Colonization of Rice Leaf Blades by an African Strain of *Xanthomonas oryzae* pv. *oryzae* Depends on a New TAL Effector That Induces the Rice Nodulin-3 *Os11N3* Gene

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African strains of *Xanthomonas oryzae* pv. *oryzae* contain fewer TAL effectors than Asian strains, and their contribution to pathogenicity is unknown. Systematic mutagenesis of *tal* genes was used to decipher the contribution of each of the eight TAL effector paralogs to pathogenicity of African *X. oryzae* pv. *oryzae* BAI3. A strain mutated in *talC* was severely affected in the production of disease symptoms. Analysis of growth in planta upon leaf-clip inoculation showed that mutant bacteria multiplied only at the site of inoculation at the apex of the leaf, suggesting a requirement for *talC* during colonization of vascular tissues. Such tissue-specific effect of a *tal* mutant is a novel phenotype, which has not yet been characterized in other xanthomonads. Microarray experiments comparing the host response of rice leaves challenged with BAI3⁺ vs. BAI3^{ΔtalC} were performed to identify genes targeted by TalC. A total of 120 upregulated and 21 downregulated genes were identified, among them *Os11N3*, which is a member of the *MtN3/saliva* family. Based on semiquantitative reverse transcription-polymerase chain reaction and β-glucuronidase reporter assays, we show that *Os11N3* is directly upregulated by TalC and identify a TalC DNA target box within the *Os11N3* upstream sequence.

Phytopathogenic bacteria of the genus *Xanthomonas* cause severe diseases on many crop plants, including economically important plants such as rice, wheat, banana, beans, tomato, *Citrus* spp., and cotton. Common diseases found in nature include vascular wilt, cankers, leaf spots, fruit spots, leaf blight, and leaf streak. To colonize their host organisms, most *Xanthomonas* spp. evolved a highly conserved type III secretion sys-

tem (T3SS) that injects a cocktail of type III effectors (T3E) into plant cells, in which they interfere with immune responses or facilitate nutritional or virulence processes of benefit for the pathogen. A recent review listed nearly 40 families of T3E from *Xanthomonas* spp. (White et al. 2009).

AvrBs3 from *X. axonopodis* pv. *vesicatoria* and PthA from *X. citri* subsp. *citri* are prototypes of an intriguing family of T3E (Bonas et al. 1989; Swarup et al. 1991). Homologs of these two proteins are only found in other *Xanthomonas* species and in *Ralstonia solanacearum*. Owing to their ability to reprogram the transcriptome of the host cells by mimicking eukaryotic transcription factors, AvrBs3/PthA-like proteins are also referred to as transcription activator-like (TAL) effectors (Yang et al. 2006). TAL effectors are characterized by a central protein domain comprised of a variable number of tandemly-repeated near-identical modules of 34 or 35 amino acids. In addition, TAL effectors contain nuclear localization signals (NLS) and an acidic transcriptional activation domain (AAD) in their C-terminal region. Because the N- and C-terminal portions of the proteins are highly conserved, functional differences between members of the family are mainly due to the central repeat region. The number and order of repeats specify the virulence and avirulence activities of a given TAL effector (Boch and Bonas 2010). Recent results confirmed that the prototypic TAL effector AvrBs3 acts as a transcription factor that binds to specific DNA elements in the host cell nucleus and induces the expression of a distinct set of host genes. AvrBs3 was shown to bind directly to a so-called *UPA* (upregulated by AvrBs3) box in the promoter region of AvrBs3-dependently induced *upa* genes (Kay et al. 2007). One of the *upa* genes, *upa20*, encodes a basic helix-loop-helix transcription factor, which is sufficient to cause host-cell hypertrophy when expressed ectopically. The repeated modules of AvrBs3 are essential for DNA-binding of AvrBs3 and build up a unique type of DNA-binding domain. How this domain contacts DNA and determines specificity remained enigmatic until the recent discovery of the TAL effector's DNA recognition code (Boch et al. 2009; Moscou and Bogdanove 2009). The binding specificity is governed by so-called repeat-variable diresidues (RVD) at position 12 and 13, present in each module of the central

Nucleotide sequence data for the entire 8,901-bp sequence carrying *talC* is available in GenBank under accession number I465540 JN234375.

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure is published online and that Figures 1, 4, and 7 appear in color online.

protein domain. The number of repeated modules corresponds to the number of recognized base pairs. This knowledge of the recognition code now allows the prediction of target DNA boxes for TAL effectors.

A variety of TAL effectors are associated with biological effects and contribute to disease symptoms (White and Yang 2009). TAL effector-mediated gene induction leads, for instance, to developmental changes of the host plant, such as enhanced cell divisions in citrus canker and cell enlargement resulting in a hypertrophy of the tissue (Marois et al. 2002; Swarup et al. 1992). Other TAL effectors are required to establish the susceptibility of the host towards infection, although the molecular details of this interaction are largely unknown. When the responsible TAL effector is absent in a mutant bacterium, any susceptibility (*S*) genes cannot be induced and disease cannot be established (White and Yang 2009). Hence, lack of susceptibility is reminiscent of a classic gene-for-gene resistance (Flor 1971). However, lack of susceptibility is typically a recessive trait, while classic gene-for-gene resistance follows a dominant segregation pattern. For example, *Os8N3* was identified by comparative transcriptomics of susceptible rice challenged with *X. oryzae* pv. *oryzae* PXO99^A versus PXO99^A lacking the major virulence TAL effector gene *pthXo1*. Full virulence of PXO99^A was found to depend on *PthXo1*, which mediates the induction of the *S* gene *Os8N3*. Disease is impaired in RNAi lines in which *Os8N3* is knocked down (Yang et al. 2006). *Os8n3* is a member of the *N3* gene family, which is found throughout the plant kingdom, and with related genes in mammals, insects, nematodes, and filamentous fungi (White and Yang 2009). Little is known about its role in disease with respect to promoting susceptibility to xylem-colonizing pathogenic bacteria. Three additional TAL effectors (*pthXo2*, *pthXo3*, and *avrXa7*) with major roles in virulence have been identified in field isolates of *X. oryzae* pv. *oryzae* (Hopkins et al. 1992; Yang and White 2004). They can each restore virulence to PXO99^A lacking *pthXo1*, but they do so without inducing *Os8N3* (Yang et al. 2006). It was speculated that these effectors target other members of the *N3* family (White and Yang 2009). Aside from *pthXo1*, at least two TAL effector genes of PXO99^A have moderate effects on virulence, *pthXo6* and *pthXo7*. They respectively mediate the induction of *OsTFX1*, a bZIP transcription factor gene, and *OsTFIIAγ1*, a small subunit of the transcription factor IIA that compensates for the recessive *R* gene *xa5* mutation in *OsTFIIAγ5* (Sugio et al. 2007). Intriguingly, the *S* gene *Os8N3* is allelic to the recessive *R* gene *xa13*. Indeed, *xa13* differs from *Os8N3* by a substitution in the promoter region that prevents induction by *PthXo1*. Hence, resistance is due to impaired expression of *Os8N3* (Chu et al. 2006b; Yang et al. 2006). *xa5* is a missense mutant of the gene for the gamma subunit of the core transcription factor TFIIA (Lyer-Pascuzzi et al. 2008). It is hypothesized that the mutation provides protection against disease by blocking a function necessary for TAL effector-mediated *S* gene induction (Bogdanove et al. 2010; White and Yang 2009). These two cases illustrate well the concept that resistance may result from a lack of susceptibility. On the other side, certain plant lines have evolved a sophisticated recognition strategy using dominant resistance (*R*) gene promoters as molecular traps, taking advantage of the activity of TAL effectors (Bogdanove et al. 2010). Promoter activation by a certain TAL effector may lead to resistance gene induction and subsequent cell death or general resistance, as exemplified by pepper *Bs3* and rice *Xa27* resistance genes, which are induced by *AvrBs3* and *AvrXa27*, respectively. In both cases, the susceptible alleles differ from the resistant ones by sequence variation in their promoter region, which impairs TAL effector recognition and binding (Gu et al. 2005; Römer et al. 2007).

Bacterial leaf blight (BLB) caused by *X. oryzae* pv. *oryzae* is one of the most devastating rice diseases worldwide (Nino-Liu et al. 2006). *X. oryzae* pv. *oryzae* is a vascular pathogen, entering the plant through hydathodes or wounds. Upon entry in hydathodes, bacteria multiply in the epithem and gain access to the xylem, in which they disseminate systemically (Nino-Liu et al. 2006). In Africa, BLB has become emergent in most rice growing areas since the 1980s. Recently, we characterized new races originating from West Africa, highlighting substantial differences between Asian and African *X. oryzae* pv. *oryzae* strains (Gonzalez et al. 2007), which are also reflected in differences of their genome structures (V. Verdier and R. Koebnik, *personal communication*). Among other specific features, African strains were shown to harbor a reduced set of TAL effectors. A systematic mutagenesis was initiated in our laboratory, aiming at deciphering the contribution of each of the eight TAL effector paralogs of African *X. oryzae* pv. *oryzae* BAI3 to the pathogen's lifestyle and virulence. Several mutants were obtained, one of which (thereafter designated as *talC*) was severely affected in the production of disease symptoms on susceptible rice lines. This study reports on the functional characterization of *talC*, a new major virulence TAL effector of *X. oryzae* pv. *oryzae*, which targets *Os11N3*, a member of the *MtN3/saliva* gene family.

RESULTS

Systematic mutagenesis of *tal* genes in *X. oryzae* pv. *oryzae* BAI3^R identifies a mutant with a strong defect in virulence.

In order to analyze the function of each of the eight *tal* genes present in *X. oryzae* pv. *oryzae* BAI3^R, we followed a systematic *tal* knock-out mutagenesis strategy. For this, we constructed a one-for-all integrative suicide plasmid (named pVO-155-*tal*) harboring a 206-bp DNA fragment encoding a highly conserved region downstream of the central repeat domain and upstream of the NLS and AAD (Fig. 1A). A single integrative recombination event, therefore, disconnects these two essential protein domains, resulting in truncated, functionally inactive TAL fragments. Because the 206-bp DNA fragment displays at least 90% of identity among *X. oryzae* *tal* genes, we assumed that each of the eight BAI3^R *tal* genes can potentially be targeted during mutagenesis. We conjugated pVO-155-*tal* into *X. oryzae* pv. *oryzae* BAI3^R and obtained 16 putative *tal* mutants. All 16 mutants were shown to result from pVO-155 integration into a *tal* gene, as indicated by nested polymerase chain reaction (PCR) analysis (based on the use of pVO-155- and *tal*- specific oligonucleotides located outside of the integration region). No transconjugants were obtained using the pVO-155 empty vector. Next, we performed pathogenicity assays by leaf-clip inoculation. Only one transconjugant (named BAI3^RΔ*talC*) consistently led to a clear reduction of BLB symptoms, as shown by the measurement of lesion length on susceptible rice lines scored 2 weeks after leaf-clip inoculation (Fig. 1B). Sequencing of PCR amplicons from this mutant confirmed that BAI3^RΔ*talC* was indeed inactivated in one of the eight BAI3^R *tal* genes (not shown).

Molecular characterization of BAI3^RΔ*talC*.

To decipher the identity of the affected *tal* gene, we examined the BAI3^RΔ*talC* mutant by Southern blot hybridization. Genomic DNA was digested with *Bam*HI, which cleaves outside of the central repeat region (at the ATG start codon and 149 bp upstream of the stop codon) of almost all *tal* genes, and was probed with the *avrXa7* 3' region. Since *tal* genes mainly differ in the number and order of repeat modules in the central region, it is possible to distinguish different *tal* genes accord-

ing to the size of their corresponding *Bam*HI fragment. Comparing *Bam*HI-digested genomic DNA of wild-type BAI3^R with that of mutant strain BAI3^R Δ *talC* revealed that a 3.7-kb band was absent in BAI3^R Δ *talC* (Fig. 2, lanes 1 and 2), suggesting that inactivation of the 3.7-kb corresponding *tal* gene is responsible for the observed decreased virulence phenotype

Fig. 1. Phenotype of *Xanthomonas oryzae* pv. *oryzae* mutant strain BAI3^R Δ *talC* upon leaf-clip inoculation. **A**, Schema of the strategy followed towards the systematic knock-out of *tal* genes in *X. oryzae* pv. *oryzae* BAI3^R. A map of a typical *tal* gene is depicted, highlighting the repeat modules in the center and C-terminal nuclear localization signals (black boxes) and an acidic activation domain. The conserved 206-bp sequence that was amplified from BAI3^R genomic DNA and cloned into pVO-155 yielding pVO-155-*tal* is represented in gray. White and black arrows in pVO-155 indicate *nptII* and *uidA* genes, respectively. **B**, Four-week-old Nipponbare and Azucena lines were challenged with water (1), *X. oryzae* pv. *oryzae* wild-type strain BAI3^R (2), and *X. oryzae* pv. *oryzae* mutant strain BAI3^R Δ *talC* (3) and were photographed 14 days after leaf-clip inoculation. This experiment was reproduced three times with similar results.

of BAI3^R Δ *talC*. We next constructed a BAI3 genomic DNA cosmid library in order to clone the corresponding *tal* gene. A total of 51 *tal*-containing cosmids were identified upon colony hybridization, and 18 cosmid clones were digested with *Bam*HI and were analyzed by Southern blot hybridization using the *avrXa7* 3' region as probe. Cosmid 13H5 was found to carry a 3.7-kb *Bam*HI fragment (Fig. 2, lane 3) and was digested with *Eco*RI for subcloning into pBBR1MCS-5 (Kovach et al. 1995). Restriction, PCR, and DNA sequence analysis identified one clone with a 8,901-kb *Eco*RI insert encompassing the complete coding sequence of the candidate *tal* gene with its native promoter (details below and in Figure 3A). This clone was named pBBR-13H5 and was used for complementation studies.

Transfer of pBBR-13H5 into BAI3^R Δ *talC* restores virulence.

To demonstrate that the reduced virulence phenotype of BAI3^R Δ *talC* is due to the inactivation of the identified *tal* candidate, plasmid pBBR-13H5 was conjugated into BAI3^R Δ *talC*. Two independent transconjugants were subjected to pathogenicity assays using syringe-infiltration and leaf-clip inoculation. Infiltration of the parental strain BAI3^R resulted in water-soaked lesions located inside and around the area of inoculation, while BAI3^R Δ *talC* was highly reduced in water-soaking (Fig. 4A, leaves 1 and 2). As shown in Figure 4A, transfer of pBBR-13H5 into BAI3^R Δ *talC* restored water-soaking (leaves 4 and 5), in contrast to that of the empty vector pBBR1MCS-5 (leaf 3). In a more quantitative assay, leaf-clip inoculation also demonstrated the potential of pBBR-13H5 to restore virulence, albeit partially (Fig. 4B). To better understand why BAI3^R Δ *talC* is limited in its capacity to lead to disease, we quantified bacterial populations within the top 15 cm at 0, 4, 8, and 12 days after clip inoculation. Each leaf was cut into three 5-cm segments (A, B, and C), with A being close to the site of inoculation (Fig. 4C). Growth-curve profiles for BAI3^R and BAI3^R Δ *talC* pBBR13H5 were similar in each of the three leaf segments, while those for BAI3^R Δ *talC* demonstrated that the BAI3^R Δ *talC* population remained at the leaf apex in segment A. Intriguingly, while BAI3^R Δ *talC* had apparently lost the ability to colonize the rice leaf at sites distant to the inoculation area, bacteria were still able to multiply at near wild-type levels in leaf fragment A. In contrast, no multiplication could be observed

Fig. 2. Southern hybridization analysis of BAI3^R Δ *talC* genomic DNA. Genomic DNA of *Xanthomonas oryzae* pv. *oryzae* strains BAI3^R (lane 1) and BAI3^R Δ *talC* (lane 2) and DNA of cosmid clone 13H5 (lane 3) were digested with *Bam*HI, were separated by gel electrophoresis, and were probed with a 725-bp 3' fragment of *avrXa7*. Size markers are indicated at the right side.

upon leaf-clip inoculation of mutant strain BAI3^RΔ*hrcC* with a defect in the T3SS (data not shown). Altogether, these data demonstrate that deletion of a *tal* in BAI3^RΔ*talC* is associated with reduced virulence and loss of the capacity to spread through the leaf blade. Introduction of pBBR-13H5 into BAI3^RΔ*talC* complemented for colonization along the rice leaf and partially for disease symptom development.

***talC* encodes a new TAL effector with a unique order of 21.5 repeats.**

Sequence analysis of the 8,901-kb insert in pBBR-13H5 revealed an open reading frame of 3,825 bp encoding a predicted 1,274-amino acid (aa) protein with strong similarity to *X. oryzae* pv. *oryzicola* TAL effector proteins. Southern blot hybridization experiments showed that our *tal* of interest was the third one in terms of *Bam*HI band size among the eight identified genes (Fig. 2, lane 1) and was therefore named *talC*. TalC is made up of a unique order of 21.5 repeats composed of mainly 34-aa modules and two 33-aa modules. As do almost all functional TAL effectors, TalC carries predicted NLS and an AAD in the C-terminal region. Immediately upstream and downstream of the *talC* locus are open reading frames encoding transposases of the *IS1595* and *ISXo17* families of insertion

elements (IS) as well as a portion with similarity to IS5 transposases (Fig. 3A). To get insight into the evolutionary history of *talC*, we compared its nucleotide sequence (excluding the central repeat coding region) to those of different members of the *tal* family. Interestingly, as shown in a phylogenetic tree based on a maximum likelihood analysis (Fig. 5), *talC* clusters together with *tal* genes from *Xanthomonas oryzicola* pv. *oryzicola*. This is in agreement with previous data showing that *X. oryzae* pv. *oryzae* African strains are more closely related to *X. oryzicola* pv. *oryzicola* rather than to *X. oryzae* pv. *oryzae* strains originating from Asia (Gonzalez et al. 2007).

TalC drives the induction of a member of the *nodulin 3 (N3)* gene family.

In order to identify rice genes directly targeted by TalC, we performed transcriptome analysis using the Affymetrix full genome array of rice. We compared RNA obtained from rice leaves challenged with BAI3^R vs. BAI3^RΔ*talC* strains and harvested 24 h postinoculation of susceptible Nipponbare plants. Genes were analyzed according to fold increase, and some of the most strongly induced genes were validated using semi-quantitative reverse transcription (sqRT)-PCR analysis (Table 1; Supplementary Fig. S1). Overall, 120 and 21 genes were found

Fig. 3. Genetic organization of *talC* and candidate target DNA boxes. **A**, Annotation of the 9,005-kb *Eco*RI-fragment from pBBR-13H5 containing *talC*; upstream and downstream sequences include open reading frames (ORF) coding for transposases of the *IS1595* and *ISXo17* families, respectively. Immediately upstream of *talC* and to the 3' end of the insert, portions of ORF with homology to *IS5* transposases and to *Xanthomonas oryzae* pv. *oryzae* MAFF 311018 oxidoreductase (YP_452848.1), respectively, are also represented. **B**, Hypervariable amino acids at position 12 and 13 (RVD) of the 21.5 TalC repeats are aligned to the consensus target DNA box for TalC and candidate TalC box identified in the region upstream of *Os11N3*. R, Y, and N stand for G or A, C or T, and A, T, C, or G, respectively. Bold font indicates the single position where the predicted recognized nucleotide differs from the one of the candidate box for TalC. **C**, The sequence of the *Os11N3* upstream region is depicted with the candidate TalC box framed and the AvrXa7 box highlighted in solid gray (Römer et al. 2010). The start codon is indicated in bold font. The 5' terminal nucleotide of each target box is indicated in white font.

to be up- and down-regulated, respectively. Interestingly, the gene *Os11g31190* (hereafter *Os11N3*), which displayed the highest fold induction, is a member of the *MtN3/saliva* gene family, which also includes the *pthXo1*-dependently activated susceptibility gene *Os8N3* (Chu et al. 2006b; Yang et al. 2006). Moreover, the upstream region of *Os11N3* was recently shown to carry a functional DNA target box recognized by AvrXa7 of *X. oryzae* pv. *oryzae* PXO86 (Römer et al. 2010). We therefore considered *Os11N3* to be a potentially good direct target of TalC and first focused on this gene. *Os11N3* is represented in the GenBank database by a full-length cDNA of 1,494 bp (accession AK101913) with five exons leading to a predicted coding sequence of 912 bp (Fig. 6A). The increase in *Os11N3* expression was confirmed by sqRT-PCR analysis, using oligonucleotides designed to specifically amplify a unique region in the 3' untranslated region and cDNA prepared from total RNA of Nipponbare leaves upon infiltration of BAI3^R, BAI3^R Δ *talC*, BAI3^R Δ *hrcC*, and water. *Os11N3* was only induced by wild-type bacteria and not by inoculation of rice plants with water, with the T3SS-deficient strain BAI3^R Δ *hrcC* (not shown) or with BAI3^R Δ *talC* (Fig. 6B).

The region upstream of *Os11N3* is a direct target of TalC.

TAL effectors bind to DNA target boxes located upstream of target genes and induce their expression. The target box of a given TAL effector can be predicted from the order and identity

of residues 12 and 13 of each repeat, referred to as RVD (Fig. 3A) (Boch et al. 2009; Moscou and Bogdanove 2009). Analysis of the *Os11N3* promoter region identified a putative target box for TalC located 317 bp upstream of the predicted start codon (Fig. 3B). Our data thus suggest that TalC directly regulates *Os11N3* expression upon binding to the target box in front of *Os11N3*. The major virulence TAL effector AvrXa7 was recently reported to drive the expression of *Os11N3* as well, and a target box residing 38 bp downstream of the box of TalC was identified (Fig. 3C) (Römer et al. 2010). To test functionality, the predicted TalC box of *Os11N3* as well as an optimized version according to the TalC RVD-binding specificities were introduced into the minimal pepper *Bs4* promoter and were cloned in front of a *uidA* reporter gene. The reporter constructs were agro-infiltrated into *Nicotiana benthamiana* leaves in combination with *talC* or *hax3*, which are expressed under the control of a 35S promoter. β -Glucuronidase (GUS) assays showed that the *Bs4* promoter derivatives containing the TalC boxes were specifically recognized by TalC but not by Hax3 (Fig. 7A to C). Finally, a 341-bp fragment upstream of the *Os11N3* coding sequence and including the predicted TalC box was cloned in front of the GUS reporter gene. Codelivery of this 341-bp fragment with *talC* led to even stronger GUS activity as compared with that when the target TalC box is embedded into the *Bs4* minimal promoter (Fig. 7D). Altogether, this strongly suggests that *Os11N3* is directly activated by TalC in planta.

Fig. 4. Complementation analysis of mutant strain *Xanthomonas oryzae* pv. *oryzae* BAI3^R Δ *talC*. **A**, Leaves of 3-week-old Nipponbare plants were infiltrated, using a needleless syringe, with *X. oryzae* pv. *oryzae* strains BAI3^R (1), BAI3^R Δ *talC* (2), BAI3^R Δ *talC* (pBBR1MCS-5) (3), BAI3^R Δ *talC* (pBBR-13H5#1) (4), and BAI3^R Δ *talC* (pBBR-13H5#2) (5). Leaves were photographed 5 days after inoculation. **B**, Lesion length was measured 14 days postinoculation of rice Azucena leaves with *X. oryzae* pv. *oryzae* strains BAI3^R (1), BAI3^R Δ *talC* (2), BAI3^R Δ *talC* (pBBR1MCS-5) (3), BAI3^R Δ *talC* (pBBR-13H5#1) (4), and BAI3^R Δ *talC* (pBBR-13H5#2) (5). Means with the same letters are not statistically different based on a Tukey's honest significant difference test ($\alpha = 0.05$). **C**, Bacterial growth in 4-week-old rice Azucena leaves. Five-centimeter leaf segments A (proximal inoculation site), B, and C were scored at 0, 4, 8, and 12 days postinoculation. The experiment was repeated three times, using three leaves per tested condition. Error bars represent standard error.

Because BAI3 is avirulent on IRBB7 lines containing the *avrXa7*-matching resistance gene *Xa7*, we investigated the *avrXa7*-like activities of *talC*. To this end, pBBR-13H5 was conjugated into strain PXO99^A, which is virulent on IRBB7. However, no gain of avirulence could be observed upon leaf-clip inoculation of IRBB7 leaves with PXO99^A (pBBR-13H5) (Table 2). Similarly, since BAI3 is also avirulent on IRBB4 and IRBB5 rice lines, we analyzed PXO99^A (pBBR-13H5) for a potential gain of avirulence, but again, transconjugants remained fully virulent. This suggests that, although both TalC

and AvrXa7 target the susceptibility gene *Os11N3*, TalC does not trigger expression of the resistance gene *Xa7*.

DISCUSSION

This study reports on the functional analysis of TalC, a new member of the TAL-AvrBs3/PthA family of type III effectors. TalC was isolated from *X. oryzae* pv. *oryzae* BAI3, which originates in Burkina Faso in West Africa. While first reported in Africa in the 1980s, bacterial blight of rice is nowadays pre-

Fig. 5. *talC* clusters with *tal* genes of *Xanthomonas oryzae* pv. *oryzicola*. A neighbor-joining tree of TalC and related TAL effector proteins was constructed using ClustalW2 and approximate likelihood ratio test (aLRT)-PhyML. The branch length is proportional to divergence, and the 0.02 scale represents 2% change. Relevant aLRT support values are indicated as numbers (0 to 1) at branching points. Protein names of *Xanthomonas oryzae* pathovars follow a pattern using an abbreviation of the species and pathovar, strain, and protein names. *Xoo* = *X. oryzae* pv. *oryzae*, MAFF = strain MAFF 311018, KACC = strain KACC10331, *Xoc* = *X. oryzae* pv. *oryzicola*, BLS = strain BLS256, *Xav* = *X. axonopodis* pv. *vesicatoria*, *Xca* = *X. campestris* pv. *armoraciae*, *Xac* = *X. axonopodis* pv. *citri*, *Xcm* = *X. campestris* pv. *malvacearum*. The tree was rooted using protein sequences of the non-*X. oryzae* pathovars as outgroup.

sent in most rice-producing West African countries and continues to spread along with the intensification of rice cultivation, thus causing a major concern for food security. Recently, we characterized new *X. oryzae* pv. *oryzae* races originating from West Africa (Gonzalez et al. 2007). A specific feature of African *X. oryzae* pv. *oryzae* strains, as compared with Asian strains, is a reduced number of *tal* genes. To gain insight into the pathogenicity of African *X. oryzae* pv. *oryzae* strains and to anticipate durable and broad resistance strategies, we evaluated the function of TAL effectors, some of which had been shown previously to be essential for *X. oryzae* pv. *oryzae* pathogenicity (White and Yang 2009).

TalC is a new major virulence gene in *X. oryzae* pv. *oryzae* BAI3.

Systematic mutagenesis of *tal* genes in strain BAI3^R led to the generation of 16 mutants, only one of which was severely

impaired in virulence. Molecular characterization revealed that the mutant was affected in one of the eight *tal* genes, referred to as *talC*. It remains to be investigated which *tal* genes were inactivated in the other mutants; however, none of them was impaired in virulence under our laboratory settings. Although we probably did not saturate the mutagenesis of all *tal* genes and some with detectable virulence function might have been missed, our data are consistent with the hypothesis that possibly all *X. oryzae* pv. *oryzae* strains, including those of African origin, strictly depend on only one or two members of the *tal* effector gene family for pathogenicity (Yang and White 2004). Inactivation of *talC* dramatically reduced lesion length (Fig. 1B) and water soaking (Fig. 4A), indicating that *talC* is a major virulence gene in *X. oryzae* pv. *oryzae* BAI3. Indeed, these results are reminiscent of *pthXo1*, *pthXo2*, *pthXo3*, and *avrXa7*, which encode major virulence determinants in *X. oryzae* pv. *oryzae* strains PXO99^A, JXO1^A, PXO61, and

Table 1. Fold increase and database matches for top 10 target sequences

Rank ^a	Probe set ID	code AGI	Ratio ^b	P value ^c	Similarity ^d	RT-PCR ^e
1	Os.4974.1.S1_at	gb AK101913.1	7.34	0.00 ^E +0	Os11N3 nodulin MtN3 family protein	+
2	Os.4974.1.S1_x_at		6.76	0.00 ^E +0		
3	Os.57323.1.S1_at	gb AK111303.1	4.89	0.00 ^E +0	HLH DNA-binding domain containing protein	+
4	Os.9823.1.S1_at	gb AK061798.1	3.01	0.00 ^E +0	Cyclase family protein	+
5	Os.4020.1.S1_at	gb AK059636.1	2.68	0.00 ^E +0	Oxidoreductase, aldo/keto reductase	nd
6	Os.17271.1.S1_at	gb AK059809.1	2.54	0.00 ^E +0	Citrate-binding protein precursor	+
7	Os.55065.1.S1_at	gb AK107405.1	2.5	0.00 ^E +0	DNA binding protein	+
8	OsAffx.13648.1.S1_a	tigr 9632.m00293	2.49	0.00 ^E +0	Subtilisin homologue	nd
9	OsAffx.16605.1.S1_at	tigr 9635.m0377	2.12	0.00 ^E +0	Cytochrome P450 51	nd
10	Os.6043.1.S1_at	gb AB040744.1	2	0.00 ^E +0	Basic helix-loop-helix family protein	+

^a Probe set references were ranked according to the fold increase of average value of the three replicates with BAI3^R compared with the average value of the three replicates with BAI3^R Δ *talC*.
^b Log₂ ratio of treatment with *Xanthomonas oryzae* pv. *oryzae* BAI3^R versus the value for treatment with *X. oryzae* pv. *oryzae* BAI3^R Δ *talC*.
^c A gene is declared differentially expressed if the Bonferroni *P* value is less than 0.05.
^d Similarity matches were provided at the PLEXdb database.
^e Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to validate gene expression. + = *TalC*-dependent gene expression was confirmed; nd = not tested.

Fig. 6. TalC drives the induction of *N3* gene member *Os11N3*. **A**, *Os11N3* is represented by the full-length cDNA AK101913. Exons are represented by solid dark boxes and introns as thin lines above. Numbers indicate the number of nucleotides. The 5' and 3' untranslated regions were inferred from the alignment of the genomic sequence with the cDNA sequence. Affymetrix probe set ID numbers are indicated below the diagram. **B**, *Os11N3* is induced in a *talC*-dependent manner. Semiquantitative reverse transcription-polymerase chain reaction of *Os11N3* (26 cycles) and actin (28 cycles) was performed with RNA purified 24 h postinfiltration of Nipponbare rice leaves with water (lane 2) and *X. oryzae* pv. *oryzae* strains BAI3^R (lane 3) and BAI3^R Δ *talC* (lane 4). Nipponbare genomic DNA (lane 1) was used as positive control.

PXO86, respectively, representing different *X. oryzae* pv. *oryzae* lineages from Asia (Yang and White 2004). Earlier studies of our group had identified three new races of *X. oryzae* pv. *oryzae* originating from West Africa, A1 to A3, with strain BAI3 belonging to race A1, which is incompatible in the near-isogenic rice lines IRBB4, IRBB5, and IRBB7, but not in the parental line IR24. Surprisingly, analysis of the molecular diversity indicated that African *X. oryzae* pv. *oryzae* strains form a clearly separated genetic group distant to the Asian strains and more closely related to *X. oryzae* pv. *oryzicola*, the causal agent of bacterial leaf streak of rice (Gonzalez et al. 2007). Accordingly, phylogenetic analysis demonstrated that *talC* clusters together with *tal* genes of *X. oryzicola* pv. *oryzicola* BLS256 (Fig. 5). In addition, comparison of *X. oryzicola* pv. *oryzicola* BLS256 *tal* genes with those of *X. oryzae* pv. *oryzae* strains available in GenBank indicated a greater similarity among paralogues than among orthologues, suggesting that the expansion of *tal* gene families and repertoires in these two pathovars probably occurred after their divergence.

BAI3^R*ΔtalC* is restricted for movement along the leaf but multiplies at the site of inoculation.

Analysis of bacterial multiplication and movement in planta showed that BAI3^R*ΔtalC* was dramatically affected in its abil-

ity to spread through the rice leaf blade. This behavior likely reflects the ability of the bacteria to move along the vascular tissues (Fig. 4C). Surprisingly, the mutant strain was still able to multiply at almost wild-type levels in the vicinity of the inoculation site (proximal 5-cm segment A). We hypothesize that, upon leaf-clip inoculation, *X. oryzae* pv. *oryzae* popula-

Table 2. Reactions of near-isogenic lines (NIL) IRBB4, IRBB5, IRBB7, and IRBB13 with single bacterial-resistance genes (*Xa*) against *Xanthomonas oryzae* pv. *oryzae* strains^a

NIL	<i>Xa</i> gene	BAI3	PXO99 ^A	PXO99 ^A pBBR1-5	PXO99 ^A pBBR-13H5
IR24		S	S	S	S
IRBB4	<i>Xa4</i>	R	S	S	S
IRBB5	<i>xa5</i>	R	S	S	S
IRBB7	<i>Xa7</i>	R	S	S	S
IRBB13	<i>xa13</i>	S	R	R	R

^aSix-week-old IRBB lines were leaf clip-inoculated with *X. oryzae* pv. *oryzae* strains BAI3^R, PXO99^A, PXO99^A (pBBR1-5), and PXO99^A (pBBR-13H5). Resistance or susceptibility of rice plants to *X. oryzae* pv. *oryzae* is expressed in lesion lengths measured 14 days after inoculation. Resistant (R) < 5 cm, moderately resistant (MR) = 5 to 10 cm, moderately susceptible (MS) = 10 to 15 cm, and susceptible (S) > 15 cm. This experiment was reproduced three times, giving similar results.

Fig. 7. The upstream region of *Os11N3* is transcriptionally activated by TalC. **A**, Amino acids 12 and 13 of the TalC and Hax3 repeats and predicted target DNA specificities (TalC box P, native box; TalC box O, optimized box). **B**, TalC and Hax3 boxes cloned in front of the minimal *Bs4* promoter and a 341-bp fragment of the *Os11N3* region upstream of the coding region were cloned into a β-glucuronidase (GUS) reporter vector. **C**, Specific inducibility of TalC box O and TalC box P by TalC. **D**, Specific inducibility of the *Os11N3* 341-bp upstream region (rice) by TalC. GUS reporter constructs were codelivered via *Agrobacterium tumefaciens* into *Nicotiana benthamiana* with 35S-driven *talC*, *hax3*, and empty T-DNA (ev) (error bars indicate standard deviation; *n* = 3). MU = 4-methyl-umbelliferone. 35S::uidA (+) served as control. Leaf disks were stained with X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide).

tions gain access to vascular tissues but also to the leaf mesophyll apoplast. While a loss of *talC* probably impairs bacterial multiplication in the xylem, and therefore colonization of distal leaf segments (B and C), bacteria having entered the mesophyll tissue may be able to multiply, although remaining restricted to tissues close to the inoculation site. This suggests that TalC is specifically required for the interaction with xylem parenchyma cells by either one or both suppressing vascular-specific defense responses or promoting the spread of the bacteria from local infection sites, i.e., by delivery of sufficient amounts of essential nutrients. Such tissue-specific virulence function of a TAL effector is a novel phenotype and has not yet been characterized for other xanthomonads. Interestingly, however, AvrAC_{Xcc8004} is an Avr protein from *X. campestris* pv. *campestris* that was shown to elicit a hypersensitive response only when bacteria were inoculated into the vascular system by piercing the central veins of leaves (Xu et al. 2008). In a conceptually comparable study, Iyer and associates (2008) reported on the effect of the recessive resistance gene *xa5* on *X. oryzae* pv. *oryzae* bacterial population dynamics. By analyzing bacterial growth and movement in isogenic rice lines containing *xa5* or *Xa5*, the authors showed that resistant plants restricted the progression of bacteria along the leaf without impairing their multiplication close to the inoculation site, similar to what we observed when comparing BAI3^R and BAI3^R Δ *talC*. *X. oryzae* pv. *oryzae* infects leaves primarily by vascular colonization through hydathodes, in natural settings (Nino-Liu et al. 2006). Future experiments will, therefore, involve alternative inoculation methods, such as hydathode invasion (Hugouvieux et al. 1998), that better mimic the natural route of infection.

TalC targets a gene of the N3 nodulin family.

By microarray experiments and sqRT-PCR comparing the transcriptomes of rice leaves challenged with *X. oryzae* pv. *oryzae* BAI3^R versus BAI3^R Δ *talC*, we identified several rice genes to be specifically induced by *X. oryzae* pv. *oryzae* BAI3^R, thus most likely being under control of *talC* (Table 1). One of the most strongly induced genes was *Os11N3*, a member of the *MtN3/saliva* family related to nodulin 3 (N3) protein of *Medicago truncatula* (White and Yang 2009). This family consists of approximately 20 members in rice and includes *Os8N3*, also known as *Xa13* (Chu et al. 2006a). Interestingly, *Os8N3* was identified by Yang and associates (2006) using similar microarray profiling experiments comparing the host response upon challenge with PXO99^A versus the mutant strain PXO99^A ME2 defective in *pthXo1*. Like *talC*, *pthXo1* encodes a major virulence gene and activation of *Os8N3* transcription results in susceptibility of rice to *X. oryzae* pv. *oryzae* PXO99^A. *Os8N3* was therefore considered to be a *S* gene (Yang et al. 2006). Our data demonstrate that *Os11N3* is a direct target of TalC. Visual inspection of the *Os11N3* upstream region revealed a potential DNA target box for TalC (Fig. 3), which was confirmed to be functional using GUS transient assays. Moreover, use of a 341-bp sequence upstream of *OS11N3*, including the TalC target box, led to even stronger enzymatic activity of the reporter (Fig. 7). Similar to our finding, another major virulence TAL effector, AvrXa7, was shown by electrophoretic mobility shift and GUS reporter assays to bind upstream of and to activate expression of *Os11N3* (Römer et al. 2010). As depicted in Figure 3B, a DNA target box for AvrXa7 resides 38 bp downstream of the TalC box. Altogether, these data suggest functional convergence for virulence in the rice-*X. oryzae* pv. *oryzae* pathosystem whereby several strains with different geographical origins and belonging to different lineages rely on a single major virulence TAL, the function of which is to target members of the N3 gene family.

How the function of N3 genes relates to bacterial leaf blight disease of rice remains an open question. *Os8N3* and *OS11N3* are probably functionally redundant disease-promoting genes, since the *pthXo1*-defective strain PXO99^A ME2 can be complemented via the introduction of *avrXa7* (Yang and White 2004). This finding suggests that lack of *Os8N3* induction can be overcome by *avrXa7*-dependent induction of *Os11N3*. Surprisingly, introduction of *talC* into PXO99^A did not restore virulence on *xa13* lines (Table 2), suggesting that the expected *talC*-dependent transcription activation of *Os11N3* either did not occur or was insufficient to promote virulence. Similarly, introduction of *pthXo1* or *avrXa7* into BAI3^R Δ *talC* did not restore virulence on susceptible rice lines (not shown). The molecular basis for this absence of complementation between major virulence TAL genes originating from Asia and Africa is so far unknown.

Finally, *X. oryzae* pv. *oryzae* BAI3^R is incompatible on the IRBB7 rice line, which contains the matching *Xa7* resistance gene (Gonzalez et al. 2007). This suggests that BAI3^R harbors *avrXa7* activity. Our data show that PXO99^A expressing *talC* is not able to trigger *Xa7*-mediated resistance (Table 2). Either this *avr* activity is repressed in PXO99^A, possibly by other T3E, or another TAL effector of BAI3^R is involved, possibly related to the 4.2-kb *Bam*HI fragment that was detected in Southern blots. Future analysis of the other BAI3^R *tal* members will help us to elucidate how virulence evolved in African strains and to which extent each *tal* gene contributes to compatible and incompatible interactions.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains used in this study were *Escherichia coli* DH5 α (Stratagene, La Jolla, CA, U.S.A.) and *X. oryzae* pv. *oryzae* strains BAI3 (Gonzalez et al. 2007) and PXO99^A (Hopkins et al. 1992). In order to enable BAI3 for triparental conjugation, rifampicin-resistant clones were selected upon plating on PSA medium (10 g of peptone, 10 g of sucrose, 1 g of glutamic acid, 16 g of agar per liter of H₂O) supplemented with 100 μ g/ml of rifampicin. Each of five isolates showing resistance to rifampicin was inoculated into rice and was confirmed to be as pathogenic as the wild-type strain. One, referred to as BAI3^R, was chosen for further experiments. *E. coli* cells were cultivated at 37°C in lysogenic broth (LB) medium, *X. oryzae* pv. *oryzae* strains at 28°C in PSA medium and *Agrobacterium tumefaciens* GV3101 at 28°C in LB medium. Plasmids were introduced into *E. coli* and *Agrobacterium* strains by electroporation and into *X. oryzae* pv. *oryzae* using pRK2073 as a helper plasmid in triparental mating (Figurski and Helinski 1979). Antibiotics were added in the medium at the following final concentrations: kanamycin, 50 μ g/ml; rifampicin, 100 μ g/ml; gentamycin, 20 μ g/ml; and spectinomycin, 50 μ g/ml.

Plant material and plant inoculations.

Experiments were performed under greenhouse conditions at 26°C and 80% relative humidity. Rice lines Nipponbare and Azucena were used for virulence assays, while line IR24 and near-isogenic rice lines IRBB4, IRBB5, IRBB13, and IRBB7 containing the resistance genes *Xa4*, *xa5*, *xa13*, and *Xa7* respectively, were used for avirulence tests (Ogawa et al. 1987). Leaves of 3-week-old plants were infiltrated with a bacterial suspension resuspended at an optical density at 600 nm (OD₆₀₀) of 0.5 using a needleless syringe, as previously described (Reimers and Leach 1991). Leaf-clip inoculation was performed on 4- to 6-week-old rice plants using a bacterial suspension resuspended at an OD₆₀₀ of 0.2 (Kauffman et al. 1973). Symptoms were scored by observation of water-soak-

ing upon syringe infiltration and by measuring lesion lengths upon leaf-clip inoculation, 5 and 14 days postinoculation, respectively. Significance between treatments was assessed using the Tukey honest significant difference test for post-analysis of variance pair-wise comparisons, set at 5% ($P < 0.05$). *Nicotiana benthamiana* plants were grown with 16 h of light, 40 to 60% humidity, at 23°C (day) and 19°C (night) in the greenhouse. Leaves of 5- to 7-week-old plants were inoculated with *Agrobacterium* strains using a needleless syringe. Inoculated plants were transferred to a Percival growth chamber (BioRad, Munich) with 16 h of light at 22°C (day) and 18°C (night).

Generation of *tal* mutants.

To construct plasmid pVO-155-*tal*, a 206-bp *avrXa7* fragment was amplified by PCR using primers Tal-Ct-F (5'-GAA TCAATCGCCGATTCCCGA-3') and Tal-Ct-R (5'-ACTCGA AGCCCGCGGTGGAACG-3') and pH81 (Hopkins et al. 1992) as DNA template. The amplification product was ligated into T-tailed pBluescript II KS(+) (Marchuk et al. 1990) and was subcloned as a 242-bp *Sall/XbaI* fragment into suicide vector pVO-155 (Oke and Long 1999), leading to pVO-155-*tal*. pVO-155-*tal* was introduced into recipient strain BAI3^R by triparental mating, thus disrupting *tal* genes upon a single recombination event. Sixteen transconjugants obtained upon a single conjugation event were streaked twice consecutively on PSA medium supplied with rifampicin and kanamycin and were grown accordingly. To confirm the integration of pVO-155-*tal* into *tal* genes, nested PCR assays were performed by means of primers Tal-Ct-Fw2 (5'-GCGTTGGCCGCGTTGACCAA-3') and Tal-Ct-Fw3 (5'-GCCGATCAGGGCGAGATAACT-3'), and primers pVO-155-Rev (5'-TGTAAGAAACCCCAACCCGTGAA-3') and pVO-155-Rev2 (5'-GCGCGTTACAAGAAAGCCGG-3'), annealing to sequences upstream of the 206-bp *tal* fragment carried by pVO-155-*tal* and in pVO-155, respectively. Primers Tal-Ct-Rev2 (5'-GGGGCCGCATCTTGTTCCTCA-3') and Tal-Ct-Rev3 (5'-CAGATCGTCCCTCCGACTGA-3') and primers pVO-155-FW (5'-GAGATCCCCAGCCCGCCTAATG-3') and pVO-155-FW2 (5'-CATGCCTGCAGGTTCGAGATC-3') were used to analyze integration of pVO-155-*tal* downstream of the 206-bp *tal* fragment.

Construction of BAI3^RΔ*hrcC*.

A *hrcC* internal fragment (nucleotide 486 to 1,375) was obtained by PCR amplification using BAI3^R genomic DNA and was cloned into nonreplicative suicide plasmid pK18mob::sacB, yielding pK18mob::sacB-*hrcC*486-1375. Upon triparental conjugation, primary transformants were selected on GYE medium (20 g glucose, 10 g yeast extract per liter) supplemented with kanamycin. Next, kanamycin-resistant colonies were plated on GYE medium containing 5% sucrose to select for second recombination. Colonies found to be sensitive to kanamycin and resistant to high sucrose concentration were assayed by PCR for disruption of *hrcC*, using primer *hrcC*-up (5'-TGAGACAT TCCCGGCTTTAC-3'), corresponding sequence upstream of the insert in pK18mob::sacB-*hrcC*486-1375, and primer *hrcC*-down (5'-TCAGGGCGACACCAATG-3'), which corresponds to the 3' end of *hrcC*.

In planta growth experiments.

Infected leaves were collected at 0, 4, 8, and 12 days after leaf-clip inoculation and were briefly rinsed in 70% ethanol, followed by submersion in sterile water for 10 s. Leaves were cut into three segments of 5 cm from the apex, were put into 2-ml Eppendorf tubes containing two metallic beads ($\Phi = 3$ mm), were frozen by submersion into liquid nitrogen, and were ground into a fine powder, using the Qiagen TissueLyser system (30 rounds per second for 2 min). Ground material was resus-

pended in 1 ml of sterile water and 10- μ l drops of a dilution series were spotted onto PSA medium as triplicates. This experiment was performed three times.

Southern blot hybridization.

Standard procedures for Southern blots were utilized (Ausubel et al. 1988). Genomic DNA from *X. oryzae* pv. *oryzae* strains was extracted using Wizard genomic DNA purification kit (Promega, Madison, WI, U.S.A.), was digested with *Bam*HI, and was migrated on a 0.8% agarose gel at 50 V for 36 h. Filters were probed with a ³²P-labeled 725-bp C-terminal *avrXa7* fragment that was obtained by PCR using primers Tal-Ct-Fw2 and Tal-Ct-Rev2.

Construction of a cosmid library and pBBR-13H5.

A cosmid library of *X. oryzae* pv. *oryzae* BAI3^R genomic DNA was generated using the pWEB-TNC cosmid cloning kit (Epicentre Biotechnologies, Madison, WI, U.S.A.). Approximately 1,500 clones were obtained and stored. Dot-blot colony hybridization was performed using a ³²P-labeled 725-bp C-terminal *avrXa7* PCR product, leading to the identification of 51 positive clones. *Bam*HI digestion of 18 potential *tal*-positive cosmids were analyzed by Southern-blot hybridization, thus identifying clones corresponding to the eight *tal* genes of strain BAI3^R. Cosmid 13H5 was chosen for subcloning, as it was to contain a *Bam*HI-hybridizing band corresponding to the expected molecular weight of *talC*. Cosmid 13H5 was digested with *Eco*RI and was directly ligated with *Eco*RI-linearized pBBR1MCS-5 (Kovach et al. 1995). Upon PCR-based screening, positive clones with a 8,901-kb insert fragment were identified and sequenced, yielding pBBR-13H5.

Phylogenetic analysis.

Amino-acid sequences of TAL effectors were retrieved from GenBank upon BlastP analysis, using TalC as query. When possible, proteins were chosen for representing the diversity of lineage and strain origin, especially among the *Xanthomonas oryzae* species. N- and C-terminal regions were concatenated and aligned using ClustalW2. Sequence alignments were further processed manually to eliminate gaps due to indels. A neighbor-joining tree was constructed using PhyML with program SeaView version 4, and bootstrap analyses were based on 1,000 replicates. The tree was rooted using protein sequences of the non-*X. oryzae* pathovars as outgroup.

Microarray experiments.

Six fully expanded leaves of six 3-week-old independent individuals (cv. Nipponbare) were infiltrated with the tested strains (OD₆₀₀ = 0.5) and were pooled for RNA isolation at 24 h postinoculation. Total RNA was extracted using the RNeasy plant mini kit (Qiagen, Hilden, Germany), and on-column DNase digestion was performed. All RNA samples were checked for their integrity on The Agilent 2100 bioanalyzer according to the Agilent Technologies (Waldbronn, Germany). Total RNA (1 μ g) was used to synthesize biotin-labeled cRNAs with the One-Cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA, U.S.A.). The processed cRNA was used for hybridization to Affymetrix GeneChip Rice genome array on the Affymetrix GeneChip instrument system at the Institut National de la Recherche Agronomique Unité de Recherche en Génomique Végétale (Evry, France). The .raw CEL files were imported in R software for data analysis. All raw and normalized data are available through the CATdb database (AFFY_*X. oryzae* pv. *oryzae*_Rice; Gagnot et al. 2008) and from the Gene Expression Omnibus repository at the National Center for Biotechnology Information (Barrett and Edgar 2006), accession number GSE19844.

The data were normalized with the gcrma algorithm (Irizarry et al. 2003), available in the Bioconductor package (Gentleman et al. 2004). To determine differentially expressed genes, we performed a usual two group *t*-test that assumes equal variance between groups. The variance of the gene expression per group is a homoscedastic variance, where genes displaying extremes of variance (too small or too large) were excluded. The raw *P* values were adjusted by the Bonferroni method, which controls the family wise error rate (Ge et al. 2003). A gene is declared differentially expressed if the Bonferroni *P* value is less than 0.05. Similarity matches were provided at PLEXdb (Plant Expression database).

SqRT-PCR experiments.

RT-PCR experiments were performed using primers yielding a 300- to 400-bp product for each candidate target gene. RT-PCR templates were produced as described for microarrays. RNA quality and quantity was estimated using a ND-1000 Nanodrop spectrophotometer. cDNA was synthesized with 1 µg of total RNA by means of the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA, U.S.A.). Specific primers amplifying the actin transcript were used as control. Appropriate PCR cycle numbers specific to each gene and to the primer combination were determined by testing a wide range of cycle numbers and choosing one for which DNA amplification that was still in the exponential phase. PCR conditions appropriate for each gene and the corresponding primer pair sequences are available in Supplementary Table S1.

TalC and GUS reporter constructs.

The *talC* coding region was amplified by PCR from pBBR-13H5 and inserted into pENTR/D-TOPO (Invitrogen). Sequencing indicated no PCR errors. *talC* was transferred by LR recombination into pGWB6 (Nakagawa et al. 2007) to generate a green fluorescent protein–TalC fusion. Nuclear localization of the fusion protein was confirmed by confocal laser scanning microscopy. Derivatives of the TalC box were placed at the 5' end of the minimal *Bs4* promoter from tomato, using PCR, and were inserted into pENTR/D-TOPO (Invitrogen) as described previously (Boch et al. 2009). For GUS reporter constructs, a 341-bp fragment of the *Os11N3* promoter region upstream of the coding region was subcloned into pENTR/D-TOPO. The predicted TalC box is located at position –319 to –297. The promoter constructs were transferred by LR recombination into pGWB3 (Nakagawa et al. 2007) containing a promoterless *uidA* gene. All entry fragments were sequenced. Primers used for cloning of these constructs can be obtained upon request.

GUS assays.

GUS assays were done essentially as described previously (Boch et al. 2009). Briefly, *Agrobacterium* strains delivering TAL effector constructs and GUS reporter constructs were mixed 1:1 and were inoculated into *Nicotiana benthamiana* leaves with a total OD₆₀₀ of 0.8. At 2 days postinfiltration, leaf disks (0.9 cm diameter) were sampled and GUS activity was determined. For qualitative GUS assays, leaf disks were incubated in X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) staining solution, were destained in ethanol, and were dried. Quantitative GUS activities were determined using 4-methyl-umbelliferyl-β-D-glucuronide as substrate. Proteins were quantified using Bradford assays (BioRad). Data are compiled from triplicate samples from different plants.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

ClustalW2 software: www.ebi.ac.uk/Tools/clustalw2/index.htm
 National Center for Biotechnology Information website: www.ncbi.nlm.nih.gov
 PLEXdb (Plant Expression database): www.plexdb.org/index.php