

A transcription activator-like effector from *Xanthomonas oryzae* pv. *oryzicola* elicits dose-dependent resistance in rice

AARON W. HUMMEL¹†, KATHERINE E. WILKINS^{2,3}, LI WANG², R. ANDRES CERNADAS²‡ AND ADAM J. BOGDANOVE^{1,2,*}

¹Department of Plant Pathology and Microbiology, Iowa State University, 351 Bessey Hall, Ames, IA 50011, USA

²Plant Pathology and Plant–Microbe Biology Section, School of Integrative Plant Science, Cornell University, Ithaca, NY 14853, USA

³Graduate Field of Computational Biology, Cornell University, Ithaca, NY 14853, USA

SUMMARY

Xanthomonas spp. reduce crop yields and quality worldwide. During infection of their plant hosts, many strains secrete transcription activator-like (TAL) effectors, which enter the host cell nucleus and activate specific corresponding host genes at effector binding elements (EBEs) in the promoter. TAL effectors may contribute to disease by activating the expression of susceptibility genes or trigger resistance associated with the hypersensitive reaction (HR) by activating an executor resistance (*R*) gene. The rice bacterial leaf streak pathogen *X. oryzae* pv. *oryzicola* (Xoc) is known to suppress host resistance, and no host *R* gene has been identified against it, despite considerable effort. To further investigate Xoc suppression of host resistance, we conducted a screen of effectors from BLS256 and identified Tal2a as an HR elicitor in rice when delivered heterologously by a strain of the closely related rice bacterial blight pathogen *X. oryzae* pv. *oryzae* (Xoo) or by the soybean pathogen *X. axonopodis* pv. *glycines*. The HR required the Tal2a activation domain, suggesting an executor *R* gene. Tal2a activity was differentially distributed among geographically diverse Xoc isolates, being largely conserved among Asian isolates. We identified four genes induced by Tal2a in next-generation RNA sequencing experiments and confirmed them using quantitative real-time reverse transcription-polymerase chain reaction (qPCR). However, neither individual nor collective activation of these genes by designer TAL effectors resulted in HR. A *tal2a* knockout mutant of BLS256 showed virulence comparable with the wild-type, but plasmid-based overexpression of *tal2a* at different levels in the wild-type reduced virulence in a directly corresponding way. Overall, the results reveal that host resistance suppression by Xoc plays a critical role in pathogenesis. Further, the dose-dependent avirulence activity of Tal2a and the apparent lack of a single canonical target that accounts for HR point to a novel, activation domain-dependent

mode of action, which might involve, for example, a non-coding gene or a specific pattern of activation across multiple targets.

Keywords: bacterial leaf streak of rice, resistance, TAL effector, *Xanthomonas*.

INTRODUCTION

Xanthomonas is a large group of bacteria that collectively infect a variety of important crop species, including soybean, cassava, cotton, banana, wheat and rice, several of which are staple foods in the developing world. The rice (*Oryza sativa*) pathogens *Xanthomonas oryzae* pv. *oryzicola* (Xoc) and *Xanthomonas oryzae* pv. *oryzae* (Xoo) cause bacterial leaf streak and bacterial blight by colonizing the leaf mesophyll and xylem, respectively, and cause yield losses as high as 30% and 50%, respectively (Niño-Liu *et al.*, 2006). Several *Xanthomonas* species and pathovars, including Xoc and Xoo, rely on transcription activator-like (TAL) effectors to promote disease (Al-Saadi *et al.*, 2007; Antony *et al.*, 2010; Athinuwat *et al.*, 2009; Cernadas *et al.*, 2014; Kay *et al.*, 2007; Sugio *et al.*, 2007; Swarup *et al.*, 1991; Wichmann and Bergelson, 2004; Yang and White, 2004; Yang *et al.*, 1994, 2006; Yu *et al.*, 2011).

Functioning as heterologous transcription factors, TAL effectors (Yang *et al.*, 2006) are delivered via the bacterial type III secretion system into host cells (Szurek *et al.*, 2002), where C-terminal nuclear localization signals direct them to the nucleus (Gurlebeck *et al.*, 2005; Szurek *et al.*, 2001, 2002; Van den Ackerveken *et al.*, 1996; Yang and Gabriel, 1995). There, a central domain of highly conserved, 33–35-amino-acid repeats, each containing hypervariable residues at positions 12 and 13 (also called the repeat-variable diresidue or RVD), directs the recognition of specific host gene promoter sequences called effector binding elements (EBEs) (Boch *et al.*, 2009; Moscou and Bogdanove, 2009). Each TAL effector wraps the DNA in a right-handed superhelix, positioning the second residue of each RVD into the major groove, where it contacts an individual nucleotide in the forward strand (Deng *et al.*, 2012; Mak *et al.*, 2012). Collectively, these interactions define, in a predictable way, the number and identity of adjacent

*Correspondence: Email: ajb7@cornell.edu

†Present address: KWS SAAT SE, Gateway Research Center, 1005 N. Warsaw Rd., St. Louis, MO 63132, USA

‡Present address: Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453 – C1417DSE, Buenos Aires, Argentina

nucleotides that constitute the EBE. A C-terminal acidic activation domain (AD) then activates or enhances transcription, presumably by directly engaging the host RNA polymerase complex (Gu *et al.*, 2005; Kay *et al.*, 2007; Marois *et al.*, 2002; Römer *et al.*, 2007, 2009b, 2010; Sugio *et al.*, 2007; Szurek *et al.*, 2001; Yang *et al.*, 2000, 2006; Zhu *et al.*, 1998, 1999).

TAL effectors promote disease by inducing host susceptibility (*S*) genes, which produce conditions favourable for the pathogen or otherwise contribute to symptom development (Antony *et al.*, 2010; Cernadas *et al.*, 2014; Cohn *et al.*, 2014; Hu *et al.*, 2014; Streubel *et al.*, 2013; Sugio *et al.*, 2007; Yang *et al.*, 2006; Yu *et al.*, 2011). The modular mechanism by which TAL effectors recognize specific DNA sequences facilitates the identification of such genes by enabling not only the prediction of EBEs in host genomes, but also the design of artificial repeat arrays to bind user-defined DNA sequences (Boch *et al.*, 2009; Christian *et al.*, 2010; Morbitzer *et al.*, 2010; Moscou and Bogdanove, 2009). Using any of several rapid assembly methods for designer TAL effectors (dTALs) (Cermak *et al.*, 2011; Li *et al.*, 2011; Morbitzer *et al.*, 2011; Weber *et al.*, 2011; Zhang *et al.*, 2011), it is possible to target novel sites in the promoter of a gene up-regulated by a TAL effector to activate the gene in isolation from other targets of that effector and assess whether the gene is important for disease susceptibility. This approach has been used for bacterial leaf streak and bacterial blight in rice, canker in citrus and bacterial blight in cassava to distinguish important host *S* genes up-regulated by the major virulence TAL effectors of each pathogen (Cernadas *et al.*, 2014; Cohn *et al.*, 2014; Hu *et al.*, 2014; Streubel *et al.*, 2013).

Plants resist attack by TAL effector-wielding pathogens in some cases with executor resistance (*R*) genes (Bogdanove *et al.*, 2010). Like *S* genes, these are directly and specifically transcriptionally activated by TAL effectors, but they encode proteins that cause the hypersensitive reaction (HR) and arrest disease progression (Gu *et al.*, 2005; Römer *et al.*, 2007). Because executor genes depend on TAL effector function, the requirement for a specific sequence of RVDs and a functional AD in the effector is a hallmark of this resistance mechanism. The five characterized executor genes, *Bs3* and *Bs4c* from pepper (*Capsicum annum*), and *Xa27*, *Xa10* and *Xa23* from rice, recognize the activity of AvrBs3 and AvrBs4 of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) and AvrXa27, AvrXa10 and AvrXa23 of Xoo, respectively (Gu *et al.*, 2005; Römer *et al.*, 2007; Strauss *et al.*, 2012; Tian *et al.*, 2014; Wang *et al.*, 2015). None has similarity to other plant *R* genes, although *Bs3* is homologous to the flavin-dependent monooxygenases (Gu *et al.*, 2005; Römer *et al.*, 2007; Strauss *et al.*, 2012) and *Xa10* has low similarity to bacterial Orai and Na⁺/Ca²⁺ antiporters (Tian *et al.*, 2014). Another rice bacterial blight *R* gene, *Xa7*, depends for recognition on the specific repeat region and AD of the cognate TAL effector, AvrXa7 (Yang *et al.*, 2000; Zhu *et al.*,

1998, 1999), and is therefore probably an executor *R* gene, but has yet to be cloned.

It is noteworthy that no *R* gene effective against Xoc has been discovered in rice, despite substantial effort and in contrast with the more than 30 *R* genes that have been identified against Xoo. This could be a result of an observed ability of Xoc to suppress resistance. The Xoo effectors AvrXa7 and AvrXa10 expressed heterologously in Xoc strain BLS303 from a low-copy plasmid failed to confer avirulence on rice plants with the corresponding *R* gene, despite confirmed delivery (Makino *et al.*, 2006). However, when the Avr gene was expressed from a high-copy plasmid, the HR was observed, indicating that resistance suppression by Xoc is quantitative (Makino *et al.*, 2006). Consistent with this finding, the maize *Rxo1* gene, a member of the nucleotide-binding site leucine-rich repeat class of *R* genes, as a transgene in rice, provided HR-associated immunity against strains of Xoc expressing the non-TAL effector AvrRxo1 (Zhao *et al.*, 2004, 2005). Also, *Xa27* engineered to contain EBEs for multiple Xoc TAL effectors in its promoter, again as a transgene, conferred sufficiently strong resistance to be effective (Hummel *et al.*, 2012).

To investigate the importance of suppression of host resistance by Xoc in bacterial leaf streak, we initiated a screen (in rice cv. Nipponbare plants) of full-length TAL effectors of strain BLS256 for resistance-eliciting activity when delivered heterologously by strain EB08 of the soybean (*Glycine max*) pathogen *Xanthomonas axonopodis* pv. *glycines* (Xag) (Hummel *et al.*, 2012), which causes no symptoms and no HR in rice, or by strain POX99^A of Xoo. This screen identified Tal2a (and none of 16 other TAL effectors tested) as an elicitor of the rice HR, suggesting a role in avirulence for this TAL effector that is masked by suppression of host resistance during the natural infection process. HR required the Tal2a AD. However, assays of Tal2a avirulence activity in Xoc by knockout and overexpression and characterization of Tal2a targets using dTALs revealed that Tal2a avirulence activity is quantitative, and suggested a novel mode of action distinct from canonical activation of a single, executor *R* gene.

RESULTS

A Xoc TAL effector delivered heterologously elicits an AD-dependent HR

Xag strain EB08 expressing *tal2a* of Xoc strain BLS256 (Bogdanove *et al.*, 2011), carried on a plasmid, elicited HR within 48 h after inoculation by syringe infiltration into rice (cv. Nipponbare) leaves. Delivery of Tal2a by Xoo PXO99^A elicited a similar phenotype, although the HR was visibly weaker than that elicited by Xag delivering Tal2a. A truncated Tal2a lacking the AD (Tal2a-AD), expressed in either Xag or Xoo, did not trigger the HR (Fig. 1), suggesting that the response depends on gene activation by Tal2a.

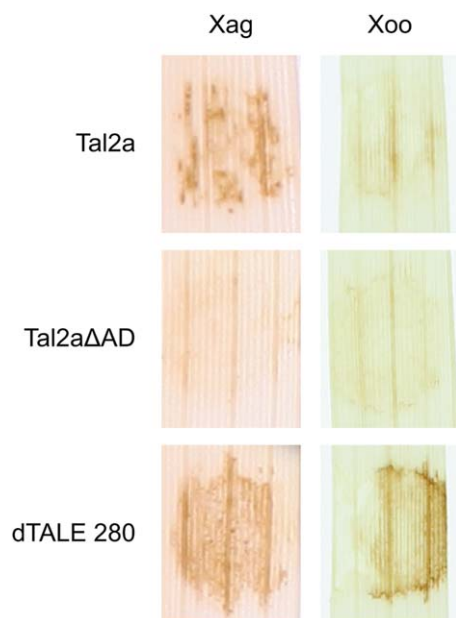


Fig. 1 *Xanthomonas axonopodis* pv. *glycines* EB08 (Xag) and *Xanthomonas oryzae* pv. *oryzae* PXO99^A (Xoo) delivering Tal2a induce activation domain-dependent hypersensitive reaction (HR) in rice. Leaves were cleared in ethanol 72 h after syringe infiltration of bacteria transformed to express Tal2a or Tal2a missing its activation domain (Tal2a Δ AD) from plasmid pKEB31 (see text). HR is visible as browning. Experiments were repeated independently a minimum of three times and showed consistent results.

Tal2a targets a ubiquitin carboxy-terminal hydrolase gene, but activation is insufficient for HR

To better understand the apparent suppression of Tal2a-triggered HR by Xoc, and because the activation domain dependence of the HR to Tal2a is characteristic of an executor *R* gene, we sought to identify the activated target(s) of Tal2a. We predicted Tal2a EBEs in the rice promoterome, which we defined as the sequences 1000 bp upstream of every annotated gene (Doyle *et al.*, 2012; gene models from the MSU Rice Genome Annotation Project, version 6.1, <http://rice.plantbiology.msu.edu>), and cross-referenced them with genes significantly induced by Xoc BLS256 in a previous microarray-based experiment (Cernadas *et al.*, 2014). Two of the Xoc-induced genes contained a predicted EBE for Tal2a (Table S1, see Supporting Information), a putative ubiquitin carboxy-terminal hydrolase gene (*UCH*; Os02g43760; $q = 0.005$) and a putative phosphatidylinositol-4-phosphate 5-kinase gene (*PPK*; Os06g14750; $q = 0.196$).

To determine whether *UCH* and *PPK* are true targets of Tal2a, we analysed their expression patterns by quantitative real-time reverse transcription-polymerase chain reaction (qPCR) during infection of rice leaves with PXO99^A delivering Tal2a or Tal2a-AD. Relative to mock-inoculated tissue, *PPK* transcript was induced significantly in tissue inoculated with PXO99^A delivering

Tal2a, but induction in tissue inoculated with PXO99^A delivering Tal2a Δ AD was similar (Fig. S1, see Supporting Information), suggesting that activation is not dependent on Tal2a activity and may be a non-specific response to *X. oryzae* infection. In contrast, *UCH* mRNA was significantly more abundant in leaves infiltrated with PXO99^A expressing Tal2a compared with Tal2a Δ AD (Fig. 2a), suggesting that Tal2a activity is necessary for its induction. To confirm Tal2a as the sole activator of *UCH* in BLS256, we generated a BLS256 knockout mutant of *tal2a* (strain M169) and found that it did not activate the *UCH* gene. Re-introduction of *tal2a* on a plasmid restored *UCH* activation (Fig. 2b), confirming that Tal2a is responsible for *UCH* induction by Xoc BLS256.

To assess whether *UCH* activation is responsible for the HR elicited by Tal2a, we designed (Doyle *et al.*, 2012) and constructed (Cermak *et al.*, 2011) four dTALEs – 280, 611, 612 and 613 – targeted to the *UCH* promoter, overlapping or proximal to the predicted Tal2a EBE (Fig. 2c and Table S2, see Supporting Information). Although qPCR indicated that all four dTALEs specifically activated *UCH* compared with mock-inoculated tissue (Fig. 2d), only dTALE 280, which was targeted to the last 15 nucleotides of the Tal2a EBE, triggered the HR (Fig. 2d). This HR was visibly stronger than that elicited by Tal2a (Fig. 1 and Fig. 2d). Because dTALEs 611, 612 and 613 each activated *UCH* to a similar extent as dTALE 280 without triggering the HR, *UCH* activation alone is not sufficient to cause HR. Instead, a different gene or set of genes activated by Tal2a and dTALE 280, but not by the other dTALEs, probably contributes to or causes the phenotype.

Tal2a activity is differentially distributed across geographically diverse isolates and these reveal three more specific rice targets

To determine whether another gene, potentially missed in the predictions based on the microarray data, might underlie the HR elicited by Tal2a, we examined RNAseq-based global gene expression profiles in rice leaves inoculated individually with 11 different field isolates (Wilkins *et al.*, 2015 and Gene Expression Omnibus accession GSE67588). Taking *UCH* activation as a marker for the presence of Tal2a, we classified the 11 field isolates into 'Tal2a⁺' and 'Tal2a⁻' groups. This revealed broad distribution among Asian isolates, with the Tal2a⁺ group including all of three Chinese isolates, an isolate from Malaysia and two of three isolates from the Philippines. The Tal2a⁻ group included the remaining Philippines strain, an Indian strain and all of three African strains.

In addition to *UCH*, five other genes were activated by the six Tal2a⁺ strains that were not activated by strains in the Tal2a⁻ group (Table S3, see Supporting Information). To assess Tal2a dependence of activation for these genes, we generated and cross-referenced RNAseq data for rice leaves treated with PXO99^A expressing Tal2a or dTALE 280. Compared with leaves treated

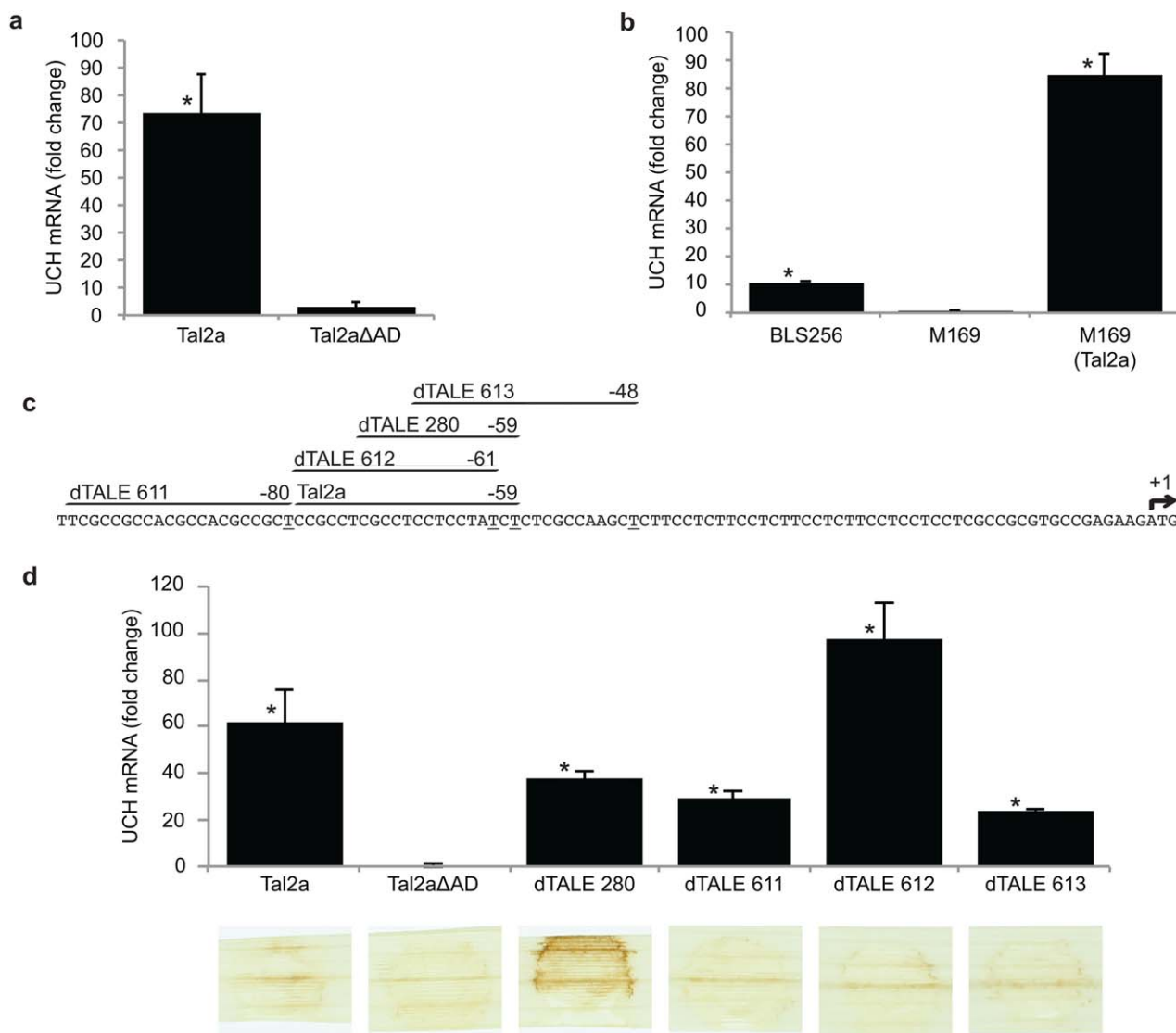


Fig. 2 A ubiquitin carboxy-terminal hydrolase (*UCH*) gene is activated by Tal2a, but is insufficient for the hypersensitive reaction (HR). (a) Quantitative real-time reverse transcription-polymerase chain reaction (qPCR) assay of expression of the *UCH* gene in response to Tal2a or Tal2aΔAD delivered by *Xanthomonas oryzae* pv. *oryzae* PXO99^A (Xoo), relative to mock-inoculated tissue. Tal2a was expressed from plasmid pKEB31. Tissue was sampled at 48 h after syringe infiltration of bacterial suspensions. Each value shown is the average of two technical replicates of three biological replicates. Capped vertical lines show the standard deviation. The relative fold change was calculated by the $2^{-\Delta\Delta C_t}$ method. Asterisks indicate values significantly different ($P \leq 0.05$) from mock as determined by two-tailed, heteroscedastic *t*-tests. This experiment was repeated three times with similar results. (b) qPCR assay of *UCH* expression as in (a) in response to wild-type *X. oryzae* pv. *oryzicola* BLS256, *tal2a* mutant M169 and M169 carrying the *tal2a* gene in plasmid pKEB31. Independent experiments were repeated twice with similar results. (c) The 101 nucleotides of the *UCH* gene upstream of the translational start site showing the predicted effector-binding element (EBE) for Tal2a and EBEs targeted by each of four designer transcription activator-like effectors (dTALEs) designed to activate transcription of the gene. Numbers indicate the position of the last (3') nucleotide of each EBE (underlined) relative to the translational start (+1, arrow). (d) qPCR results as in (a) showing the activation of the *UCH* gene by each of the four dTALEs delivered by Xoo and, below, the response of rice leaves (as in Fig. 1) to each. Each dTALE activated the *UCH* gene, but, of these, only dTALE 280, the EBE of which is contained entirely within the Tal2a EBE, triggered an HR, and this HR was stronger than that induced by Tal2a. Experiments were repeated at least twice with similar results.

with PXO99^A expressing Tal11b, another BLS256 effector, 347 annotated genes were significantly up-regulated at least 2.0-fold ($q < 0.05$) in response to both Tal2a and dTALE 280 (Gene Expression Omnibus accession GSE67958). Two of the five genes that

were up-regulated in addition to *UCH* by the Tal2a⁺ field isolates of Xoc were absent from this list, leaving a total of four genes (including *UCH*) uniquely activated by the Xoc strains and by Xoo carrying Tal2a (or dTALE 280). The four genes actually comprise

two pairs of adjacent genes, *UCH* and *Os02g43770*, oriented towards each other, and *Os10g40130* and *Os10g40120*, oriented in the same direction (5' to 3'; Fig. 3a). Only one member of each pair, *UCH* and *Os10g40130*, contains a predicted EBE for Tal2a and dTALE 280 (Doyle *et al.*, 2012), raising the intriguing possibility that the others are activated by an enhancer function originating from TAL effector activity at that EBE.

To confirm whether *Os02g43770* (the Tal2a-activated gene lacking a predicted EBE but opposite *UCH*) was expressed from the sense strand, and to account for any possible non-annotated targets of Tal2a that may have been missed during transcript assembly, we analysed the RNAseq data again using Cufflinks with novel transcript discovery (Trapnell *et al.*, 2010). Nearly all reads mapping to *Os02g43770* indeed corresponded to the sense strand, and we found no additional sequences up-regulated by PXO99^A expressing Tal2a and by all Tal2a⁺ Xoc strains.

To confirm the Tal2a dependence of the activation of *Os02g43770*, *Os10g40120* and *Os10g40130*, we carried out qPCR and found that all three were specifically activated in rice leaves inoculated with BLS256 relative to M169 (Fig. S2, see Supporting Information), and also by the PXO99^A strain expressing Tal2a compared with mock-inoculated leaves (Fig. 3b).

The four identified Tal2a targets activated individually or collectively are insufficient for HR

To determine whether any of the three additional Tal2a-responsive genes is responsible for the HR elicited by Tal2a, we individually induced each with dTALEs (612 for *UCH*, 1066 for *Os02g43770*, 990 for *Os10g40120* and 1170 for *Os10g40130*; Table S2) delivered by Xoo PXO99^A. Although qPCR indicated that each dTALE uniquely activated its intended target in comparison with mock-inoculated tissue, none was sufficient to elicit the HR (Fig. 4).

To assess whether any of the genes act in concert to elicit the HR, we inoculated a mixture of the four strains each delivering one of the dTALEs. Despite the activation of all four genes, this also failed to result in the HR. As a control, the strain expressing dTALE 280 diluted to the same titre as an individual strain in the mixture elicited a strong HR (Fig. 4b).

Tal2a exhibits dose-dependent avirulence activity

As our target characterization did not reveal an executor *R* gene, we chose to further characterize the Tal2a-triggered resistance mechanism in rice and the apparent ability of Xoc to overcome it by examining the virulence of the pathogen expressing different levels of Tal2a. We compared wild-type with M169 and with M169 expressing Tal2a or dTALE 280 from a low- or a high-copy plasmid. We measured virulence using a previously described lesion length assay (Wang *et al.*, 2007), and assessed the activity of Tal2a or dTALE 280 by measuring the activation of *UCH* using

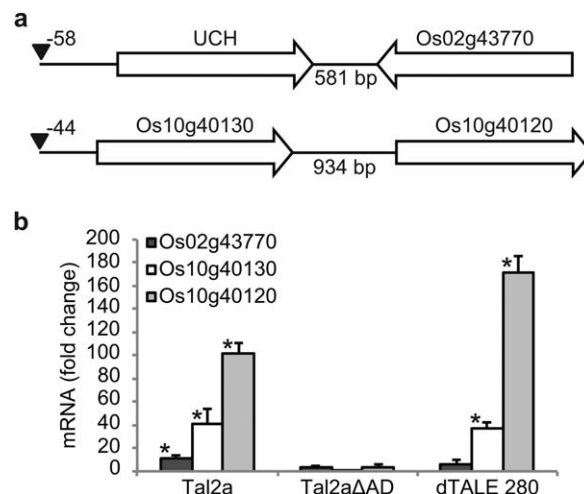


Fig. 3 Three additional genes activated in common by strains that activate the ubiquitin carboxy-terminal hydrolase (*UCH*) gene are activated by Tal2a. (a) Schematic diagram of the four genes activated in common by *Xanthomonas oryzae* strains that activate the *UCH* gene (Table S2). Genes are represented as block arrows oriented 5' to 3' and are not to scale. Introns are not depicted. Candidate effector-binding elements (EBEs) for Tal2a are labelled with black triangles with the position indicated as in Fig. 2. (b) Expression of the genes in response to Tal2a or Tal2a missing its activation domain (Tal2aΔAD) assayed by quantitative real-time reverse transcription-polymerase chain reaction (qPCR), as described for Fig. 2. Experiments were repeated twice with similar results.

qPCR (Fig. 5). The average lesion length for M169 with no plasmid was slightly longer than that for the wild-type strain, but this was significant in only one experiment ($P = 0.031$), suggesting, at most, a nominal negative effect of Tal2a on BLS256 virulence when expressed from the chromosome. Tal2a or dTAL280 in M169 expressed from the low-copy plasmid resulted in a 1.79- or 2.28-fold higher *UCH* induction, respectively, than did Tal2a expressed from the chromosome in the wild-type (calculated from two independent qPCR experiments). When the TAL effectors were expressed from the high-copy plasmid, *UCH* fold inductions were higher still (6.07- and 7.75-fold higher than the wild-type). Relative to the wild-type, M169 expressing Tal2a from the low-copy plasmid showed no significant difference in virulence. However, M169 with Tal2a expressed from the high-copy plasmid showed a 30% reduction. M169 expressing dTALE 280 from the low-copy vector showed roughly a 10% reduction in virulence relative to the wild-type in two of four independent experiments conducted (one of these two is shown in Fig. 5; all four are shown in Fig. S3, see Supporting Information). M169 expressing dTALE 280 from the high-copy plasmid showed a 90% reduction in lesion length relative to the wild-type. The greater reductions in virulence resulting from dTALE 280 relative to Tal2a are consistent with the visibly stronger HR elicited by this effector compared

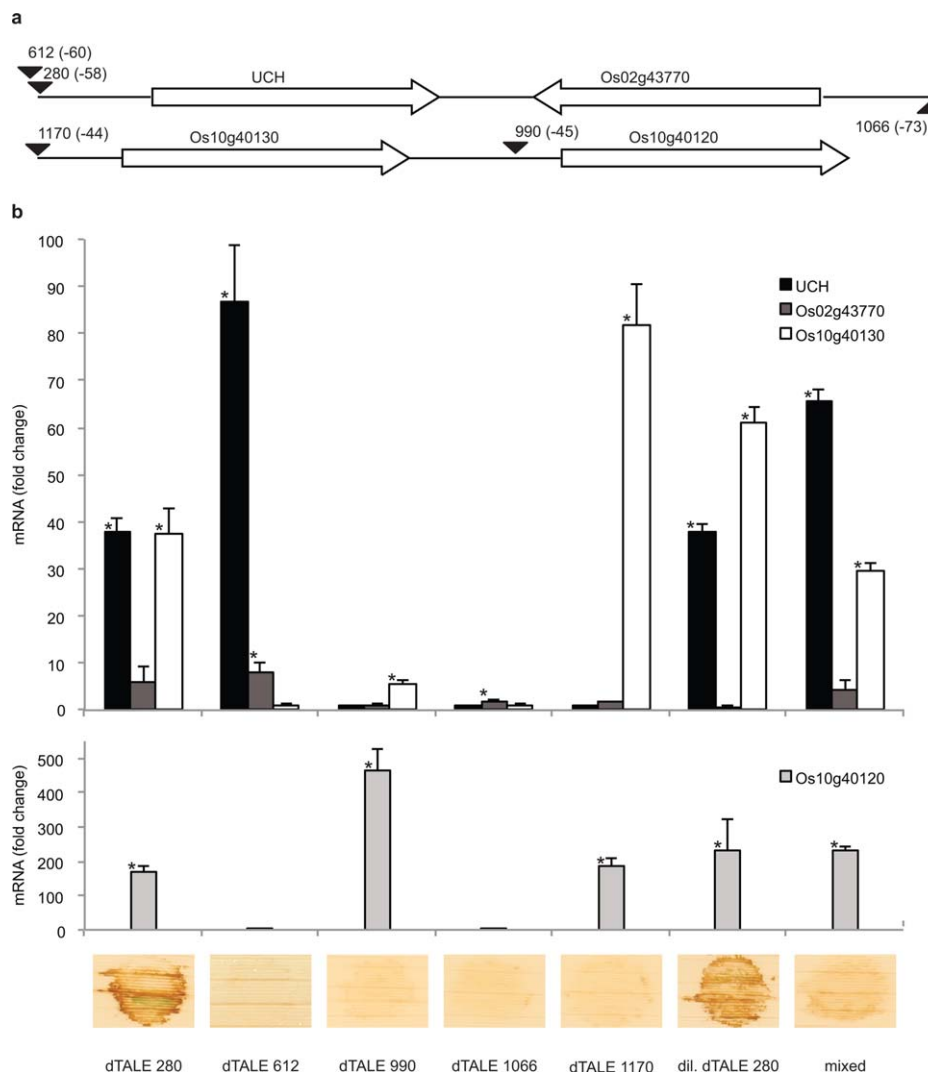


Fig. 4 The four identified Tal2a-activated genes activated in subsets or collectively are insufficient for HR. (a) Schematic of the Tal2a-activated genes, as in Fig. 3, showing the locations (parentheses) of effector-binding elements (EBEs) targeted by designer transcription activator-like effectors (dTALs) 280, 612, 990, 1066 and 1170. dTALE 1170, which binds an EBE with the same 3' end as the Tal2a EBE but a longer 5' end, is not predicted to bind at the Tal2a EBE upstream of the ubiquitin carboxy-terminal hydrolase (*UCH*) gene. (b) Quantitative real-time reverse transcription-polymerase chain reaction (qPCR) results, as in Fig. 2, showing the expression of the genes in response to each of the dTALs delivered by *Xanthomonas oryzae* pv. *oryzae* PXO99^A (*Xoo*), and (below) the response of rice leaves (as in Fig. 1). All experiments were repeated at least twice with similar results; 'mixed' indicates co-inoculation with the strains expressing dTALs 612, 990, 1066 and 1170; 'dil. dTALE 280' indicates inoculation with the dTALE 280 strain diluted to the same titer as each of the strains included in the 'mixed' suspension.

with Tal2a when either is expressed from Xag or Xoo. Finally, the virulence of M169 expressing Tal2a Δ AD from either vector was the same as the wild-type (and *UCH* was not induced). The data overall show a quantitative, inverse correlation between Tal2a activity and virulence.

As an additional test for a role of *UCH* in the avirulence activity of Tal2a, although the gene was insufficient for HR, we included virulence assays for M169 expressing dTALs 611, 612 or 613 from the high-copy vector. No effect on virulence could be detected for dTALs 611 or 613, despite robust activation of *UCH* by both effectors, providing further evidence that the *UCH* gene is

not the relevant or sole relevant target of Tal2a. Unexpectedly, dTALE 612 caused a 47% reduction in the virulence of M169 when expressed from the high-copy vector, although we did not observe a visible HR when it was delivered by PXO99^A. dTALE 612 shares 13 of the 19 nucleotides in its *UCH* EBE with the *UCH* EBE for dTALE 280, and all of its EBE with the *UCH* EBE for Tal2a. Thus, it is possible that dTALE 612 triggers resistance at a level below the threshold required for the visible HR, but sufficiently high to impair virulence. Alternatively, or in addition, a distinct target of dTALE 612 might contribute to this avirulence effect.

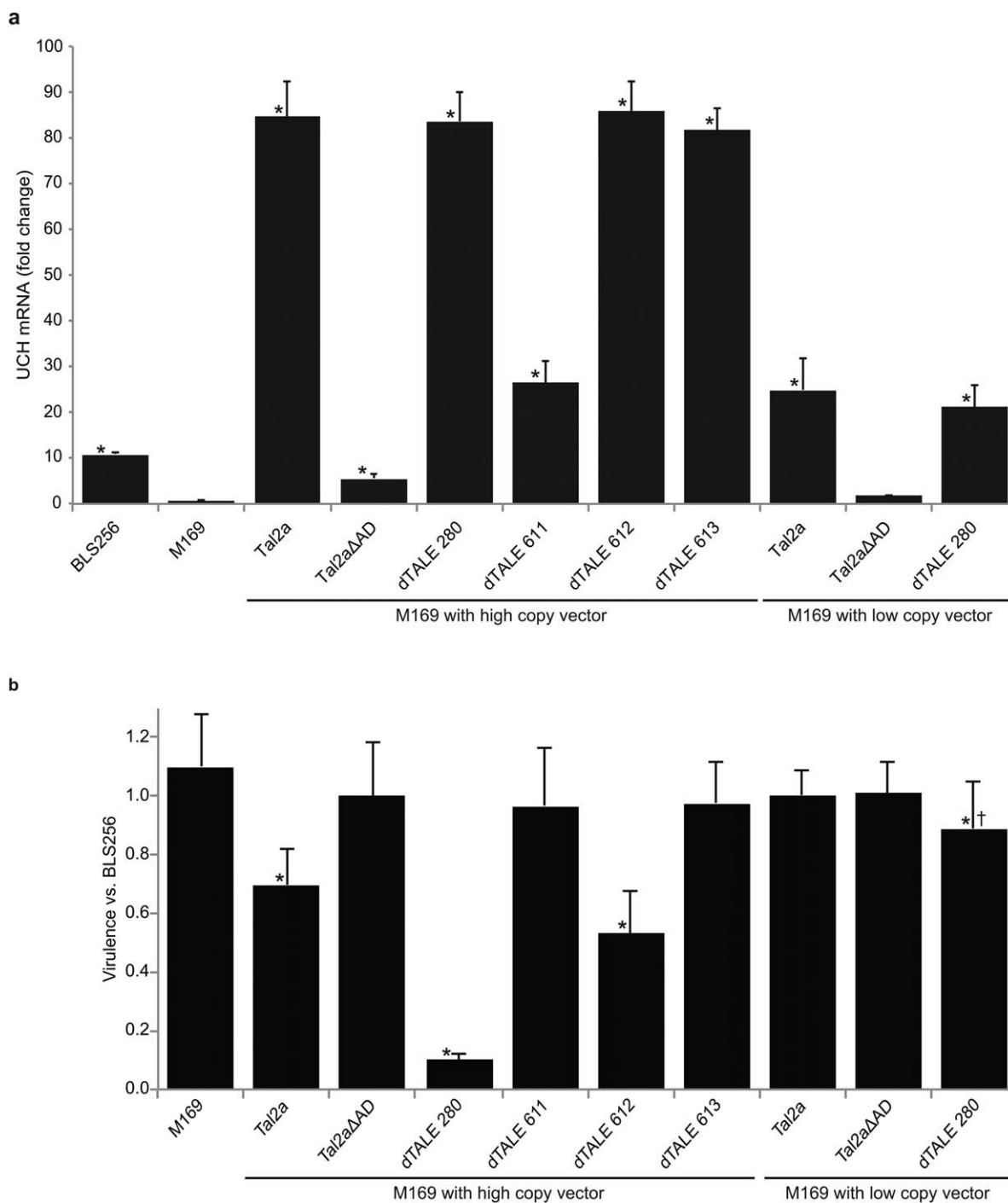


Fig. 5 Tal2a inhibits the virulence of *Xanthomonas oryzae* pv. *oryzicola* (Xoc) in a dose-dependent manner. (a) Quantitative real-time reverse transcription-polymerase chain reaction (qPCR) results, as in Fig. 2, showing the expression of the ubiquitin carboxy-terminal hydrolase (*UCH*) gene in response to wild-type Xoc strain BLS256, the Tal2a mutant derivative M169 and M169 transformed to express Tal2a, Tal2a missing its activation domain (Tal2aΔAD) or one of several designer transcription activator-like effectors (dTALEs), with the TAL effector genes borne on a high-copy (pKEB31) or low-copy (pHM1) vector. Experiments were repeated twice with similar results. (b) Virulence of the BLS256-derived strains in (a) relative to BLS256. Vertical bars represent ratios of lesion lengths resulting from side-by-side inoculation of the indicated strain and BLS256 at 10 days post-inoculation (Cernadas *et al.*, 2014). Each is the average of at least eight biological replications. Experiments were repeated at least twice with similar results. Capped vertical lines show the standard deviation. Asterisks indicate values significantly different ($P \leq 0.05$) from 1.0 (no change). M169 expressing dTALE 280 from the low-copy vector † resulted in an average lesion length ratio of < 1.0 in each of the four independent experiments in which it was tested (Fig. S3), but this was statistically significant in only two of these experiments.

Finally, we examined the influence of Tal2a and dTALE 280 on the virulence of PXO99^A. Consistent with the results in M169, expression of Tal2a or dTALE 280 in PXO99^A from the high-copy vector resulted in reduced lesion lengths following leaf clip inoculation (Kauffman *et al.*, 1973), relative to strains expressing Tal2a-AD or dTALEs 611 or 613; dTALE 612, in contrast with its effect in M169, had no measurable effect on PXO99^A virulence (Fig. S4, see Supporting Information).

DISCUSSION

The lack of characterized major gene resistance to Xoc in rice is an increasingly serious problem as bacterial leaf streak becomes more widespread. A marked ability of Xoc to suppress host defence (Makino *et al.*, 2006) may account for the failure of breeders and pathologists so far to identify an effective native *R* gene. Our discovery of the avirulence function of Tal2a and the apparent widespread distribution of this effector reveals that the suppression of effector-triggered host resistance by Xoc plays a critical role during infection.

Our finding that elicitation of the HR by (heterologously expressed) Tal2a requires the AD suggests an executor *R* gene. However, dTALE-driven activation of the four targets of Tal2a identified, individually or together, does not recapitulate the HR. A *tal2a* knockout mutant of BLS256 shows virulence comparable with the wild-type, but plasmid-based overexpression of *tal2a* at different levels in the wild-type reduces virulence in a directly corresponding way. The apparent lack of a canonical target that can account for the HR to Tal2a and the AD and dose dependence of Tal2a avirulence activity point to a novel mechanism of defence elicitation distinct from the activation of a single, executor *R* gene.

Because TAL effectors typically act by binding to and activating target promoters, molecular cloning of an executor *R* gene that corresponds to an avirulence TAL effector can reasonably be approached by gene expression analysis and EBE prediction (Boch *et al.*, 2014). For example, RNAseq of host genes in the presence and absence of the TAL effector AvrBs4 was used to identify the *Bs4c* executor *R* gene in pepper (Strauss *et al.*, 2012). Our inability to pinpoint the source of Tal2a-triggered resistance using this approach reveals its limitations. These limitations may stem from an oversimplified view of the effects of TAL effectors on the host transcriptome. In addition to canonical, activated genes, some targets might be directly down-regulated by a TAL effector that binds in such a way as to block transcript elongation or otherwise prevent expression. TAL effectors may induce the expression of small RNAs that would not be captured in a polyA-enriched cDNA library. The activation of long non-coding RNAs or unannotated genes might also occur and would not be detected using annotation-based methods of mapping RNAseq reads. To account for the possibility of novel transcripts, we performed a second analysis on the aligned RNAseq reads using Cufflinks (Trapnell

et al., 2010), but found no sequences up-regulated in common by all Tal2a⁺ strains.

Limitations might also stem from the specific parameters applied in a search, or the experimental design. For example, we applied a cutoff of a two-fold minimum induction compared with controls. This leaves the formal possibility that a very low level of induction of the putative target, driven by weak binding to a highly imperfect EBE, is responsible for the HR, which would have caused us to overlook the source of resistance. Although unlikely, the possibility also exists that one or more required, but insufficient, mediators of the HR are activated by PXO99^A and Xag EB08 independently of Tal2a. This scenario would have resulted in exclusion from consideration as a Tal2a target in our analysis. In future studies, an RNAseq-based comparison of wild-type BLS256 with M169, a mutant that we succeeded in isolating only after the RNAseq experiments presented here were completed, might better reveal all relevant targets of Tal2a. The dataset would be strengthened if rice varieties differentially responsive to Tal2a could be identified and included in the gene expression analysis. Nonetheless, partial redundancy of BLS256 TAL effectors for the activation of some Tal2a targets could yet confound the results.

Complexity in the gene expression pattern required for resistance might have played a role in our inability to pinpoint the genetic source of the HR to Tal2a. Specifically, the phenotype might be the result not only of the activation of two or more genes by Tal2a (or dTALE 280), but of a certain proportional pattern of activation across the targets that we failed to recapitulate with our mixed dTALEs. Or, elicitation of the HR by Tal2a and dTALE 280 might depend on an alternative transcript for one or more targets that results from TAL effector-dependent repositioning of the transcriptional start site, which can occur as a function of the site at which a TAL effector binds (Antony *et al.*, 2010; Hummel *et al.*, 2012; Kay *et al.*, 2007, 2009; Römer *et al.*, 2009a,b). This mechanism would depend on the exact position of the activating TAL effector on the DNA and would be difficult to replicate by targeting dTALEs to different sequences in the promoter. It may be significant that the only two TAL effectors sufficient to elicit the HR in this study, Tal2a and dTALE 280, share EBEs that terminate at the same nucleotide in the *UCH* promoter.

Should any of these scenarios turn out to be true for Tal2a recognition, it would expand the current model of TAL effector-mediated avirulence, i.e. activation of a single, protein-coding, executor *R* gene by binding to the promoter of that *R* gene. Already, an example of target activation that may depart from the general model for TAL effectors was provided in this study by *Os02g43770*, which is downstream of *UCH* and inversely oriented (Fig. 3), and is activated by Tal2a despite having no predicted EBE in its promoter. The *UCH* promoter bound by Tal2a may act as an enhancer to drive the expression of *Os02g43770*. Another possible example is the Tal2a-activated gene *Os10g40120*, which lacks

a predicted Tal2a EBE and may be driven similarly by an enhancer effect of Tal2a binding at the neighbouring upstream gene *Os10g40130*.

We have reported recently complete genome sequences for all but one of the Xoc strains used in this study. These genome sequences confirmed our classification of the strains as Tal2a⁺ or Tal2a⁻ (Wilkins *et al.*, 2015). Given its avirulence function and apparent dispensability for virulence, why is Tal2a so broadly distributed among Asian isolates? Perhaps, in some rice genotypes, activation of one or more of the Tal2a targets identified here or other targets specific to these genotypes contributes to virulence. Although we did not assay for a virulence contribution of Tal2a in cultivars other than Nipponbare, we did observe that Tal2a delivered by Xag elicits a strong HR not only in Nipponbare, but also in *japonica* cv. Kitaake and *indica* cv. IR24 (data not shown). The cultivars *indica* and *japonica* represent two of the three major lineages of rice, suggesting broad conservation of Tal2a recognition among cultivated rice varieties. Therefore, persistence of Tal2a almost certainly depends on the ability of Tal2a-expressing strains to suppress host resistance. Perhaps, without a detectable fitness cost for the level of expression of Tal2a that occurs with the endogenous, chromosomal gene, there is insufficient selective pressure to eliminate the gene, even without it conferring any selective advantage.

This study provides the first example in Tal2a of an Xoc TAL effector that functions as an avirulence factor. The quantitative nature of Tal2a activity sets it apart from the avirulence factors characterized to date. Although activity varies across avirulence factors, we are unaware of any with a dose-dependent effect such as observed for Tal2a. One possible exception is AvrBs3, which triggers resistance mediated by the nucleotide-binding, leucine-rich repeat tomato R protein Bs4 only when the bacterial protein is expressed highly in plant cells via *Agrobacterium*-mediated t-DNA delivery (Schornack *et al.*, 2005). In that case, presumably direct interaction of the effector and the R protein triggers the response. The fact that overexpression of AvrBs3 is necessary suggests that the interaction is relatively low affinity. The same may be true of Tal2a and its resistance-mediating DNA target(s). This supposition is strengthened by our observation that dTALE 280, which, as described earlier, was designed to optimally target a sequence within the Tal2a EBE in the UCH promoter, exhibits avirulence activity that is overall stronger than that of Tal2a but similarly dose dependent. Future identification of the genetic source of the resistance response to Tal2a might afford the opportunity, through genome editing, to increase the affinity of the target(s) for Tal2a and increase the plant response beyond the capacity of Xoc to suppress it, providing a new resource for the management of bacterial leaf streak. As the rice response triggered by Tal2a expressed from a high-copy vector in Xoo reduced bacterial blight symptoms as well, the targets might additionally be edited to trap

a conserved Xoo TAL effector for control of that disease, or a set of TAL effectors for broad-spectrum control of both diseases, in a manner similar to the recent modifications of *Xa27* and *Xa10* (Hummel *et al.*, 2012; Zeng *et al.*, 2015). Identification of the genetic basis for the Tal2a-triggered HR will also be important to understand the quantitative nature of Tal2a function itself, and may help to further elucidate the reason for the lack of observed major gene resistance to bacterial leaf streak in rice.

EXPERIMENTAL PROCEDURES

Plant growth, plant inoculations, virulence assays and qPCR

Rice (*Oryza sativa* L. cv. Nipponbare) was grown and inoculated as described by Hummel *et al.* (2012), except that, for Xoc virulence assays, virulence of M169 strains was expressed as the lesion length relative to that caused by wild-type BLS256 inoculated on the opposite side of the leaf midrib in each case. Xoo PXO99^A virulence assays were conducted as described by Hummel *et al.* (2012), except that virulence was expressed as the ratio of the lesion length to the total leaf length. qPCR was performed as described previously (Hummel *et al.*, 2012; Livak and Schmittgen, 2001; Schmittgen and Livak, 2008), using gene-specific primers (Table S4, see Supporting Information), except total reaction volumes were reduced to 25 μ L, total RNA template was reduced to 50 ng and a minimum of two qPCR technical replicates was performed for each independent biological sample.

TAL effector clones, bacterial transformation and *tal2a* mutagenesis

The *tal2a* gene was cloned from Xoc BLS256 as described by Hummel *et al.* (2012). dTALEs were assembled, and then transferred via the Gateway LR II Clonase enzyme kit (Life Technologies, Grand Island, NY, USA) into the broad-host-range and moderately high-copy plasmid pKEB31, as described by Cermak *et al.* (2011). For low copy, the pKEB31-derived plasmids were digested with *Hind*III and the fragment containing the *lac* promoter and TAL effector gene was ligated into the *Hind*III site of pHM1 (Hopkins *et al.*, 1992). The growth and transformation of *Xanthomonas* were performed as described by Hummel *et al.* (2012). To generate the *tal2a* knockout mutant, the suicide plasmid pAH412 was manufactured by replacing the first 19.5 repeats of the central repeat region of the *tal2a* clone with the Kan^R gene of the EZ-Tn5 Transposon (Epicentre, Madison, WI, USA). This clone is in pBlueScript II (Agilent Technologies, Santa Clara, CA, USA), and flanking the modified central repeat region are 5' and 3' sequences from the Xoc TAL effector genes *tal2a* and *tal1c* (Hummel *et al.*, 2012). M169 was generated by transformation of Xoc BLS256 by electroporation with pAH412, followed by selection on plates amended with kanamycin. In M169, the N-terminal region of the knockout cassette was mapped to the *tal2a* genomic locus by PCR and sequencing. Knockout of *tal2a* was confirmed by qPCR showing loss of *UCH* induction by M169.

Next-generation RNA sequencing

For Xoo strains expressing Tal2a and dTALE 280, rice leaves were inoculated and total RNA was isolated for qPCR as described by Hummel *et al.* (2012). Activation of *UCH* by Xoo PXO99^A expressing Tal2a and dTALE 280, and not by Xoo PXO99^A expressing Tal11b, was confirmed by qPCR. For each of the Xoc field isolates, plus a mock inoculum, eight leaves from four 15-day-old rice plants were syringe infiltrated and pooled, and total RNA was extracted for qPCR as described previously (Hummel *et al.*, 2012; Wilkins *et al.*, 2015). Independent experiments were repeated three times. The Iowa State University DNA Facility prepared mRNA libraries using the Illumina (San Diego, CA, USA) TruSeq RNA Sample Preparation Kit v2, according to the manufacturer's protocol. For the PXO99^A experiment, two independent biological replicates of each treatment were indexed and multiplexed into a single flow cell lane; for the Xoc field isolate experiment, three independent biological replicates of each treatment were indexed and filled the remaining lanes of the flow cell.

Sequencing was performed on a HiSeq 2000 (Illumina) by the Iowa State University DNA Facility, yielding 178.8 million and 912.4 million high-quality reads for the Xoo and Xoc experiments, respectively; 177.2 million and 901.4 million reads, respectively, passed all filtering steps. Before the reads were aligned, adapter sequences were removed using the Trimmomatic (Lohse *et al.*, 2012) Illumina-Clip trimming step with the following settings: seedMismatches = 2, palindromeClipThreshold = 40 and simpleClipThreshold = 15. Low-quality read ends (bases with a phred quality score of less than 20) were then removed using BRAT (Harris *et al.*, 2010) with default settings, because independent evaluation suggests that this step improves alignment quality (Yu *et al.*, 2012). Alignment and splice site identification were completed using TopHat (Kim *et al.*, 2013; Trapnell *et al.*, 2009) with a maximum intron length of 10 000 and default settings otherwise. Reads were aligned to the Os-Nipponbare-Reference-IRGSP-1.0 rice genome using the MSU Rice Genome Annotation Project version 7.0 as a reference annotation (Kawahara *et al.*, 2013). The aligned reads were assembled into a minimal set of transcripts using Cufflinks (Trapnell *et al.*, 2010) with the option '-multi-read-correct', the same reference annotation used for alignment and default settings otherwise.

On a first run, the reference annotation was passed to Cufflinks using the '-G' flag, which requires that Cufflinks ignore reads that are incompatible with existing transcripts. When none of the previously annotated genes was confirmed as the Tal2a target, this step was repeated using the '-g' flag instead, allowing Cufflinks to assemble novel transcripts to explain the observed reads. In order to extract non-normalized read counts for each of the resulting transcripts, the HTSeq function htseq-count (<http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html>) was used with default settings for non-stranded reads. Differentially expressed genes were then identified using Quasiseq (Lund *et al.*, 2012). Genes were considered to be differentially expressed if they had a *q* value of <0.05 and at least a two-fold change in expression. The analysis described above was performed separately for the RNAseq reads obtained from rice inoculated with Xoo PXO99^A and the reads obtained from rice inoculated with the Xoc isolates.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Similar activation of a phosphatidylinositol-4-phosphate 5-kinase gene (*PPK*) predicted to be a target of Tal2a by *Xanthomonas oryzae* pv. *oryzae* (Xoo) expressing Tal2a or Tal2a lacking its activation domain.

Fig. S2 Tal2a-dependent activation of three additional rice genes by *Xanthomonas oryzae* pv. *oryzicola* (Xoc).

Fig. S3 Virulence of *tal2a* mutant M169 expressing dTALE 280 from the low-copy vector pHM1.

Fig. S4 Inhibition of *Xanthomonas oryzae* pv. *oryzae* (Xoo) virulence by Tal2a.

Table S1 Targets of Tal2a predicted using microarray data of Cernadas *et al.* (2014).

Table S2 Repeat-variable diresidue (RVD) and effector-binding element (EBE) sequences for Tal2a and designer transcription activator-like effectors (dTALEs) that activate Tal2a-responsive genes.

Table S3 Genes up-regulated by all Tal2a⁺ field isolates of *Xanthomonas oryzae* pv. *oryzicola* (Xoc).

Table S4 Gene-specific primers used for quantitative real-time reverse transcription-polymerase chain reaction (qPCR).