v I family
12g36850 pathogenesis-related Bet v I family
03g55240 cytochrome P450
08g42800 expressed protein
01g06740 ribosome inactivating protein
IV
07g18750 LTPL42 - Protease inhibitor/seed storage
01g68589 LTPL39 - Protease inhibitor/seed storage
06g37690 S-locus-like receptor protein kinase
02g41890 phytosulfokine receptor precursor
V
09g34150 NBS-LRR disease resistance protein
02g50340 membrane attack/perforin/complement
07g03920 lectin-like receptor kinase 7
p2h
EV


Red
Green
Merged
mRFP / GFP-Xo1
mRFP-Tal1c / GFP-Xo1
mRFP-Tal2h / GFP-Xo1
50 μm



	Carolina Gold										Nipponbare					
Input			Tal2h		Tal2h + Tal3c		Tal1c + Tal3c		Tal1c + Tal2h				Tal2h + Tal3c		Tal1c + Tal3c	
Experiment	#1	#2	#1	#2	#1	#2	#1	#2	#1	#2	#1	#2	#1	#2	#1	#2
Tal2h	-	-	5	16	7	13	-	-	-	-	-	-	8	15	-	-
Tal3c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tal1c	-	-	-	-	-	-	5	14	7	9	-	-	-	-	-	11
Xo1	-	-	4	6	3	6	-	-	-	-	-	-	-	-	-	-