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Ectopic activation of the rice NLR heteropair RGA4/RGA5 confers resistance to bacterial blight and bacterial leaf streak diseases

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SUMMARY

Bacterial blight (BB) and bacterial leaf streak (BLS) are important diseases in *Oryza sativa* caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), respectively. In both bacteria, transcription activator-like (TAL) effectors are major virulence determinants that act by transactivating host genes downstream of effector-binding elements (EBEs) bound in a sequence-specific manner. Resistance to *Xoo* is mostly related to the action of TAL effectors, either by polymorphisms that prevent the induction of susceptibility (*S*) genes or by executor (*R*) genes with EBEs embedded in their promoter, and that induce cell death and resistance. For *Xoc*, no resistance sources are known in rice. Here, we investigated whether the recognition of effectors by nucleotide binding and leucine-rich repeat domain immune receptors (NLRs), the most widespread resistance mechanism in plants, is also able to stop BB and BLS. In one instance, transgenic rice lines harboring the *AVR1-CO39* effector gene from the rice blast fungus *Magnaporthe oryzae*, under the control of an inducible promoter, were challenged with transgenic *Xoo* and *Xoc* strains carrying a TAL effector designed to transactivate the inducible promoter. This induced *AVR1-CO39* expression and triggered BB and BLS resistance when the corresponding *Pi-CO39* resistance locus was present. In a second example, the transactivation of an auto-active NLR by *Xoo*-delivered designer TAL effectors resulted in BB resistance, demonstrating that NLR-triggered immune responses efficiently control *Xoo*. This forms the foundation for future BB and BLS disease control strategies, whereupon endogenous TAL effectors will target synthetic promoter regions of *Avr* or *NLR* executor genes.

INTRODUCTION

Plant–pathogen interactions are governed by specificity. Plant pathogens possess precisely adapted infection strategies that allow them to colonize particular tissues of a generally limited range of host plant species. For this purpose, they deploy specialized virulence factors enabling them to overcome the physical and chemical barriers of the plant, access nutrients and suppress or escape host immune responses. Among them, effectors are of central importance. They are pathogen proteins that are secreted into the host–pathogen interface or directly inside host cells during infection, and mediate immune suppression or manipulation of plant physiology and metabolism (Hogenhout *et al.*, 2009; Jones and Dangl, 2006).

Plant resistance is also highly specific and adapted to the type of pathogen. It largely relies on inducible defense responses activated upon the recognition of pathogen-derived signals by plant immune receptors. Most of them are either cytoplasmic proteins of the class of nucleotide-binding and leucine-rich repeat domain (NLR) proteins (Takken and Goverse, 2012) or plasma membrane-located proteins that possess an extracellular receptor domain, often coupled to an intracellular kinase domain (Böhm

et al., 2014). NLR proteins recognize cytoplasmic effector proteins in a direct or indirect manner, whereas membrane immune receptors recognize many different types of pathogen-derived signals, such as effector proteins that act in the plant–pathogen interface, outside of host cells, general microbe-associated molecular patterns, such as cell wall-derived fungal chitin or bacterial lipo-polysaccharides, or plant-derived compounds released during infection and acting as danger signals (Cook *et al.*, 2015; Jones and Dangl, 2006). Immune receptor activation triggers rapid defense responses that are accompanied by an oxidative burst, and often involve a localized programmed cell death called the hypersensitive response (HR). Generally, the activation of these plant immune responses stops or attenuates colonization by the pathogen; however, in certain cases it is exploited by pathogens to promote their development (Faris *et al.*, 2010).

Oryza sativa is the staple food for nearly half of the global population. Its production is constantly threatened by many different diseases, among which the most devastating is blast, caused by the ascomycete fungus *Magnaporthe oryzae*. Other important diseases include bacterial blight (BB) and bacterial leaf streak (BLS), caused by the gram-negative bacteria *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), respectively. These three pathogens infect leaves but their tissue specificity differs. *M. oryzae* attacks epidermal and mesophyll cells, *Xoc* enters through stomata or wounds, and colonizes the mesophyll apoplast, and *Xoo* occupies the xylem upon entry through hydathodes or wounds (Nino-Liu *et al.*, 2006).

In both blast and BB, genetic resistance is crucial for disease control, but interestingly the genes and mechanisms conferring resistance to *M. oryzae* and *Xoo* fundamentally differ (Liu *et al.*, 2014). Most of the more than 100 blast *R* genes that have been genetically identified and mapped in rice act exclusively in a dominant manner (Ballini *et al.*, 2008; Liu *et al.*, 2014). Among the 24 *R* genes that have been cloned, 23 code for NLRs. This shows that rice blast resistance is mainly conferred by dominant resistance (*R*) genes coding for NLR immune receptors. Ten *avirulence* (*Avr*) genes have been cloned from *M. oryzae*, and all but one code for effector proteins that are secreted and translocated into the cytoplasm of host cells during infection (Giraldo and Valent, 2013; Wu *et al.*, 2015). With the exception of PWL2, the cognate *R* gene of which is unknown, these translocated Avr effectors from *M. oryzae* are recognized by NLR proteins, indicating that cytoplasmic effector recognition by NLR immune receptors is the main mechanism in rice blast resistance. Interestingly, in many cases, rice blast resistance involves paired NLR proteins that are both necessary and sufficient to recognize one or multiple Avrs (Cesari *et al.*, 2014a,b).

Among them, the NLR pair RGA4/RGA5 that interacts functionally and physically to recognize the *M. oryzae* effectors AVR1-CO39 and AVR-Pia became a model for understanding their mode of action in monocots (Cesari *et al.*, 2014b, 2013). *RGA4* and *RGA5* are tightly linked at the *Pi-CO39*/*Pia* resistance locus in an inverted tandem orientation with a small shared 5′ intergenic region of 3.5 kb. RGA4 acts as a constitutively active activator of disease resistance signaling, and is repressed, in the absence of pathogen, by RGA5 (Figure S1). Consistent with this, *RGA4* overexpression or *RGA5* knock-down leads to cell death in the absence of recognized Avrs (Cesari *et al.*, 2014b). In addition to its repressor function, RGA5 acts as a receptor of AVR1-CO39 and AVR-Pia. It binds physically to these effectors through an unconventional C-terminal domain related to the copper chaperone ATX1 (RATX1 domain) that is required for effector recognition, and acts as an integrated decoy domain (Cesari *et al.*, 2014a, 2013). This recognition event de-represses RGA4 and triggers immune responses that lead to the inhibition of pathogen growth and the induction of HR. The molecular details of the interaction between RGA4 and RGA5 are still poorly defined but may rely on higher-order complex formation. Indeed, homo- and heterotypic interactions of RGA4 and RGA5, involving in particular their N-terminal coiled-coil domains, have been detected (Cesari *et al.*, 2014b).

While no BLS *R* gene has yet been identified in rice, 41 BB *R* genes have been characterized genetically. Among them, about a third act in a recessive manner (Liu *et al.*, 2014). Ten BB *R* genes have been cloned so far, including six genes with action mediated by transcription activator-like (TAL) effectors (Chu *et al.*, 2006; Gu *et al.*, 2005; Hutin *et al.*, 2015b; Liu *et al.*, 2011; Tian *et al.*, 2014; Wang *et al.*, 2014). TAL effectors are major virulence determinants in many plant pathogenic *Xanthomonas* species. They act as bona fide plant transcription factors able to localize to the host cell nucleus and regulate the host transcriptome (Boch and Bonas, 2010). TAL effector proteins have a conserved modular structure, including an N-terminal type-3 secretion signal, a central region made of tandem repeated sequences involved in host DNA-binding, two or three nuclear localization signals and an acidic transcription activation domain (AD) located in the C terminus. The central region is composed of a nearly identical 33–35 amino acids repeat, where most of the polymorphism lies within residues at positions 12 and 13, also referred to as hyper variable di-residues (RVDs). The RVDs array in a TAL effector defines its DNA-binding specificity, and therefore its capacity to bind to a host promoter through specific target sequences referred to as effector-binding elements (EBEs). The TAL code that governs this interaction was elucidated, demonstrating that each repeat recognizes one single nucleotide through its RVD (Boch *et al.*, 2009; Moscou and Bogdanove, 2009). This allows us to scan host promoteromes for the preferential EBEs of a given TAL effector based on its RVDs sequence (Noel *et al.*, 2013), and to design so-called designer/artificial

TAL effectors to specifically induce genes of interest (Bogdanove and Voytas, 2011).

In a susceptible host, TAL effectors mediate the induction of susceptibility (*S*) genes, which are essential for disease development (Hutin *et al.*, 2015a). In rice, the best characterized are clade-III members of the SWEET family of sugar transporter that determine host susceptibility to *Xoo* (Streubel *et al.*, 2013). Several studies showed that the lack of induction of these *SWEET* genes as a result of polymorphisms in the EBEs leads to resistance by loss of susceptibility (Hutin *et al.*, 2015a). This is exemplified by the three cloned recessive genes *xa13*, *xa25* and *xa41* that present polymorphisms in their promoter, resulting in the impaired induction of *OsSWEET11*, *OsSWEET13* and *OsSWEET14*, respectively (Chu *et al.*, 2006; Hutin *et al.*, 2015b; Liu *et al.*, 2011). The action of the recessive resistance gene *xa5* is also TAL effector-dependent. It encodes for the small γ-subunit of the general transcription factor TFIIA that differs from Xa5 by a single amino acid substitution (Iyer and McCouch, 2004). Physical interaction between Xa5 and TAL effectors was not demonstrated; however, several studies suggest that this general transcription factor could be required for TAL effector-dependent *trans*-activation of target host genes (Gu *et al.*, 2009; Sugio *et al.*, 2007; Tian *et al.*, 2014).

The remaining six cloned BB *R* genes are dominant. Three of them are triggered by *Xoo* TAL effectors, including AvrXa27, AvrXa10 and AvrXa23, which lead to resistance through transcriptional activation of their cognate *R* genes *Xa27*, *Xa10* and *Xa23*, respectively (Zhang *et al.*, 2015). All three code for proteins of unknown functions that act as uncommon activators of resistance responses and cell death, and the corresponding genes were therefore called executor (*E*) genes (Boch *et al.*, 2014). *Xa21* (Song *et al.*, 1995) and *Xa3/Xa26* (Sun *et al.*, 2004) code for receptor-like protein kinases, and the tyrosine-sulfated peptide RaxX has recently been identified as the presumable ligand of Xa21 (Pruitt *et al.*, 2015). Interestingly, *Xa1* is the only BB *R* gene that encodes an NLR (Yoshimura *et al.*, 1998). Its mode of action and its corresponding *Avr* gene are not known. Overall, it clearly appears that on the contrary to rice blast resistance, resistance to *Xoo* relies on many different mechanisms but not or only marginally on effector recognition by NLR receptors. In fact, whether or not immunity triggered by NLR-dependent effector recognition can control *Xoo* has not yet been investigated.

RESULTS

AVR1-CO39 expression triggers cell death and an oxidative burst in *Pi-CO39* rice lines

To investigate the impact of NLR-triggered immunity on *Xoo* and *Xoc* infection, we generated transgenic rice lines expressing *AVR1-CO39* in an inducible manner, and verified that *AVR1-CO39* expression triggers *Pi-CO39*-dependant defense responses and cell death. *AVR1-CO39* was cloned in the pINDEX2 vector under the control of the $pUAS_{gal4}$ promoter ($pUAS_{gal4}$*::AVR1-CO39*) that is activated by the pINDEX2-encoded GVG transcription factor after perception of the glucocorticoid hormone dexamethasone (DEX; Ouwerkerk *et al.*, 2001). The plasmid was stably introduced into the rice cultivars Kitaake, which possesses the *Pi-CO39* locus allowing the recognition of AVR1-CO39 (Cesari *et al.*, 2013), and Kanto51 (*pi-co39*) used as a negative control. To verify the tight regulation of the $pUAS_{gal4}$ promoter, control rice lines were generated with a pINDEX2 plasmid carrying the *uidA* gene cloned downstream of the $pUAS_{gal4}$ sequence ($pUAS_{gal4}$*::uidA*). These lines showed high beta-glucuronidase (GUS) activity after transplantation into DEX-containing medium, but no GUS activity when transplanted into DEX-free medium (Figure 1a). Transgenic lines carrying the $pUAS_{gal4}$*::AVR1-CO39* construct did not show any GUS activity with or without DEX treatment. These results indicate that the $pUAS_{gal4}$ promoter is tightly regulated in the rice accessions used. It is not active prior to DEX induction and it allows strong gene expression after induction.

To determine whether $pUAS_{gal4}$ also drives *AVR1-CO39* expression in a DEX-inducible manner, *AVR1-CO39* transcript levels were determined by qRT-PCR using total RNA extracted from leaves of DEX- or mock-treated transgenic Kitaake and Kanto51 rice lines carrying the $pUAS_{gal4}$*:AVR1-CO39* or the $pUAS_{gal4}$*::uidA* construct (Figure 1b). *AVR1-CO39* transcripts were not detected in DEX- or mock-treated $pUAS_{gal4}$*::uidA* or mock-treated $pUAS_{gal4}$*:AVR1-CO39* plants, whereas DEX-treatment induced *AVR1-CO39* expression in all transgenic lines carrying the $pUAS_{gal4}$*:AVR1-CO39* construct (Figure 1b). Interestingly, the *AVR1-CO39* transcript level was three times higher in one Kitaake $pUAS_{gal4}$*:AVR1-CO39* line (Kitaake line 1) compared with the other (Kitaake line 2). Taken together, these results indicate that the $pUAS_{gal4}$ promoter drives the expression of *AVR1-CO39* after DEX treatment only; however, different levels of expression are observed among independent transgenic lines.

Remarkably, the expression of *AVR1-CO39* in Kitaake plants, carrying the resistance locus *Pi-CO39*, induced necrosis in the entire plant less than 4 days after transplantation in the DEX-containing medium (Figure 2a). This phenotype was more pronounced in Kitaake line 1, which expressed *AVR1-CO39* at the highest level, compared with line 2 (Figure 1b). No cell death was observed in Kitaake plants that were transplanted to the mock medium (Figure 2a). Kanto51 $pUAS_{gal4}$*::AVR1-CO39* plants did not die with or without DEX induction, indicating that the observed necrosis results from the specific recognition of the effector by the products of *Pi-CO39* (Figure 2a). Control Kitaake transgenic lines carrying the $pUAS_{gal4}$*:uidA* construct did not show any cell death after DEX treatment.

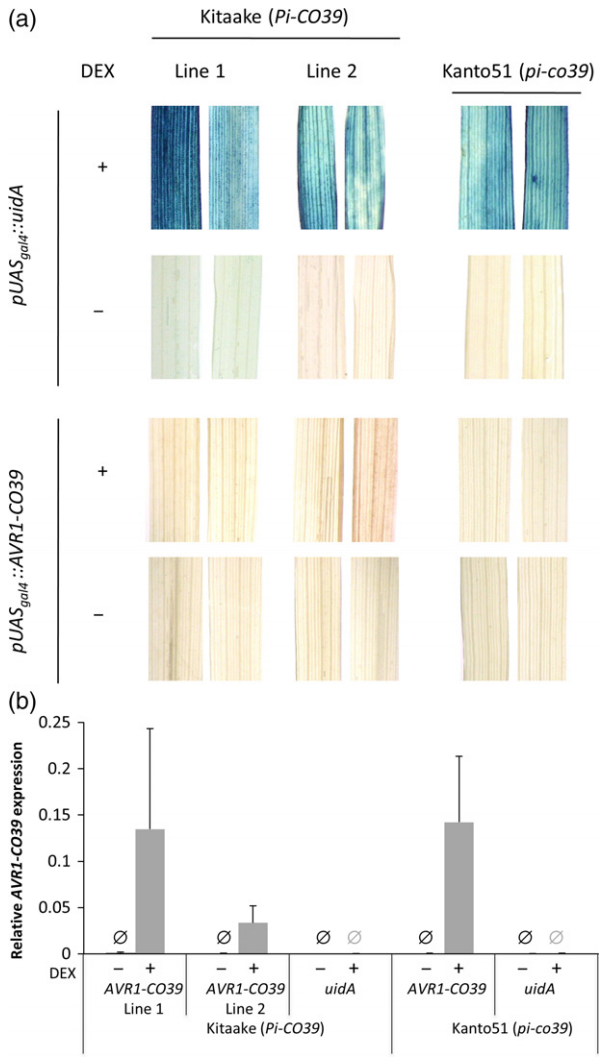

Figure 1. The *AVR1-CO39* and *uidA* genes are induced in transgenic *Oryza sativa* (rice) lines upon treatment with dexamethasone (DEX). (a) Rice cultivars Kanto51 and Kitaake were transformed with the $pUAS_{gal4}$*::uidA* or $pUAS_{gal4}$*:AVR1-CO39* constructs. Two independent transgenic Kitaake lines and one Kanto51 line were selected for each construct. Transgenic plants were treated with 100 μM DEX (+) or no DEX (–) at 2 weeks of age. GUS activity was determined 24 h after DEX treatment and pictures of the leaves of two representative plants were taken after clearing. (b) Leaf samples were harvested 24 h after DEX treatment and used for the determination of *AVR1-CO39* transcript levels by qRT-PCR. The relative expression level of *AVR1-CO39* was determined by using the constitutively expressed *actin* gene as a reference. The graph shows means and standard errors calculated from the values obtained for three independent biological samples per condition. In some samples, *AVR1-CO39* transcripts were not detectable (Ø) because its abundance was below the detection threshold.

We noted an overall reduction of plant growth after transplantation in the DEX-containing medium compared with plants transferred to mock medium (Figure 2a). A similar phenotype was observed previously in *Arabidopsis thaliana* and rice, and is associated with the non-specific activation of off-targets by the GVG transcription factor after its DEX-mediated nuclear re-localization (Kang *et al.*, 1999; Ouwerkerk *et al.*, 2001).

Reactive oxygen species (ROS) play a major role in plant defense against pathogens, and the production of hydrogen peroxide (H_2O_2) has been recognized as an important feature of plant cells that undergo programmed cell death during host–pathogen interaction. To determine whether the cell death phenotype induced by AVR1-CO39 recognition is linked to the production of H_2O_2, 3,3′-diaminobenzidine (DAB) staining was performed on DEX- or mock-treated $pUAS_{gal4}$*::uidA* or $pUAS_{gal4}$*::AVR1-CO39* lines (Figure 2b). DAB polymerizes instantly and locally into a stable brown polymer when it comes into contact with H_2O_2 in the presence of peroxidase, and has been widely used to detect H_2O_2 accumulation in plant cells undergoing an HR after pathogen recognition (Thordal-Christensen *et al.*, 1997). Following DEX treatment, only leaves from transgenic Kitaake lines expressing AVR1-CO39 showed a strong DAB staining (Figure 2b). Mock-treated Kitaake $pUAS_{gal4}$*::AVR1-CO39* lines as well as DEX- or mock-treated Kitaake $pUAS_{gal4}$*::uidA* and Kanto51 $pUAS_{gal4}$*::uidA* or $pUAS_{gal4}$*::AVR1-CO39* lines were not stained. This shows that the production of H_2O_2 occurs specifically after the induction of *AVR1-CO39* expression, and only when the effector is recognized by the products of *Pi-CO39* (Figure 2b).

Taken together, these results indicate that a burst of reactive oxygen species (ROS) followed by an HR occurs at the whole plant level following AVR1-CO39 recognition in plants carrying *Pi-CO39*.

A designer TAL effector transformed in *Xoo* and *Xoc* allows for the specific induction of the $pUAS_{gal4}$ promoter

To investigate whether the immune response triggered by the *Pi-CO39*-mediated recognition of AVR1-CO39 confers resistance to *X. oryzae* pathovars, a designer TAL effector was engineered to specifically target the $pUAS_{gal4}$ promoter ($dTALE_{UAS}$) and induce *AVR1-CO39* expression (Figure S1; Table S1). $dTALE_{UAS}$ was transformed into *Xoo* strain $PXO99^A$ and *Xoc* strain BLS256, which are both virulent on Kitaake and Kanto51 rice cultivars.

To verify that the $PXO99^A$ and BLS256 strains expressing $dTALE_{UAS}$ are able to specifically induce the $pUAS_{gal4}$ promoter, they were infiltrated in leaves of transgenic rice lines carrying the $pUAS_{gal4}$*::uidA* construct (Figure 3a). Strong GUS activity was detected after infiltration with *Xoo* ($dTALE_{UAS}$) and *Xoc* ($dTALE_{UAS}$), whereas no GUS activity was observed after infiltration with strains carrying the empty vector (*EV*) (Figure 3a). This indicates that $dTALE_{UAS}$ transactivates the $pUAS_{gal4}$ promoter. To analyze the induction of *AVR1-CO39* by $dTALE_{UAS}$ in $pUAS_{gal4}$*::AVR1-CO39* lines, *AVR1-CO39* transcript levels were determined by qRT-PCR in leaves of transgenic rice lines carrying the $pUAS_{gal4}$*::AVR1-CO39* or the $pUAS_{gal4}$*::uidA*

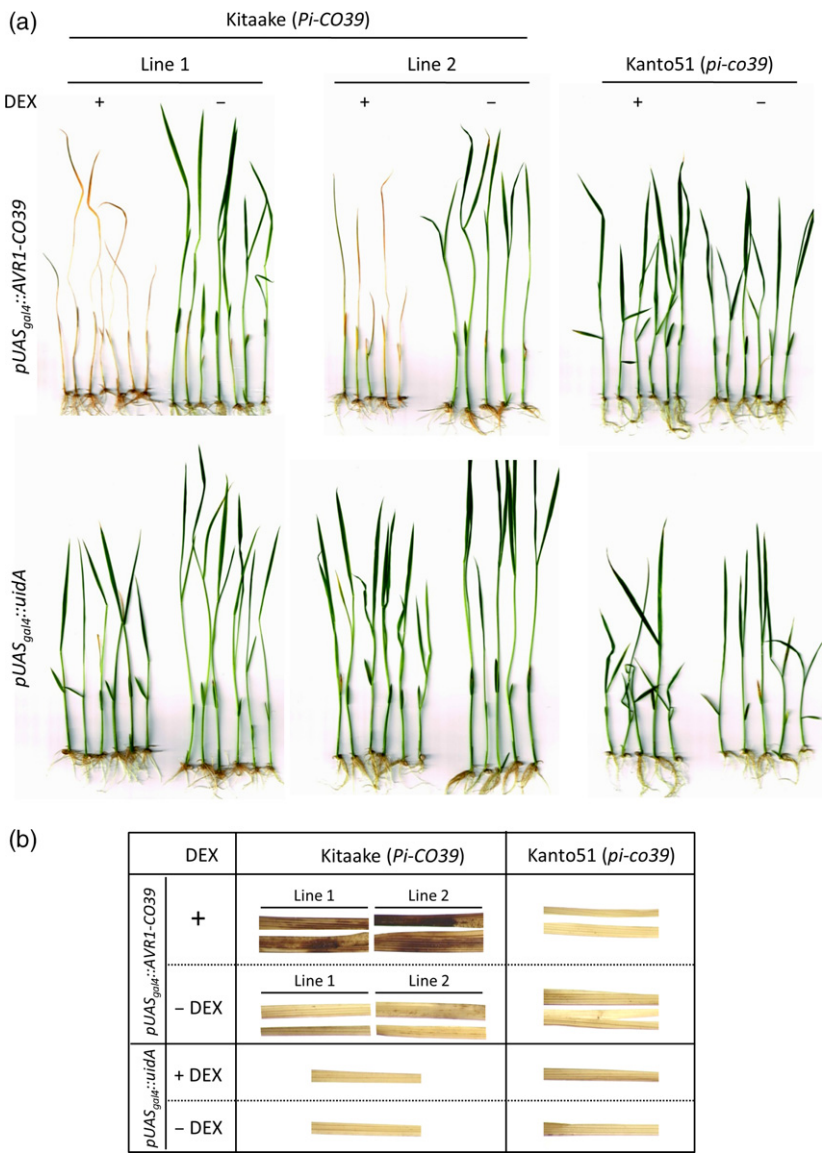

Figure 2. Induction of *AVR1-CO39* expression triggers cell death and the production of reactive oxygen species (ROS) in Kitaake lines. Transgenic Kanto51 and Kitaake plants carrying either *pUAS-gal4:uidA* or *pUASgal4:AVR1-CO39* were transferred to MS/2 media containing 100 μM dexamethasone (DEX; +) or no DEX (–) at 2 weeks of age. (a) DEX-treated Kitaake plants expressing *AVR1-CO39* died and cell death was observed 4 days after contact with DEX. Pictures were taken 4 days after the initiation of DEX treatment. (b) Leaves were sampled 2 days after the beginning of DEX treatment and stained with DAB solution. Only Kitaake DEX-treated plants expressing *AVR1-CO39* showed DAB staining indicative of H_2O_2 accumulation.

construct infiltrated with mock, PXO99^A (*EV*) or PXO99^A (*dTALE$_{UAS}$*) (Figure 3b). *AVR1-CO39* was weakly or not at all expressed upon infiltration of water or PXO99^A (*EV*), and was induced in *pUAS$_{gal4}$::AVR1-CO39* lines infiltrated with PXO99^A (*dTALE$_{UAS}$*), but not in *pUAS$_{gal4}$::uidA* lines. Interestingly, and consistent with the level of transcriptional induction after DEX treatment (Figure 3b), *AVR1-CO39* transcript levels were higher in Kitaake *pUAS$_{gal4}$::AVR1-CO39* line 1 than in Kitaake *pUAS$_{gal4}$::AVR1-CO39* line 2. Taken together, these results indicate that the *pUAS$_{gal4}$* promoter is specifically induced by both pathovars of *X. oryzae* carrying *dTALE$_{UAS}$*.

AVR1-CO39 induction in *Pi-CO39* rice lines confers resistance to *X. oryzae*

As a next step, it was investigated whether the immune response triggered by the *Pi-CO39*-mediated recognition of AVR1-CO39 confers resistance to the vascular *Xoo* or mesophyll-restricted *Xoc* pathogens. *Xoo* PXO99^A and *Xoc* BLS256 derivative strains carrying the empty vector (*EV*) or *dTALE$_{UAS}$* were infiltrated in leaves of transgenic *pUAS-gal4::AVR1-CO39* or *pUASgal4::uidA* lines, and water-soaking disease lesions were recorded 5 days after inoculation (Figure 4). PXO99^A (*EV*) led to water-soaking symptoms on all transgenic Kitaake and Kanto51 lines, showing that PXO99^A is fully virulent on these rice cultivars. As expected, PXO99^A (*dTALE$_{UAS}$*) also caused strong disease symptoms on Kanto51 lines, as well as on the Kitaake *pUAS$_{gal4}$::uidA* line. In contrast, water-soaking symptoms were strongly reduced upon inoculation of Kitaake *pUAS-gal4::AVR1-CO39* lines with PXO99^A (*dTALE$_{UAS}$*), suggesting that the immune response activated by the recognition of AVR1-CO39 by the products of the *Pi-CO39* resistance locus prevented BB.

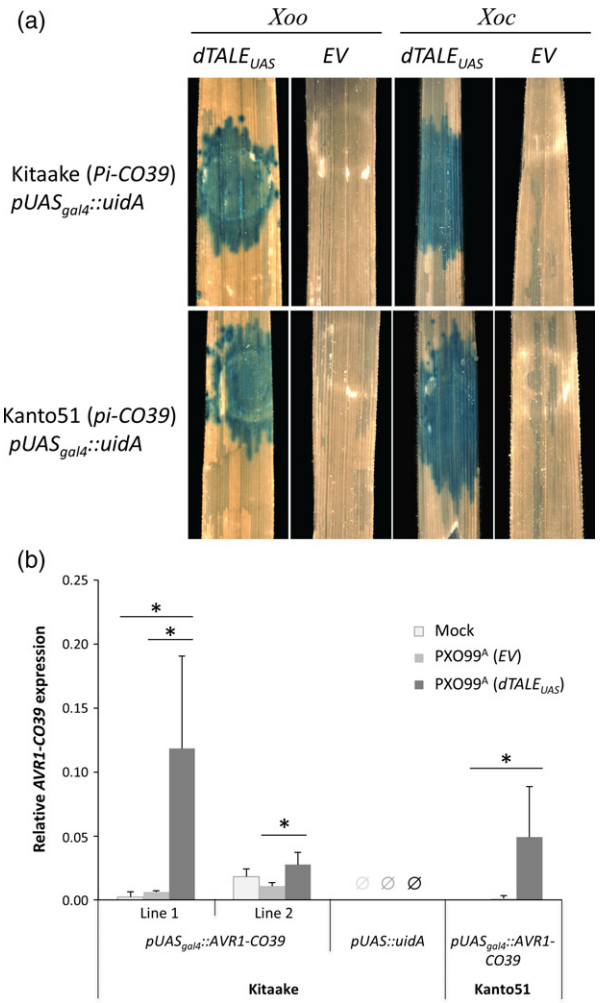

Figure 3. dTALE$_{UAS}$-expressing *Xanthomonas oryzae* transactivates *UAS$_{gal4}$* promoter sequences in transgenic *Oryza sativa* lines. (a) Rice cultivars Kanto51 and Kitaake carrying the *pUAS$_{gal4}$:uidA* construct were infiltrated with *Xoo* PXO99^A and *Xoc* BLS256 strains carrying an empty vector (*EV*) or a plasmid encoding the designer TAL effector dTALE$_{UAS}$, which specifically targets the *UAS$_{gal4}$* promoter sequence. GUS activity was determined 4 days after infiltration using X-Gluc substrate. Pictures were taken after leaf clearing using ethanol. (b) Leaves of Kanto51 and Kitaake *pUAS$_{gal4}$:AVR1-CO39* or *pUAS$_{gal4}$:uidA* lines were harvested 24 h after infiltration with PXO99^A (*EV*) or PXO99^A (*dTALE$_{UAS}$*), and used for the determination of *AVR1-CO39* transcript levels by qRT-PCR. The relative expression level of *AVR1-CO39* was determined by using the constitutively expressed *actin* gene as a reference. The graph shows means and standard errors calculated from the values obtained for three independent biological samples per condition. In some samples, the *AVR1-CO39* transcript was not detectable (Ø) because its abundance was below the detection threshold. *Mean values that significantly differ from the mean value of the corresponding empty vector or mock samples ($P < 0.05$ in a Student's *t*-test).

To determine more precisely the level of *Xoo* resistance, the transgenic Kitaake and Kanto51 lines carrying *pUAS-gal4::AVR1-CO39* or *pUASgal4::uidA* were inoculated with PXO99^A (*EV*) and PXO99^A (*dTALE$_{UAS}$*) by leaf clipping, and the length of disease lesions was measured 2 weeks later. As expected, PXO99^A (*EV*) caused disease on all Kitaake and Kanto 51 lines tested, and there was no difference in lesion length between Kitaake *pUAS$_{gal4}$::AVR1-CO39* and *pUAS$_{gal4}$::uidA* lines (Figure 5a). In contrast, PXO99^A (*dTALE$_{UAS}$*) was avirulent on Kitaake *pUAS$_{gal4}$::AVR1-CO39* where lesion length was drastically reduced as compared with Kitaake *pUAS$_{gal4}$::uidA*. Kitaake *pUAS$_{gal4}$::AVR1-CO39* line 1 exhibited a higher level of resistance than line 2, which is consistent with the higher induction of *AVR1-CO39* in line 1 as compared with line 2 (Figure 3b).

To determine whether resistance to PXO99^A (*dTALE$_{UAS}$*) in Kitaake *pUAS$_{gal4}$::AVR1-CO39* is correlated with reduced bacterial growth *in planta*, we quantified *Xoo* populations 8 days after leaf-clip inoculation. PXO99^A (*dTALE$_{UAS}$*) grew at similar or slightly higher levels than PXO99^A (*EV*) in most rice lines, but its titer was drastically reduced or undetectable in Kitaake *pUAS$_{gal4}$::AVR1-CO39* (Figure 5b). This demonstrates that the immune response activated by *Pi-CO39*-mediated recognition of AVR1-CO39 prevents leaf colonization by *Xoo*.

To test whether the *Pi-CO39*-mediated immune response is also effective against mesophyll-restricted *Xoc*, the ability of BLS256 (*dTALE$_{UAS}$*) to cause leaf streaks on Kanto51 and Kitaake *pUAS$_{gal4}$::AVR1-CO39* or *pUAS$_{gal4}$::uidA* lines was assayed. Again, all rice lines exhibited typical BLS symptoms, except Kitaake *pUAS$_{gal4}$::AVR1-CO39* leaves inoculated with BLS256 (*dTALE$_{UAS}$*) (Figure 4). The level of bacteria was measured *in planta*, 6 days after leaf infiltration. As expected from the observed disease symptoms, the titer of *Xoc* strain BLS256 (*dTALE$_{UAS}$*) is significantly lower on Kitaake *pUAS$_{gal4}$::AVR1-CO39* (Figure 5c). Overall, our results show that the immune response triggered upon recognition of the fungal effector AVR1-CO39 by the products of the *Pi-CO39* rice blast *R* locus, the NLR hetero-pair RGA4/RGA5, is effective against rice pathogenic Xanthomonads, regardless of their tissue specificities.

dTALE-mediated induction of *RGA4* confers resistance to *Xoo* and *Xoc* in *Pi-CO39* rice cultivars

To further strengthen our finding that NLR-mediated immune responses prevent infection by *Xoo*, two designer TAL effectors, dTALE$_{RGA4_1}$ and dTALE$_{RGA4_2}$, were generated to target specifically two distinct binding sites in the promoter of *RGA4* (Figures S1D, S2), the product of which is a constitutively active inducer of immune responses (Cesari *et al.*, 2014b). In the absence of any pathogen, RGA4 is repressed by RGA5 and disease resistance signaling is only activated when RGA5 binds AVR1-CO39 and AVR-Pia, and RGA4 is de-repressed. A balanced *RGA4*/*RGA5* expression is therefore important for proper RGA4 regulation, and *trans*-activation of *RGA4* should trigger the induction of immune responses and resistance to *Xoo*.

PXO99^A strains carrying either *dTALE$_{RGA4_1}$* or *dTALE$_{RGA4_2}$* were fully virulent on the rice variety Nipponbare

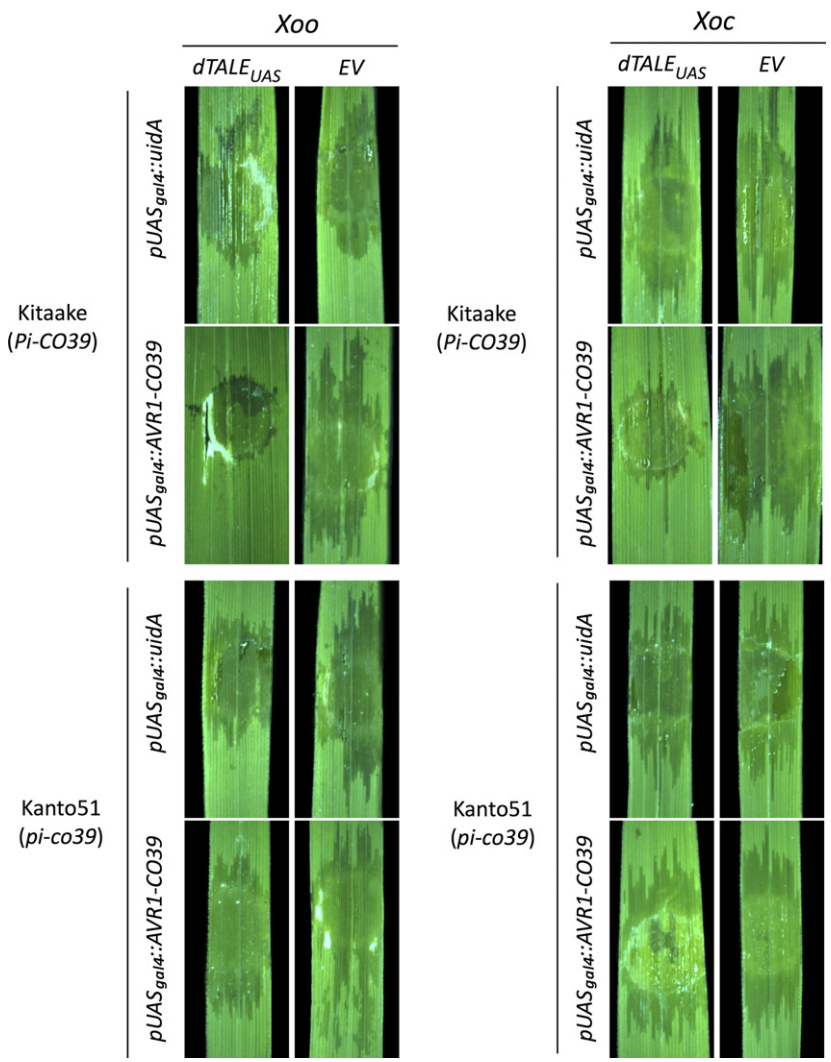

Figure 4. *AVR1-CO39* induction in *Pi-CO39 Oryza sativa* lines confers resistance to *Xoo* and *Xoc* in syringe infiltration assays. Leaves of Kanto51 and Kitaake *pUAS$_{gal4}$:uidA* or *pUAS$_{gal4}$:AVR1-CO39* lines were infiltrated with *Xoo* PXO99^{A} and *Xoc* BLS256 strains carrying an empty vector (*EV*) or a plasmid encoding *dTALE$_{UAS}$*. Leaves showed characteristic water-soaking lesions expanding from the inoculation site, with the exception of Kitaake *pUAS$_{gal4}$:AVR1-CO39* infiltrated with *Xoo* PXO99^{A} (*dTALE$_{UAS}$*) and *Xoc* BLS256 (*dTALE$_{UAS}$*), which showed no disease symptoms. Photos were taken 5 days after infiltration. This experiment was reproduced three times with similar results.

that does not contain *RGA4* (Cesari *et al.*, 2013), but did not cause disease symptoms on leaves of the rice varieties Kitaake, Sasanishiki and CO39 that all contain *RGA4* (Figure 6a). To document more precisely this avirulence on *Pi-CO39* varieties, lesion lengths after leaf-clip inoculation were compared between PXO99^{A} (*dTALE$_{RGA4_1}$*) and PXO99^{A} (*EV*). On all varieties that carry *RGA4*, the lesion length of PXO99^{A} (*dTALE$_{RGA4_1}$*) was drastically reduced when compared with PXO99^{A} (*EV*), whereas there was no difference between both strains in Kanto51 that do not possess *RGA4* (Figure 6b).

Taken together, these results show that *RGA4*-driven immune responses are sufficient to control *Xoo*.

DISCUSSION

Recognition of a fungal effector triggers resistance to bacterial pathogens in rice

In this study, we demonstrate that the resistance response triggered by the recognition of the *M. oryzae* effector AVR1-CO39 by the rice NLR pair RGA4/RGA5 is effective not only against this fungal pathogen but also against the bacterial rice pathogens *Xoo* and *Xoc*. Naturally occurring cases of NLR proteins conferring resistance to multiple pathogens are thought to rely on the recognition of different AVR effectors produced by the distinct recognized pathogens. For instance, the Arabidopsis NLR pair *RPS4*/*RRS1* confers resistance to the bacterial pathogens *Pseudomonas syringae* and *Ralstonia solanacearum* through the recognition of the effectors AvrRps4 and PopP2, respectively (Deslandes *et al.*, 2003; Gassmann *et al.*, 1999). In addition, this NLR pair confers resistance to the fungus *Colletotrichum higginsianum* through the recognition of a yet unknown effector that presumably differs from the bacterial effectors (Birker *et al.*, 2009). Another example is the tomato *Mi-1* gene that confers resistance to both root-knot nematodes and aphids through the recognition of unknown effectors that might differ between the two parasites (Vos *et al.*, 1998). In contrast to these examples, our analysis shows that the recognition of a single AVR

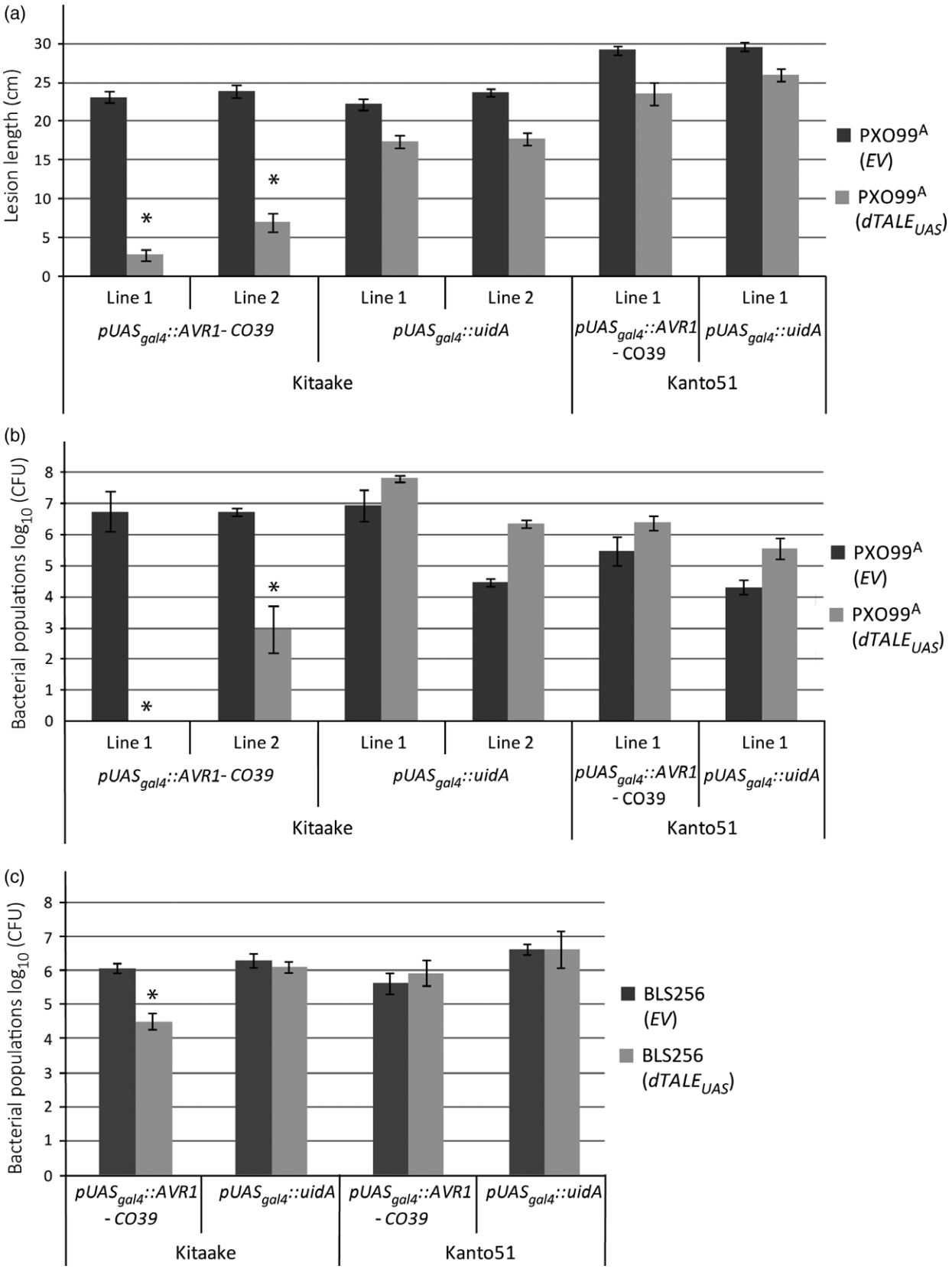

Figure 5. Induction of *AVR1-CO39* in *Pi-CO39* rice lines confers resistance to *Xoo* and *Xoc* upon quantitative leaf clipping and bacterial titer measurements. (a, b) Leaves of Kanto51 and Kitaake $pUAS_{gal4}$*:uidA* or $pUAS_{gal4}$*:AVR1-CO39* lines were leaf-clip inoculated with *Xoo* strain PXO99^{A} carrying an empty vector (dark grey) or $dTALE_{UAS}$ (light grey). (a) Lesion length was measured 15 days post-inoculation. Mean values and standard deviations were calculated from measurements of eight leaves from eight independent individuals. This experiment was repeated three times with similar results. *Significantly different values ($P < 5.10^{-5}$) by Mann–Whitney *U*-test, comparing treatments with PXO99^{A} (*EV*). (B) Bacterial titers of a 7.5-cm leaf segment located 7.5 cm distal to the inoculation site were determined 8 days after inoculation. Mean values and standard deviations were calculated based on three independent leaf samples. *Significant difference ($P < 0.002$) by Mann–Whitney *U*-test, compared with PXO99^{A} (*EV*). (C) Leaves of Kanto51 and Kitaake $pUAS_{gal4}$*:uidA* or $pUAS_{gal4}$*:AVR1-CO39* line 1 were infiltrated with *Xoc* BLS256 strains carrying an empty vector (*EV*) or a plasmid encoding $dTALE_{UAS}$. *Xoc* BLS256 titers of a 3-cm leaf segment around the infiltration site were determined 6 days after inoculation. Mean values and standard deviations were calculated based on three independent leaf samples. *Significant difference ($P < 0.005$) by Mann–Whitney *U*-test, compared with BLS256 (*EV*). This experiment was reproduced three times with similar results.

effector by the NLR heteropair RGA4/RGA5 is efficient in preventing infection by pathogens belonging to distinct kingdoms, using different infection strategies and colonizing distinct plant tissues. This indicates that defense responses activated by RGA4/RGA5 are broadly effective and are not specific to *M. oryzae*.

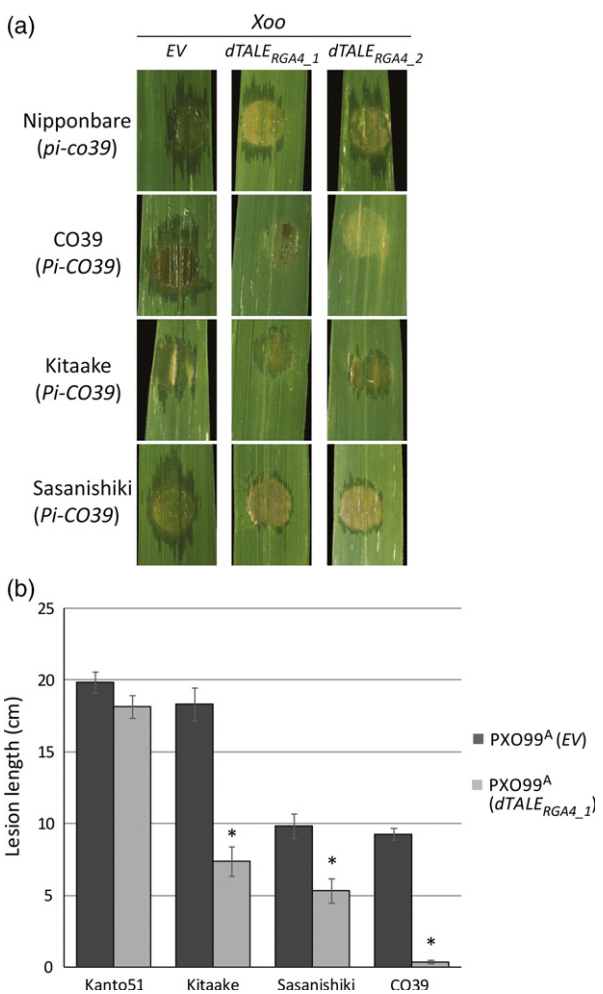

Figure 6. The induction of *RGA4* in *Pi-CO39 Oryza sativa* lines confers resistance against *Xoo*. (a) Leaves of the rice varieties carrying a functional *RGA4* (CO39, Kitaake and Sasanishiki), or not (Nipponbare), were infiltrated with strains of *Xoo* PXO99^{A} transformed with an empty vector (*EV*), or with a plasmid encoding $dTALE_{RGA4\_1}$ or $dTALE_{RGA4\_2}$. Photos were taken 5 days after infiltration. (b) Leaves of *Pi-CO39* (Kitaake, Sasanishiki and CO39) and *pi-CO39* (Kanto51) rice varieties were inoculated by leaf-clipping with *Xoo* derivative strains PXO99^{A} carrying an empty vector (dark grey) or $dTALE_{RGA4\_1}$ (light grey). Lesion length was measured 15 days post-inoculation. Mean values and standard deviations were calculated from measurements of at least 10 independent leaves. *Significant difference ($P < 10^{-4}$) by Mann–Whitney *U*-test, compared with PXO99^{A} (*EV*). This experiment was repeated twice with similar results.

Such broad activity of an NLR-triggered immune response has previously been reported in *A. thaliana*, but not yet in monocotyledonous plants. Indeed, the expression of the effectors ATR1 and ATR13 from the oomycete pathogen *Hyaloperonospora parasitica* fused to bacterial type-III secretion signals in *P. syringae* triggered resistance to this bacterial pathogen in *A. thaliana* accessions carrying the corresponding *R* genes *RPP1* or *RPP13* (Rentel *et al.*, 2008; Sharma *et al.*, 2013; Sohn *et al.*, 2007). In addition, the introduction of ATR13 into turnip mosaic virus rendered the recombinant virus avirulent on *A. thaliana* accessions possessing *RPP13* (Rentel *et al.*, 2008). It therefore becomes clear that, upon effector recognition, NLRs from mono- and dicotyledonous plants activate immune responses with a very large spectrum of activity that are effective against a broad range of pathogenic microorganisms with very different lifestyles.

NLR-mediated immune responses prevent colonization by xylem- and mesophyll- colonizing *X. oryzae* pathovars

It is striking that despite the thorough investigation of BB and BLS no case has been discovered where rice resistance is based on NLR-mediated effector recognition (Boch *et al.*, 2014; Liu *et al.*, 2014). A potential exception is the BB resistance gene *Xa1* that encodes an NLR, but its mode of action remains elusive because no corresponding *AVR* gene has been described and downstream responses have not yet been characterized (Yoshimura *et al.*, 1998). *Xa1* was described to be induced after infection with an avirulent *Xoo* isolate; however, there is no evidence that it acts as an *E* gene as it is also expressed during infection with a virulent isolate and under control conditions. Moreover the semi-quantitative RT-PCR experiments used in the study document *Xa1* expression only in an approximate manner (Yoshimura *et al.*, 1998). The *Xoc* non-host *R* gene *Rxo1* from maize codes for an NLR that recognizes the AvrRxo1 effector from *Xoc* and confers resistance to BLS in transgenic rice (Zhao *et al.*, 2005); however, rice *R* genes against BLS coding either for NLRs or other proteins have not yet been discovered.

The reason for this marginal role of NLRs in BB and BLS resistance is not clear, and hypotheses such as the

presence of potent immunity suppressors in *Xoo* and *Xoc*, or the very pronounced tissue specificity of *Xoo* that is restricted to the xylem, where NLR-triggered immune responses could eventually be inefficient, do not seem to hold true. Other xylem-colonizing vascular pathogens such as *Fusarium oxysporum*, *Ralstonia solanacearum* and *Xanthomonas campestris* pv. *campestris* are controlled by NLRs, showing that defense responses induced by NLRs effectively control a broad diversity of pathogen infection strategies, including xylem colonization (Deslandes *et al.*, 2003; Wang *et al.*, 2015; Michielse and Rep, 2009). Immune suppression has been described for *Xoc* that is able to suppress Xa10-mediated resistance triggered by AvrXa10 recognition (Makino *et al.*, 2006); however, Xa10 is not an NLR but is an executor R protein of unknown function (Tian *et al.*, 2014). Our study does not provide evidence for the suppression of NLR-triggered immunity by *Xoc* or *Xoo*, and clearly indicates that BB and BLS can be effectively controlled by immune responses triggered upon NLR-mediated effector recognition.

Therefore, screening the pool of existing NLRs from rice for receptors that recognize conserved type-III effectors from *Xoo* or *Xoc*, and in particular conserved domains of TAL effectors, or developing engineered NLRs with such specificities, appear to be innovative and promising strategies for developing novel BB and BLS resistance.

NLR and *AVR* genes can be used as executors in 'promoter trap' approaches to control BLB and BLS in rice

The crucial role of TAL effectors in the pathogenicity of *Xoo*, *Xoc* and many other devastating pathogens in the *Xanthomonas* genus has prompted biotech approaches for *Xanthomonas* resistance that copy TAL effector-based resistance occurring naturally in crops (Boch *et al.*, 2014). EBEs in the promoters of susceptibility genes have been modified by precision genome engineering to create recessive resistance based on the suppression of TAL effector virulence functions (Hutin *et al.*, 2015a; Boch *et al.*, 2014). In addition, promoter trap cassettes containing EBEs upstream of known, naturally occurring TAL effector-specific *E* genes were introduced in crop genomes (Boch *et al.*, 2014; Zhang *et al.*, 2015). In rice, for example, three different binding sites targeted by TAL effectors from *Xoo* and three binding sites of TAL effectors from *Xoc* were assembled upstream of the *Xa27 E* gene into a promoter trap construct (Hummel *et al.*, 2012). With this, resistance to *Xoo* and *Xoc* was obtained, indicating that the same *E* gene can mediate protection against pathogens with distinct tissue specificities.

Alternative approaches relying on NLR-mediated effector recognition or the use of autoactive NLRs as *E* genes have been proposed (Boch *et al.*, 2014). In our study we provide the proof of concept for both approaches, and thereby pave the way to novel biotech- and promoter engineering-based solutions to *Xanthomonas* resistance. In the first place, we show that introducing an *AVR* effector gene under the control of promoter elements targeted by a TAL effector into rice confers resistance to *Xoo* and *Xoc* isolates that possess the corresponding TAL effector. Tight regulation and high inducibility of the *AVR* gene that acts here as an *E* gene appear to be key prerequisites for the success of this strategy. Its advantage is that it can be transferred to all crops for which AVR effectors triggering strong resistance are known. Knowledge of the corresponding *NLR R* gene is not required.

In addition, we show that autoactive NLRs are well suited as *E* genes in promoter trap biotech approaches for *Xanthomonas* resistance, and in particular for resistance against BB and BLS. This greatly expands the panel of genes to be used for synthetic promoter traps mediating the recognition of TAL effectors in engineered plants. Indeed, to date, only five *E* genes have been characterized: *Bs3* and *Bs4C* from pepper (*Capsicum annuum* and *Capsicum pubescens*) and *Xa27*, *Xa23* and *Xa10* from rice (Gu *et al.*, 2005; Romer *et al.*, 2007; Strauss *et al.*, 2012; Tian *et al.*, 2014; Wang *et al.*, 2015). In particular, in plants where no TAL effector-specific *E* genes are known, precision genomic engineering offers the opportunity to introduce TAL effector-responsive elements into *NLR* promoters by non-GMO approaches and, if required, to render these NLRs autoactive. In addition, this strategy may circumvent the suppression of resistance relying on TAL effector-specific *E* genes, as has been described for *Xa10* in *Xoc*, for example (Makino *et al.*, 2006).

Taken together, our study provides significant insight into resistance against BB and BLS disease in rice. It shows that NLR-mediated effector recognition has the capacity to stop these diseases. In addition, it demonstrates the potential of manipulating defense responses induced by NLR-mediated perception of AVR proteins, or by autoactive NLRs, as a useful tool to protect plants against pathogens for which, as in the case of *Xoc*, almost no resistance sources are available. It has been suggested that such engineered defense systems could be superior to natural systems because they can be designed to confer broad spectrum, durable and pathogen-adapted resistance (Boch *et al.*, 2014); however, the durability of these activation traps will strongly depend on our capacity to assess the repertoire of TAL effectors in pathogen population. This will enable us to build optimized synthetic promoters, taking into account the diversity and evolution of TAL effectors for selecting the most conserved among multiple pathovars and/or within species.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The bacterial strains used in this study are *Xoo* PXO99^{A} and *Xoc* BLS256. They carry the empty vector pSKX1 (*EV*) or pSKX1

derivatives carrying *dTALEs*. *X. oryzae* strains were cultivated at 28°C in PSA medium (10 g peptone, 10 g glucose, 1 g glutamic acid, 16 g agar, per liter of H_2O). Rifampicin and gentamicin were added when appropriate to the medium at a final concentration of 100 μg ml^{-1} and 20 μg ml^{-1}, respectively.

Constructs for rice transformation

Details on the generation of plasmids *pUAS$_{gal4}$::uidA* and *pUAS$_{gal4}$::AVR1-CO39*, using pINDEX2 (Ouwerkerk *et al.*, 2001), are given in Table S2.

Identification of target sites and construction of designer TAL effectors

The *pUAS$_{gal4}$* and *RGA4* promoter sequences from rice variety Sasanishiki were analyzed to find the appropriate designer TAL-effector binding sites, as described previously (Streubel *et al.*, 2013). The specificity of the selected binding sites were verified with TALVEZ (http://bioinfo.mpl.ird.fr/cgi-bin/talvez/talvez.cgi; Perez-Quintero *et al.*, 2013). Designer TAL effectors were generated using the Golden TAL technology and expressed as FLAG fusions under the control of an *lac* promoter in the Golden Gate-compatible broad host range vector pSKX1 (Streubel *et al.*, 2013). The RVD sequences of the dTALEs used in this study are provided in Table S1.

Transgenic rice lines

pUAS$_{gal4}$::uidA and *pUAS$_{gal4}$::AVR1-CO39* were used for the *Agrobacterium tumefaciens*-mediated transformation (strain EH1) (Toki *et al.*, 2006) of Kanto51 and Kitaake rice cultivars. Infected calli were selected on medium containing 50 mg L^{-1} hygromycin phosphotransferase. Hygromycin-resistant calli were transferred to regeneration medium. At least six independent transgenic lines were obtained for each construct in each transformation experiment. Homozygous T_3 or T_4 generation plants were used in all experiments.

DEX treatment

Plants were grown from disinfected seeds, under sterile conditions, in half-strength Murashige and Skoog (MS) medium in a 26°C growth room with a 12-h light period. Two-week-old seedlings were transferred into Magenta boxes containing half-strength MS liquid medium supplemented with 100 μM dexamethasone (DEX; Sigma-Aldrich, https://www.sigmaaldrich.com) or mock solution (0.5% ethanol). Full plants or leaf samples were collected at appropriate times for RNA extraction, DAB staining, GUS staining or cell death assays.

DAB staining

Leaf samples were collected 2 days after DEX or mock treatment and stained in a 1-mg ml^{-1} 3′-3′-diaminobenzidine (DAB) solution, as described by Thordal-Christensen *et al.* (1997). Stained leaves were cleared from chlorophyll with a 3:1 (V/V) ethanol/acetic acid solution.

GUS staining

For the determination of GUS activity, leaves harvested 24 h after DEX treatment, or 4 days after inoculation with *Xoo* or *Xoc*, were incubated overnight at 37°C in the dark in GUS staining solution [1 mM 5-bromo-4-chloro-3-indoxyl-β-glucuronide (X-Gluc), 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.05% Triton X-100, 50 mM sodium phosphate, pH 7). Leaves were cleared using 70% ethanol.

RNA extraction and qRT-PCR analysis

RNA extraction and reverse transcription were performed as described previously (Delteil *et al.*, 2012), and quantitative PCR was performed using LC 480 SYBR Green I Master Mix (Roche, http://www.roche.ch), a LightCycler 480 instrument (Roche) and the primers listed in Table S3. The quantity of plant RNA in each sample was normalized using *actin* (*Os03 g50890*) as an internal control.

Plant inoculation assays

For infiltration assays, leaves of 3-week-old plants were infiltrated with a bacterial suspension at an optical density of 600 nm (OD_{600}) of 0.5 using a needle-less syringe, as previously described (Reimers and Leach, 1991), and symptoms of water-soaked lesions were scored 5 days post-inoculation (5 dpi). For qRT-PCR assays, leaf segments were collected 24 h post-infiltration. Leaf-clip inoculation was performed on rice plants at 4–6 weeks of age using a bacterial suspension at an OD_{600} of 0.2 (Kauffman *et al.*, 1973). Symptoms were scored by measuring lesion lengths at 14 dpi. For *Xoo in planta* growth assays, leaves of 3-week-old plants were leaf-clipped with a bacterial suspension of *Xoo* strains at an OD_{600} of 0.2, and two segments of 7.5 cm were collected at 8 dpi. For *Xoc*, leaves were infiltrated with a bacterial suspension at an OD_{600} of 0.2, and a segment of 3 cm around the infiltration site was collected at 6 dpi.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Strategy used in this study.

Figure S2. dTALEs binding sites in the *RGA4* promoter of the rice variety Sasanishiki.

Table S1. RVD sequences of the dTALEs used in this study and their target sequence.

Table S2. Constructs used in this study.

Table S3. Primers used in this study.

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