TALE1 from *Xanthomonas axonopodis* pv. *manihotis* acts as a transcriptional activator in plant cells and is important for pathogenicity in cassava plants

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SUMMARY

Many plant-pathogenic bacteria suppress pathogen-associated molecular pattern (PAMP)-triggered immunity by injecting effector proteins into the host cytoplasm during infection through the type III secretion system (TTSS). This type III secretome plays an important role in bacterial pathogenicity in susceptible hosts. *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), the causal agent of cassava bacterial blight, injects several effector proteins into the host cell, including TALE1_{*Xam*}. This protein is a member of the Transcriptional Activator-Like effector (TALE) protein family, formerly known as the AvrBs3/PthA family. TALE1_{*Xam*} has 13.5 tandem repeats of 34 amino acids each, as well as two nuclear localization signals and an acidic activation domain at the C-terminus. In this work, we demonstrate the importance of TALE1_{*Xam*} in the pathogenicity of *Xam*. We use versions of the gene that lack different domains in the protein in structure–function studies to show that the eukaryotic domains at the 3' end are critical for pathogenicity. In addition, we demonstrate that, similar to the characterized TALE proteins from other *Xanthomonas* species, TALE1_{*Xam*} acts as a transcriptional activator in plant cells. This is the first report of the identification of a TALE in *Xam*, and contributes to our understanding of the pathogenicity mechanisms employed by this bacterium to colonize and cause disease in cassava.

that are secreted and translocated to the host cell through the type III secretion system (TTSS; Büttner and He, 2009; McCann and Guttman, 2008). Most effector proteins translocated by this system act as suppressors of a general defence response of plants against microbes, called pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI; Jones and Dangl, 2006). However, many effectors are recognized in the host because of the evolution of the corresponding resistance (*R*) genes, constituting a branch of immunity in plants termed effector-triggered immunity (ETI; Flor, 1971; Jones and Dangl, 2006). When these bacterial effector proteins are recognized, they are classically called avirulence (Avr) proteins, as they betray the presence of the bacterial pathogen to the host immune system. However, in the absence of the corresponding *R* gene, these effectors can increase bacterial fitness and aggressiveness, and are therefore required for maximal virulence (Block *et al.*, 2008; Boch and Bonas, 2010).

Many effector proteins have been identified in the plant-pathogenic species of the genus *Xanthomonas*. One of the best characterized is the family of effectors called the AvrBs3/PthA (avirulence and pathogenicity) protein family. These were recently designated as TAL effectors (TALEs), for Transcriptional Activator-Like proteins, because they modulate gene expression in the host, acting like transcription factors (Boch and Bonas, 2010; Bogdanove *et al.*, 2010; Kay *et al.*, 2007). AvrBs3, the founder member of the family, has been characterized extensively in *Xanthomonas euvesicatoria* (*Xeu*), where it plays an important role in virulence of the pathogen and recognition by the host ETI system (Marois *et al.*, 2002; Minsavage *et al.*, 1990). At present, more than 40 proteins belonging to this conserved family have been identified from different species and pathovars of *Xanthomonas* and *Ralstonia solanacearum* (Heuer *et al.*, 2007; Schornack *et al.*, 2006; White *et al.*, 2009). The intriguing characteristic of this family is the presence of nearly identical tandem repeats of 34 or 35 amino acids in the central region of the protein. The C-terminal region of TALE proteins contains typical eukaryotic features, such as monopartite nuclear localization signals (NLSs) and a

INTRODUCTION

Plant-pathogenic bacteria have developed diverse strategies to colonize and cause disease in a wide range of host plants. Their pathogenicity mainly depends on an arsenal of effector proteins

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transcriptional acidic activation domain (AAD), the function of which is essential for both virulence and avirulence activities (Szurek *et al.*, 2001; Yang *et al.*, 2000). The NLSs are crucial for the interaction of AvrBs3 with the protein importin- α from pepper, indicating that the AvrBs3-like proteins recruit the host machinery to reach the nucleus (Szurek *et al.*, 2001). In addition, mutations in the NLSs or AADs of AvrBs3 abolish the recognition by the *R* gene *Bs3* in the host plant (Szurek *et al.*, 2001). Once inside the nucleus, TALE proteins regulate the transcription of specific genes, as first demonstrated for AvrBs3 (Kay *et al.*, 2007) and, more recently, for a few other members of the protein family (Boch *et al.*, 2009; Römer *et al.*, 2010). AvrBs3 activates the transcription of at least one gene involved in cell growth and proliferation, inducing developmental reprogramming in host cells by binding and activating its specific promoter target in pepper (Kay *et al.*, 2007). In the case of the incompatible interaction, this same protein induces the expression of the corresponding *R* gene *Bs3* (Römer *et al.*, 2007). Most importantly, the amino acid nucleotide sequence code that defines the target DNA sequences of TALEs in their hosts has been deciphered recently (Boch *et al.*, 2009). The variable number of repeats in the central region of this family of proteins, ranging between 1.5 and 28.5 (Boch and Bonas, 2010), as well as the specific sequence of the variable amino acids 12 and 13 in each repeat, are responsible for the binding to specific boxes in the promoters of host target genes (Boch *et al.*, 2009; Moscou and Bogdanove, 2009).

Several TALE proteins have a strong effect in bacterial pathogenesis and physiological changes in susceptible hosts. For example, PthA from *Xanthomonas axonopodis* pv. *citri* (*Xac*) is necessary to cause citrus canker disease symptoms, including cell hypertrophy, hyperplasia and cell death (Brunings and Gabriel, 2003; Duan *et al.*, 1999). Furthermore, Domingues *et al.* (2010) demonstrated that PthA and its variants PthA2 and PthA3 target citrus proteins involved in ubiquitination pathways and DNA repair. Likewise, a role in mRNA stability and translational regulation was proposed for PthA4, which targets citrus DNA- or RNA-binding proteins involved in the remodelling of chromatin and mRNA modifications (de Souza *et al.*, 2012).

Xanthomonas oryzae pv. *oryzae* (*Xoo*) also encodes several TALE family members whose inactivation results in severely reduced symptom development and bacterial growth. This is the case for *pthXo1*, which induces the rice susceptibility promoter *Os8N3* (Yang *et al.*, 2006), and *avrXa7* and *talC*, which promote the expression of the *Os11N3* promoter (Römer *et al.*, 2010; Yu *et al.*, 2011). In addition, the genes *pthXo6* and *pthXo7* induce the expression of *OsTFX1* and *OsTFIIA1*, which encode for rice transcription factors (Sugio *et al.*, 2007). Variations in these upregulated TALE (UPT) boxes account for the level of susceptibility and resistance of a particular rice cultivar to this bacterial pathogen, suggesting that the recognition of target sequences in the host is very specific, and mutations of these promoters facilitate the

development of disease resistance in the plant. Indeed, impaired induction of the *R* gene *xa13*, the recessive allele of *Os8N3*, is the result of a substitution in the gene promoter (Chu *et al.*, 2004; Yang *et al.*, 2006).

The specific recognition of sequence targets by TALE proteins provides new means for the design of mechanisms of durable resistance. Indeed, Römer *et al.* (2009) demonstrated that a chimeric promoter region combining UPT boxes recognized by TALE proteins from different pathogens retains specificity for each of them, opening up the possibility of engineering *R* genes capable of recognizing multiple TALE proteins. Recent studies have demonstrated the ability of engineered TALE nucleases (TALENs) to generate double-strand DNA breaks, which represents an accurate genetic tool to introduce site-specific modifications in eukaryotic genes (Mahfouz *et al.*, 2011, 2012). Moreover, the applications of TALEN modules for engineering modifications in plant genomes to generate resistance to plant diseases have been reported (Li *et al.*, 2012).

Xanthomonas axonopodis pv. *manihotis* (*Xam*) is the causal agent of cassava bacterial blight, the most important bacterial disease affecting this crop (Lozano, 1986). The disease is distributed worldwide, causing losses that range between 12% and 100% in affected fields (Lozano, 1986). *Xam* is a foliar and vascular pathogen, capable of inducing a wide range of symptoms, including angular leaf spots, blight, shoot wilt, dieback, stem cankers, gum exudations and vascular necrosis (Lozano and Sequeira, 1974). Bacterial penetration of the leaf tissue takes place through natural openings (stomatal apertures) or wounds (Verdier *et al.*, 2004). *Xam* multiplies in the intercellular spaces at the expense of surrounding cells and moves systemically through the xylem, causing the typical shoot wilt symptoms (Verdier *et al.*, 2004).

We have shown previously that the pathogenicity of *Xam* is associated with a plasmid (p44) that is widely distributed among *Xam* strains (Verdier, 1988). A region of 8 kb was missing from the plasmid in a nonpathogenic *Xam* strain CFBP1851 Δ p44 (previously named ORST4), suggesting its role in pathogenicity (Verdier, 1988). This 8-kb region was contained within a 12-kb *HindIII* plasmid fragment (psF2; Verdier *et al.*, 1996). A restriction map for the plasmid fragment psF2 was established and partial sequence analysis allowed the generation of a polymerase chain reaction (PCR)-based assay for the specific detection of *Xam* pathogenic strains (Verdier *et al.*, 1998). In addition, DNA probes originated from p44 were developed for *Xam* and were used to assess the population diversity in different regions worldwide, mainly South America (Gonzalez *et al.*, 2002; Restrepo *et al.*, 1999, 2000a, b; Restrepo and Verdier, 1997).

PthB_{Xam}, which is contained within the psF2 plasmid fragment, encodes a protein belonging to the TALE family in *Xam*; this effector has been renamed as TALE1_{Xam}. A conclusive demonstration of the role of TALE1_{Xam} in the pathogenicity of *Xam* on cassava is still lacking, as is an exhaustive study of the structural and

functional characteristics of this gene. Here, we demonstrate the importance of TALE1_{Xam} as a pathogenicity factor for this bacterium, assess the importance of the eukaryotic domains in bacterial fitness and disease development, and determine its role as a transcriptional activator in plant cells.

RESULTS

The *tal1*_{Xam} locus on the p44 plasmid encodes a TALE protein

A 10 736-bp fragment encompassed in the psF2 fragment of p44 was sequenced. Within this fragment, we detected TALE1_{Xam} of 3099 bp in length. Several bioinformatic analyses were carried out to analyse and characterize the *tal1*_{Xam} region. TALE1_{Xam} contains a central repeat region composed of 13.5 repeats; it also contains two NLSs, with a motif K-[KR]-X-[KR], and a transcriptional AAD, with a motif T-V-M-x-E-Q-D-[EA]-[DA]-P-F-A-G-A-A-D-D-F-P-A-F-N-E(3), at the C-terminus (Fig. 1a). The flanking sequences were analysed to detect possible rearrangements, and revealed the presence of several repetitive sequences in the region: (i) two direct imperfect repeats of 1300 bp flanking *tal1*_{Xam}; (ii) two 200-bp-long tandem repeats; and (iii) eight inverted repeats/palindromic sequences upstream and downstream of the *tal1*_{Xam} gene. An insertion element belonging to the Tn3 family and encoding a putative resolvase element is present downstream of *tal1*_{Xam} (Fig. 1b). The presence of various repetitive sequences and the resolvase flanking TALE1_{Xam}, which have also been detected in members of this family in other *Xan-*

thomonas species (Kim *et al.*, 2006; Shiotani *et al.*, 2007), suggest that this region is highly prone to horizontal transfer and recombinational events which might lead to the generation of gene homologues.

Phylogenetic relationships among TALEs

Phylogenetic relationships of TALE1_{Xam} and other members of the TALE family were established. Maximum parsimony, maximum likelihood and Bayesian analysis were used to increase the robustness of the approach, and the central repeat region was excluded because of ambiguities in the alignment. TALE1_{Xam} groups with PthC and PthB, both from *Xanthomonas citri* pv. *aurantifolii* (Xcau; Fig. 2). The diversity of the TALE was divided into two large clades: the *X. oryzae* clade, which includes TALEs from *X. oryzae* pv. *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola* (Xoc), and the *X. axonopodis*/campestris clade, which contains *Xeu* and *Xac* TALEs. In general, TALEs from the same pathovar belong to the same clade, except for AvrB6 and PthN from *Xanthomonas campestris* pv. *malvacearum* (Xcm), which cluster in the *X. axonopodis* and *X. oryzae* clades, respectively. TALE1_{Xam} shares ancestry with TALEs in the Xoc-Xoo clade, which suggests that TALE1_{Xam} could have recently diverged from this clade of effectors. The fact that TALE1_{Xam} does not group with the *campestris*/*axonopodis* clade again suggests horizontal gene transfer (HGT) events. Notably, when compared with the C-terminus, the N-terminus shows less variation among TALEs. This may be a result of selection intrinsic to the function fulfilled by these two regions, where the N-terminal region of TALEs is necessary for secretion and translocation, and

a.

MDPIRPTSPAHELLAGPQDRVQPTADRGGAAPPAGSPLDGLPARRTMSRTRLPSPPAPLPFAFSAGSFSDDLRLQFDPSSLDTSL
FNSMSAFGAPHTAASGEGDEVQSGLRADDPQATVQVAVTAARPPRAKPAAPRRRAHTSDASPAQGVDLCTLGYSQQQKEIKL
KARSTVAQHHEALIGHGFTRAHIVALSQHPAALGTAVKYQAMIAALPEATHEDIVGGKQWSGARALEALLTVSGELRGPLQLDGTG
QLLKIARGGVTAVEAVHAWRNALTGAPLN

L T P D Q V V A I A S N I G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S N G G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S N I G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S N N G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S N I G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S H D G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S N S G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S N S G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S N N G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S N G G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S H D G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S N N G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S N I G G K Q A L E T V Q R L L P V L C E Q H G
L T P D Q V V A I A S N G G G K Q A L E

STFAQLSRPDQALALTDHVALACLGRRPALEAVRKGLPHAPTILKRTNRRLPERTSHRVADHAQVAVLGFQCHSHPAQAFDE
AMTQFGMSRHGLQLFRVGVTLGLASGTLPPAPQRWHRLQASGMKRAEPSGASQTPDQASLHAFADALERELDAPSPIDRAG
QALASSRKRSRESSVTGSFAQQAVEVRVPEQRDALHUPPLSWGVKRPRTIRIGGGLPDGPMDADLAASSTVMWEQDADPFA
GAADDFFAFNEEMAWLMELFPQ

b.

Fig. 1 Analysis of the TALE1_{Xam} locus in the p44 plasmid of *Xanthomonas axonopodis* pv. *manihotis* (Xam) strain CFBP1851. (a) Amino acid sequence of TALE1_{Xam}. The hypervariable amino acids at positions 12 and 13 in each repeat are shown in bold. Nuclear localization signals (NLSs) and the acidic activation domain (AAD) are highlighted in grey and black, respectively. (b) Schematic analysis of the *Hind*III region containing the *tal1*_{Xam} gene in p44. Dotted lines indicate the 1.3-kb nearly perfect direct repeats and the upstream squares indicate the 240-bp-long tandem repeats. Diamonds indicate the inverted repeat (IR) regions. *tal1*_{Xam} and putative resolvase genes are indicated by the black and white arrows, respectively.

Fig. 2 Evolutionary relationships of 30 Transcriptional Activator-Like effector (TALE) family members. The relationships among the different TALEs were inferred using three approaches: maximum parsimony, maximum likelihood and Bayesian analysis. Maximum parsimony analysis resulted in eight trees (tree length, 1514) supported by bootstrap analysis (1000 replicates); only 725 characters were informative. Maximum likelihood was inferred using the model K81uf+G selected in ModelTest 3.7 ($\log L = -9489.03$). Bayesian analysis was run with default parameters, resulting in a mean $\ln L = -9517.65$. All the phylogenies were congruent under Kishino-Hasegawa and Shimodaira-Hasegawa tests ($P < 0.05$). The values on the branches represent bootstrap values for maximum parsimony and maximum likelihood, and posterior probabilities for Bayesian analysis. TALE genes used in this analysis are listed in Table S1.

the C-terminus plays an important role for import into the host nucleus and for transcriptional activation. Alternatively, the different phylogenetic signals between the N- and C-termini may suggest that this difference in variation could be a result of HGT and/or recombination.

We evaluated both termini independently using a partition homogeneity test. When the phylogenetic signals obtained from C- and N-termini were compared, a significant difference ($P < 0.05$) was observed, suggesting an HGT event. In addition, we performed an evaluation of the maximum likelihood tree topologies for both terminal regions independently using the Kishino–Hasegawa and Shimodaira–Hasegawa tests. Both tests showed significant differences ($P < 0.05$; data not shown), suggesting that the tree topologies were incompatible for C- and N-terminal regions. From these analyses, we make the assumption that a recombination event may have taken place between the two terminal regions of these TALEs, either by HGT or by other mechanisms.

TALE1_{xam} restores pathogenicity to the nonpathogenic strain CFBP1851Δp44

Southern blot analyses indicated that the region of 8 kb missing from the p44 plasmid in the nonpathogenic *Xam* strain CFBP1851Δp44 encompasses TALE1_{*xam*}, suggesting its role in

pathogenicity (Fig. S1, see Supporting Information). To determine whether the loss of pathogenicity observed in CFBP1851Δp44 with respect to the wild-type (WT) strain CFBP1851 was indeed caused by the lack of *tale1_{xam}*, a genetic complementation assay was carried out. Transformation of strain CFBP1851Δp44 with the full-length *tale1_{xam}* gene cloned into pBAV226 (Vinatzer *et al.*, 2006) resulted in the restoration of pathogenicity on the susceptible cassava line HMC-1 (Fig. 3a), indicating that the nonpathogenic phenotype of CFBP1851Δp44 is caused by the loss of *tale1_{xam}* in the deleted 8-kb region of the plasmid. The development of characteristic disease symptoms on cassava plants was also observed after stem inoculation with the complemented strain (Fig. S2, see Supporting Information).

To determine whether the complementation with *tale1_{Xam}* had an impact on bacterial multiplication and systemic movement *in planta*, bacterial growth was determined at different distances from the inoculation point and at 0, 4 and 8 days post-inoculation (dpi; Fig. 3b). The WT strain CFBP1851 moved through the central vein and was detected in sites 3 and 4 at 8 dpi. In contrast, the nonpathogenic derivative strain CFBP1851Δp44 was reduced in its ability to grow and move systemically through the vascular system. CFBP1851Δp44 was not detected in sites 3 and 4 at any of the time points tested. However, the complemented strain was able to grow and move at levels comparable with those of the WT

Fig. 3 TALE1_{Xam} restores the pathogenicity of the nonpathogenic strain CFBP1851Δp44, as well as its ability to grow and move systemically. (a) Disease symptoms observed on the cassava susceptible cultivar HMC-1 at 8 days post-inoculation with *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) pathogenic strain CFBP1851, nonpathogenic strain CFBP1851Δp44 and its derivative strain complemented with the *tales1*_{Xam} gene cloned into the expression vector pBAV226. (b) The growth and movement of CFBP1851, CFBP1851Δp44 and complemented strains through the mid-vein of the main leaflet of leaves from the cassava cultivar MCol1522 were monitored at 0, 4 and 8 days post-inoculation at four different sites from the inoculation point. CFU, colony-forming unit.

CFBP1851 strain (Fig. 3b). These results suggest that the absence of TALE1_{Xam} affects both bacterial growth and bacterial movement in the host plant. Hence, TALE1_{Xam} may play an important role in plant defence suppression at the vascular level, which is an important line of defence in cassava against *Xam* (Kpemoua, 1995).

The eukaryotic domains of TALE1_{Xam} are essential for pathogenicity in *Xam*

To test whether the eukaryotic domains at the C-terminus of TALE1_{Xam} are critical for pathogenicity in *Xam*, four different versions of the protein (TALE1_{Xam}ΔNLS1, TALE1_{Xam}ΔNLS2, TALE1_{Xam}ΔNLS1/2 and TALE1_{Xam}ΔAAD expressed from pBAV226) were used to complement CFBP1851Δp44. The resulting strains were inoculated on susceptible cassava plants. The strain complemented with the complete protein induced angular leaf lesions typical of the disease. However, bacteria transformed with any of the four deletion constructs did not induce the disease phenotype of the WT bacterium on host plants (Fig. 4). These results indicate that both AADs and NLSs of TALE1_{Xam} are necessary for *Xam* CFBP1851 pathogenicity.

Multiple effector genes have been shown to suppress host defence responses. We tested the ability of TALE1_{Xam} to suppress the hypersensitive response (HR) elicited by a constitutive gain-of-function version of the Pto protein (L205D; Rathjen *et al.*, 1999) and a constitutively active mitogen-activated protein kinase involved in plant defence (MEK2; Jin *et al.*, 2003). TALE1_{Xam} was unable to suppress HR under the tested conditions (data not shown).

TALE1_{Xam} acts as a transcriptional activator in plant cells

To test whether TALE1_{Xam} performs a transcriptional activator function similar to other TALEs, we first predicted the optimal putative target promoter sequence for this protein using the code recently deciphered for TALE repeats (TATAAACAAATCAAT; Boch *et al.*, 2009; Moscou and Bogdanove, 2009). The putative target promoter was cloned upstream of the minimal *Bs3* promoter and the β-glucuronidase (GUS) reporter gene. This construct was transiently co-transformed into *Nicotiana benthamiana* leaves with a construct expressing TALE1_{Xam} under the control of the 35S promoter using *Agrobacterium*-mediated transient expression. GUS activity was detected only when TALE1_{Xam} was co-expressed with the GUS construct under the control of the hybrid TALE1_{Xam} target promoter and minimal *Bs3* promoter (Fig. 5). No GUS activity was observed when the TALE1_{Xam} target promoter was absent or when TALE1_{Xam} was not co-expressed. This result suggests that TALE1_{Xam} recognizes and binds to a specific target promoter sequence in the host, activating its transcription.

Fig. 4 The eukaryotic domains of TALE1_{Xam} are necessary for the pathogenicity of *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). Disease symptoms observed on the cassava susceptible cultivar HMC-1 at 8 days post-inoculation (dpi) with *Xam* pathogenic strain CFBP1851 (a), nonpathogenic strain CFBP1851Δp44 (b) and their derivative strains complemented with TALE1_{Xam} (c), and the deletion derivatives as follows: TALE1_{Xam} with deletions in NLS1 (d), NLS2 (e), both NLSs (f) and the AAD (g). (h) MgSO₄ was inoculated as negative control. AAD, acidic activation domain; NLS, nuclear localization signal.

DISCUSSION

TALE1_{Xam}, a new member of the AvrBs3 family, is a determinant of pathogenicity in *Xam*

Several TALE family members play a role in virulence. Examples of these include *pthA* in *Xac*, which is necessary for this pathogen to cause citrus canker disease and is sufficient to cause division, enlargement and death of host cells (Duan *et al.*, 1999). Similarly, the *hax* genes (*hax2–hax4*) in *Xanthomonas campestris* pv. *armoraciae* (*Xca*) contribute additively to the aggressiveness of the pathogen in radish *Raphanus sativus* (Kay *et al.*, 2005). *tal1*_{Xam} encodes for a new member of the *Xanthomonas* TALE family and, in this study, it has been found to be a key pathogenicity determinant of *Xam* in cassava. So far, *tal1*_{Xam} has not been demonstrated to be an avirulence factor (Restrepo *et al.*, 2000a). It is important to note that most effector proteins from different phytopathogenic bacterial species only partially contribute to pathogenicity because of functional redundancy (Badel *et al.*, 2006; Kvitko *et al.*, 2009). However, we have demonstrated that TALE1_{Xam} is an effector protein crucial for pathogenicity in *Xam*, as

it complements the complete lack of pathogenicity of strain CFBP1851Δp44. This correlates well with the presence of at least one TALE in most pathogenic *Xam* strains evaluated in previous studies (Gonzalez *et al.*, 2002). *Xam* strain CFBP1851Δp44 harbours other rearrangements in various genomic locations (R. Bart and B. Staskawicz, University of California, Berkeley, personal communication) which may affect virulence; however, TALE1_{Xam} was clearly demonstrated to be a critical pathogenicity determinant.

Our analysis shows similar phylogenetic relationships between TALEs to those reported in recent studies by Moreira *et al.* (2010) and Yu *et al.* (2011). In our study, we used a larger number of genes to include all the functional members of the TALE family known so far. Phylogenetically, the group/cluster containing TALE1_{Xam} grouped with the *X. oryzae*-related clade that includes TALEs from *Xanthomonas* pathovars causing bacterial blight diseases and exhibiting a vascular lifestyle inside their hosts. Notably, TALE1_{Xam} forms a well-supported clade with TALEs from *Xanthomonas* species that affect dicot hosts, suggesting that its divergence within the *X. oryzae* clade could be a result of host adaptation. In general, we observed the maintenance of two major clades of TALEs, consistent with our data which suggest that

Fig. 5 TALE1_{Xam} is a transcriptional activator in plant cells. *Agrobacterium*-mediated transient expression of β -glucuronidase (GUS). *Nicotiana benthamiana* leaves were inoculated with *A. tumefaciens* strain GV3101 carrying pBAV139 (*tale1*_{Xam}) (a), pGWB3 (minimal *Bs3* promoter + *uidA* gene) (b), pGWB3 (TALE1_{Xam} putative target + minimal *Bs3* promoter + *uidA* gene) (c) and co-inoculated with pBAV139 (*tale1*_{Xam}) and pGWB3 (minimal *Bs3* promoter + *uidA* gene) (d) and pBAV139 (*tale1*_{Xam}) and pGWB3 (TALE1_{Xam} putative target + minimal *Bs3* promoter + *uidA* gene) (e). Transient expression was evaluated at 48 h post-inoculation (hpi).

HGT acts as a diversifying force for this family of effectors. Our hypothesis is supported by the presence of proteins, such as resolvases, which are also detected in the downstream region of other plasmid-borne TALEs, such as PthB from *Xcau*, and PthA1, PthA2, PthA3 and PthA4 from *Xac* (Brunings and Gabriel, 2003; El Yacoubi *et al.*, 2007).

The pathogenicity function of TALE1_{Xam} relies on its eukaryotic domains

Previous studies on AvrBs3 and other members of the TALE family have shown that eukaryotic domains are crucial for both virulence and avirulence activities in the host. In the case of AvrBs3, NLS motifs are critical for the interaction with the host protein importin and for subsequent translocation to the nucleus (Szurek *et al.*, 2001). Mutations in these domains disrupt the nuclear import function, and hence the modulation of the host transcriptome (Szurek *et al.*, 2001; Van den Ackerveken *et al.*, 1996). Likewise, mutations in the same regions in the *Xoo* proteins AvrXa7 and AvrXa10 impair the recognition by the cognate *R* genes *Xa7* and *Xa10*, respectively (Yang *et al.*, 2000; Zhu *et al.*, 1999), and reduce the pathogenicity function of AvrXa7 (Ponciano *et al.*, 2004). Deletions of the TALE1_{Xam} eukaryotic domains cause a loss in the

pathogenic activity of this protein. This result demonstrates that, as for other TALEs, TALE1_{Xam} must be localized inside the nucleus to bind a specific DNA target sequence and to activate the transcription of certain genes using the host transcriptional machinery. Interestingly, each single monopartite NLS alone is not sufficient for TALE1_{Xam} pathogenicity function in cassava, which suggests that the presence of both NLSs is required for TALE1_{Xam} function. One possible explanation for these observations is that the deletion mutant proteins are unstable. However, we have recently observed that TALE1_{Xam} is an autoactive protein in the yeast two-hybrid system and all the deletion mutants retain this ability (Gil *et al.*, 2011). This suggests that the proteins are stable, and therefore the most plausible explanation for these observations is that the AADs and NLSs are all required for the pathogenicity function of TALE1_{Xam}.

TALE1_{Xam} functions as a transcriptional activator

TAL-dependent transcriptional activation of host genes has been demonstrated for several bacterial TALEs to promote disease development. This is the case for *Xoo* TALEs PthXo1 and AvrXa7, which act as transcription factors modulating the expression of the N3 nodulin family glucose transporters Os8N3 and Os11N3, respectively (also called OsSWEET11 and 14; White and Yang, 2009; Yang *et al.*, 2006; Yu *et al.*, 2011). In addition, Sugio *et al.* (2007) have demonstrated that PthXo6 induces the expression of the bZIP transcription factor *OsTFX1* in rice, contributing to disease susceptibility and full bacterial virulence. As TALE1_{Xam} specifically induces the expression of a reporter gene under the control of the predicted target promoter, we can infer that this bacterial effector has a transcriptional activation function in its host when it recognizes a specific target promoter sequence through the repeat variable di-residues (RVDs) in the central repeat region of the protein.

The characterization of the *tale1*_{Xam} gene provides new insights and perspectives in the understanding of the molecular interactions between *Xam* and cassava. In addition to TALE1_{Xam}, other TALEs are present in the genome of *Xam* strains, with some strains having at least four to five TALE family members, and the most pathogenic strains carrying at least one copy (Gonzalez *et al.*, 2002). Future studies should aim to determine the importance of other TALE members and to test whether they play major or minor roles in *Xam* pathogenicity. For example, strain CFBP1851 has an additional member of the TALE family (Fig. S1; Gonzalez *et al.*, 2002) and it would be interesting to determine its role in the virulence of this strain (Gonzalez *et al.*, 2002).

The recent discovery of the code ciphered in the repeat domain from TALE proteins has allowed us to predict the optimal putative target sequences in the host (Fig. 6a). Possible binding sites for TALE1_{Xam} were predicted in the promoter regions (500 bp upstream from the translation start site) using the prediction program TALVEZ

Fig. 6 Putative target of TALE1_{Xam} in cassava.

(a) The putative target sequence in the host was predicted on the basis of the hypervariable amino acids at positions 12 and 13 in each repeat. (b) Pie chart showing the most common annotations for the top 100 predicted targets for TALE1_{Xam} in the cassava genome using the program Talvez. Annotations are based on the closest *Arabidopsis* homologue and were obtained from phytozome (<http://www.phytozome.net>).

(A. L. Pérez-Quintero *et al.*, unpublished data; <http://bioinfo.mpl.ird.fr/cgi-bin/talvez/talvez.cgi>) on the available draft genome of cassava (version Cassava 4, Cassava Genome Project; <http://www.phytozome.net/cassava>). This program uses positional weight matrices for a given RVD sequence based on known RVD-DNA specificities (Boch *et al.*, 2009; Deng *et al.*, 2012; Mak *et al.*, 2012; Moscou and Bogdanove, 2009), scores possible binding sites using a log-likelihood function and performs better than other TALE target prediction programs (Doyle *et al.*, 2012) in control tests using known TAL-DNA interactions (A. L. Pérez-Quintero *et al.*, unpublished data). Although no gene in cassava contains the optimal target site validated in this work, several genes were found to contain other possible binding sites with prediction scores as high as those obtained for known targets in other systems (Moscou and Bogdanove, 2009; Römer *et al.*, 2010). The top 100 predicted targets comprised a heterogeneous group of genes, with no over-represented gene ontology terms as determined by singular enrichment analysis using the AgriGO platform (Du *et al.*, 2010). However, some common annotations were found among the predicted targets, including transcription factors (10%) and proteins with hydrolase (7%) or kinase (5%) function (Fig. 6b). Notably, 23% of the predicted targets have no known annotation, suggesting that

TALE1_{Xam} interferes with yet unknown host functions. It should be noted that no *SWEET* genes were present in the top 100 predicted targets, indicating that *Xam* major virulence TALEs rely on different mechanisms to cause disease than those identified in *Xoo*. It is possible that phenotypic effects caused in cassava by TALE1_{Xam} are caused by the induction of more than one of the predicted targets, as has been shown in other systems (Boch and Bonas, 2010). In addition, the induction of transcription factors involved in various signalling pathways could produce large-scale effects on infection. Bioinformatic target predictions may become more accurate as a greater number of cassava varieties are sequenced. In addition, efforts to identify the target(s) of this protein in cassava using transcriptomics are underway.

The origin of TALEs, their diversity in the *Xam* population and their effect on the evolution of *Xam* genomes or the virulence of *Xam* strains are far from being understood. Answers to these and other questions might come from a deeper analysis of *Xam* genomes, as well as a deeper knowledge of the ecology of cassava bacterial blight disease. Sequencing projects focused on *Xam* strains with specific geographical origins will also improve our understanding of the interplay between the ecology and adaptation of *Xam*, and may reveal new targets for disease control.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Xam* strains CFBP1851 and CFBP1851Δp44 were grown on Luria peptone glucose agar (LPGA) plates and liquid Phi ϕ medium (Link *et al.*, 1997). Bacteria were incubated at 30 °C for 24 h in liquid medium and for 48 h in solid medium. *Escherichia coli* DH5 α was used for all cloning procedures and transformed by electroporation. Media were supplemented with kanamycin (50 μ g/mL), gentamycin (10 μ g/mL) or tetracycline (10 μ g/mL) when appropriate. Bacteria were transformed by electroporation (*E. coli*, *A. tumefaciens* and *Xam*).

Generation of deletion mutants of TALE1_{Xam}

Deletions in the eukaryotic domains (NLSs 1 and 2, and AAD) from the C-terminal region of *ta1e1*_{Xam} were produced using site-directed mutagenesis, based on overlap extension polymerase chain reaction (OE-PCR; Higuchi *et al.*, 1988). For this, all PCRs were carried out using high-fidelity Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA, USA). The flanking 3' primer contained an *Ascl* restriction site. The N-terminal domain was amplified from pF3, a pBluescript derivative vector that carries a 5.4-kb fragment issued from the native plasmid p44 in *Xam* strain CFBP1851 containing the *ta1e1*_{Xam} gene (Restrepo and Verdier, 1997), using oligonucleotides specific for this domain, and adding the CACC sequence at the 5' end for cloning into pENTR/D-TOPO (Invitrogen) and an *XhoI* restriction site at the 3' end. The different C-terminal deletion fragments were cloned into pGEM T-easy. The central repeat region was excised from pF3 with *SphI* and *XhoI*, and then ligated with the N-terminal domain in pENTR/D-TOPO, previously digested with the same enzymes. Different versions of the C-terminal fragment were excised from pGEM T-easy with *XhoI* and *Ascl*, and then ligated with the repeats and N-terminal domain in pENTR/D-TOPO, assembling the full-length gene versions. The resulting pENTR/D-TOPO clones were used in a Gateway™ LR cloning reaction in order to introduce the *ta1e1*_{Xam} versions into pBAV226, a Gateway™ bacterial expression vector with the *nptII* promoter (Vinatzer *et al.*, 2006). The *ta1e1*_{Xam} deletion mutants were then used for complementation of CFBP1851Δp44. The WT *ta1e1*_{Xam} gene was amplified by PCR from pF3 and cloned into pDONR207 vector (Invitrogen). All clones were confirmed by sequencing at MacroGen, Inc. (Seoul, South Korea).

Table 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source
<i>Xanthomonas axonopodis</i> pv. <i>manihotis</i> (<i>Xam</i>)		
CFBP1851	Wild-type carrying native plasmid p44	CFBP
CFBP1851Δ p44	CFBP1851 derivative nonpathogenic strain carrying an 8-kb deletion in plasmid p44	Verdier (1988)
Plasmids		
pENTR/D-TOPO	Gateway™ cloning vector	Invitrogen
pDONR207	Gateway™ cloning vector	Invitrogen
pBAV226	Gateway™ <i>Pseudomonas syringae</i> expression vector (<i>nptII</i> promoter and C-terminal HA-tag)	Vinatzer <i>et al.</i> (2006)
pBAV139	Gateway™ binary plant expression vector (35S promoter and C-terminal HA-tag)	Vinatzer <i>et al.</i> (2006)
pME6010	Cloning vector	Heeb <i>et al.</i> , 2000
pF3	5.4-kb <i>EcoRI</i> fragment from the p44-kb native plasmid in <i>Xam</i> ligated into pBluescript	Restrepo and Verdier (1997)

Plant material and inoculation assays

Susceptible cassava plants, from cultivars HMC-1 (a.k.a. ICA Armenia) and MCol-1522, were grown in a glasshouse at approximately 25 °C/16 °C (day/night temperatures) with a 12-h photoperiod. *Nicotiana benthamiana* plants were grown in a controlled-environment chamber at 25 °C, 85% relative humidity and with a 16-h photoperiod. Bacterial suspensions of *Xam* [optical density (OD) = 1, $\sim 3 \times 10^9$ colony-forming units (CFU)] were prepared in 10 mM MgSO₄ with a 12-h culture and were subsequently diluted to the appropriate cell concentrations for individual experiments. Young leaves from 1-month-old cassava plants were inoculated by placing 20 μ L of the bacterial suspension into 2-mm-diameter holes previously made on the leaf using a cork borer, as described previously (Restrepo *et al.*, 2000a). Symptom appearance was monitored every day up to 15 dpi.

Quantification of *in planta* bacterial growth

To study the growth and movement of *Xam* through the leaf tissue of susceptible cassava plants, the main vein of the main leaflet was pricked using a syringe needle. A 10- μ L drop of an OD = 0.2 (1×10^8 CFU/mL) bacterial suspension was deposited on the wound. The leaflets were sampled at 0, 4 and 8 dpi. The number of bacterial cells in the leaves was determined at the top 4 cm of each leaf, which was cut into four 1-cm sections, and labelled as sites 1, 2, 3 and 4 (site 1 being the section adjacent to the inoculation point). The leaf pieces were then processed individually by grinding in 100 μ L of 10 mM MgSO₄. Serial dilutions were made and spread onto LPGA plates. The plates were incubated at 28 °C until single colonies could be counted. The number of CFUs per section (equivalent to about 1 cm²) was counted and standard errors were calculated. The experiment was repeated independently three times.

Agrobacterium-mediated transcriptional activation assay

To test the transcriptional activation activity of TALE1_{Xam}, *Agrobacterium* transient expression assay was carried out. The TALE1_{Xam} predicted target was cloned upstream of the minimal *Bs3* promoter (positions –344 to –1 from the *Bs3* open reading frame in *Capsicum annuum* cv. ECW-30R) into the T-DNA vector pGWB3 (Nakagawa *et al.*, 2007) in order to fuse these elements in frame with the *uidA* gene. A construct containing only the minimal *Bs3* promoter was used as negative control. In addition, the

tale1_{Xam} gene was cloned into the binary vector pBAV139 (Vinatzer *et al.*, 2006). After transformation into *A. tumefaciens* strain GV3101, these constructs were co-inoculated or inoculated separately into *N. benthamiana* leaves. After 48 h, the inoculated leaves were collected and subjected to a qualitative GUS assay by vacuum infiltration with the GUS staining solution [50 mM sodium citrate pH 7.0, 0.1 M HCl, 0.2% Tween 20 and 1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc)]. Leaves were incubated overnight in this solution and subsequently cleared with 100% ethanol.

Bioinformatic analyses

The entire 10 736-bp sequence encompassing the region containing *tale1_{Xam}* has been deposited in GenBank (Accession number HQ113297). This replaces the former sequence AF012325 that had been deposited previously. Inverted repeats, tandem repeats and palindromic sequences in *tale1_{Xam}* were searched using the EMBOSS suite (Sarachu and Colet, 2005). The threshold values were reduced to increase the probability of repeat finding, and the output was analysed based on the size, number and score. Using the IS Finder database (<http://www-is.biotoul.fr/is.html>), BLAST searches were performed to reveal the presence of elements related to insertion sequences (ISs). The candidate ISs were confirmed with BLASTX (parameters by default) against the nonredundant protein database. As a member of the TALE family, TALE1_{Xam} nucleotide and amino acid sequences were compared against nucleotide and protein databases at GenBank (<http://www.ncbi.nlm.nih.gov/>) using BLASTX to identify the members of this family from *Xanthomonas* species. All the sequences employed for this study are summarized in Table S1 (see Supporting Information).

Phylogenetic analyses

N-terminal and C-terminal regions for all the sequences were extracted in two separate multifasta files using the web EMBOSS suite. Nucleotide sequence alignments were constructed without the central repeat region, using CLUSTALW2 (<http://www.ebi.ac.uk/Tools/clustalw2/>). A BLOSUM30 matrix was used for sequence comparisons and the alignment was manually revised using MacClade 4.08 (Maddison and Maddison, 2005).

Pairwise distance analysis was carried out using PAUP*4.0b10 (Swofford and Sullivan, 2003). The following approaches were carried out simultaneously for tree construction: neighbour-joining method (Saitou and Nei, 1987) and maximum parsimony analyses using heuristic searches, both methods employing bootstrap analysis with 1000 replicates in order to determine the reliability of the tree. PhyML was employed for maximum likelihood analysis (Guindon and Gascuel, 2003). Appropriate evolutionary models for use in maximum likelihood analysis were determined for the dataset using ModelTest version 3.7 and selected on the basis of the hierarchical likelihood ratio test (hLRT). Bayesian analysis was performed using the program MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). Trees were visualized and annotated using FigTree version 1.3.1 (Rambaut and Drummond, 2010).

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SUPPORTING INFORMATION

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

Fig. S1 Southern blot analysis of genomic DNA from *Xanthomonas axonopodis* pv. *manihotis* (Xam) strains CFBP1851, CFBP1851Δp44 and CFBP1851Δp44 + TAL1_{Xam}. Total DNA was digested with *Bam*HI and separated on a 1% agarose gel. The blot was hybridized with a 595-bp probe corresponding to the 3' end of *tale1*_{Xam}.

Fig. S2 TAL1_{Xam} restores the pathogenicity of the nonpathogenic strain CFBP1851Δp44 after inoculation on stems of cassava plants. Disease symptoms observed on cassava plants after inoculations on the stem of the cassava cultivar MCol1522, monitored at 7, 15, 20 and 28 days post-inoculation, with the *Xanthomonas axonopodis* pv. *manihotis* (Xam) pathogenic strain CFBP1851, nonpathogenic strain CFBP1851Δp44 and its derivative strain complemented with the *tale1*_{Xam} gene.

Table S1 Members of the Transcriptional Activator-Like effector (TALE) family used in the phylogenetic analyses.