The role of type III effectors from *Xanthomonas axonopodis* pv. *manihotis* in virulence and suppression of plant immunity

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SUMMARY

Xanthomonas axonopodis pv. *manihotis* (*Xam*) causes cassava bacterial blight, the most important bacterial disease of cassava. *Xam*, like other *Xanthomonas* species, requires type III effectors (T3Es) for maximal virulence. *Xam* strain CIO151 possesses 17 predicted T3Es belonging to the *Xanthomonas* outer protein (Xop) class. This work aimed to characterize nine Xop effectors present in *Xam* CIO151 for their role in virulence and modulation of plant immunity. Our findings demonstrate the importance of XopZ, XopX, XopAO1 and AvrBs2 for full virulence, as well as a redundant function in virulence between XopN and XopQ in susceptible cassava plants. We tested their role in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) using heterologous systems. AvrBs2, XopR and XopAO1 are capable of suppressing PTI. ETI suppression activity was only detected for XopE4 and XopAO1. These results demonstrate the overall importance and diversity in functions of major virulence effectors AvrBs2 and XopAO1 in *Xam* during cassava infection.

Keywords: ETI, PTI, type III effectors, virulence, *Xanthomonas axonopodis* pv. *manihotis*.

INTRODUCTION

Cassava (*Manihot esculenta*) is the third most important source of calories in the tropics (FAO, 2008; Ospina and Ceballos, 2012). Cassava production is limited by a variety of plant pathogens that compromise the food safety of millions of people around the world (Howeler *et al.*, 2013; Legg *et al.*, 2015; Lopez and Bernal, 2012; Patil *et al.*, 2015). *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), the causal agent of cassava bacterial blight (CBB), generates losses of up to 100% under appropriate climatic conditions (Lozano and Sequeira, 1974). This disease threatens food security in the tropics, where cassava constitutes a major staple food (CABI, 2015; FAO, 2008). Rapid dispersal of CBB in some cassava production regions (Joseph and Elango, 1991) and new disease reports in regions in which cassava is a staple crop (Kone *et al.*, 2015; Wonni *et al.*, 2014) underscore the importance of finding new ways to manage this plant disease (Lopez and Bernal, 2012). The management of this, as well as other, plant diseases would greatly benefit from a comprehensive knowledge of pathogen population dynamics and of the genetic bases for virulence in the pathogen and for resistance/susceptibility in the plant (Dangl *et al.*, 2013; Li Y *et al.*, 2013). Recent studies reporting the genomic sequence and identification of pathogenicity genes in *Xam* (Arrieta-Ortiz *et al.*, 2013; Bart *et al.*, 2012), as well as those on the genetic mapping and physical localization of immune-related gene products, have great potential for the development of resistant varieties in cassava (Lopez *et al.*, 2007; Soto *et al.*, 2015). However, a more detailed characterization of pathogenicity determinants in *Xam* and resistance genes against CBB in cassava is needed.

Plants have developed an immune system based on their co-evolution with microbes (Dodds and Rathjen, 2010). The plant immune system is composed of two layers: the first involves the recognition of microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) by plant pattern recognition receptors (PRRs), which triggers a moderate defense response, named PAMP-triggered immunity (PTI) (Zhang and Zhou, 2010; Zipfel, 2014). PTI is characterized by the production of reactive oxygen species (ROS), calcium-dependent signalling pathways (Stael *et al.*, 2015), activation of mitogen-activated protein kinases (MAPKs) (Meng and Zhang, 2013) and callose deposition at the cell wall (Adam and Somerville, 1996). However, successful pathogens have

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developed strategies to evade PTI and cause disease. Their mechanisms include the secretion and translocation of effector proteins, resulting in effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). In turn, plants have evolved a second layer of immunity, expressing NOD-like receptors (NLRs), classically known as resistance proteins. NLRs are mostly intracellular receptors that directly or indirectly recognize effectors, leading to a robust defence response, named effector-triggered immunity (ETI) (Cui *et al.*, 2015). ETI is characterized by ROS production (Torres *et al.*, 2006), activation of the MAPK cascade (Meng and Zhang, 2013; Teper *et al.*, 2015) and a form of programmed cell death called the hypersensitive response (HR) (Coll *et al.*, 2011). Bacteria can, in turn, use additional strategies to overcome ETI (Swords *et al.*, 1996). Hence, PTI, ETS and ETI, the result of co-evolution between plants and microbes, are the main determinants of the output of a given infection.

In plant pathogenic bacteria, type III effectors (T3Es) play a crucial role in pathogenicity (Buttner and He, 2009). T3Es are translocated into the plant cytosol by the type III secretion system (T3SS); once inside the cell, they manipulate the plant cell to favour bacterial growth (Chen *et al.*, 2010; Grant *et al.*, 2006). T3Es can be grouped into different families based on their biochemical function. A family of T3Es in *Xanthomonas* species, called transcription-activator like effectors (TALEs), are DNA-binding proteins that act as transcriptional activators of plant genes (Boch and Bonas, 2010). These plant genes include sugar transporters, which are hypothesized to provide increased levels of sucrose for pathogen growth (Chen *et al.*, 2010; Zhou *et al.*, 2015), as well as transcription factors that globally regulate physiological processes in the plant cell (Hu *et al.*, 2016; Kay *et al.*, 2007). Another diverse group of effectors comprises the *Xanthomonas* outer proteins (Xops). Xop effectors play a major role in the modulation of PTI and ETI (Schulze *et al.*, 2012; Stork *et al.*, 2015; Taylor *et al.*, 2012). Xops may carry out these functions by acting as enzymes, including E3 ubiquitin ligases (Singer *et al.*, 2013), small ubiquitin-like modifier (SUMO) proteases (Hotson *et al.*, 2003; Roden *et al.*, 2004) and uridyl transferases (Feng *et al.*, 2012). In addition, they may carry out their functions in a non-enzymatic manner.

In *Xam*, previous work has focused on the population dynamics (Restrepo *et al.*, 2004; Trujillo *et al.*, 2014a,b), strain characterization in cassava cultivars (Restrepo *et al.*, 2000; Wydra *et al.*, 2004) and the mapping of expressed sequence tags (ESTs) associated with resistance (Lopez *et al.*, 2004, 2007). However, the identification of *Xam* pathogenicity factors is an important step towards the development of new strategies against CBB. Studies characterizing pathogenicity factors in *Xam* have revealed TALE1-$_{Xam}$ as a crucial virulence factor (Castiblanco *et al.*, 2013), which induces transcriptional changes in cassava (Munoz-Bodnar *et al.*, 2014). In addition, TALE20$_{Xam668}$ acts as a gene activator for the

sugar transporter MeSWEET10 (Cohn *et al.*, 2014) and TALE14-$_{Xam668}$ promotes virulence in *Xam*668 (Cohn *et al.*, 2015). These studies have revealed the functional relevance of TALEs in the virulence of *Xam*. However, very little is known about the role of Xop effectors in *Xam*, especially when compared with homologues in other pathosystems.

The repertoire of Xop effectors from *Xam* has been reported in the reference strain CIO151 (Arrieta-Ortiz *et al.*, 2013). In addition, sequencing of 65 *Xam* genomes has allowed the identification of core T3Es among *Xam* strains from diverse geographical and temporal origins (Bart *et al.*, 2012). In this work, we studied the importance of nine of the 17 *Xam* T3Es to assess their role in virulence and plant immunity. The T3Es studied here cover two core (*xopN* and *xopV*) and seven non-core (*avrBs2*, *xopX*, *xopQ*, *xopZ*, *xopAO1*, *xopR* and *xopE4*) T3Es. Our findings reveal that four T3Es (XopZ, XopX, XopAO1 and AvrBs2) are important for full virulence when single mutants are evaluated. A redundant effect in virulence was seen between *xopN* and *xopQ*. Three T3Es (XopR, AvrBs2 and XopAO1) were able to suppress PTI and two (XopE4 and XopAO1) were able to suppress ETI. Altogether, these results are useful for our understanding of the molecular mechanisms of disease and to help guide the development of cassava varieties resistant to CBB.

RESULTS

Four different effectors are important for full virulence in *Xam* strain CIO151

To determine the role of *Xam* T3Es in virulence, T3E knockout (KO) mutants were generated and their ability to cause disease in cassava was evaluated. We were able to mutate two core (*xopN* and *xopV*) and seven non-core (*avrBs2*, *xopX*, *xopQ*, *xopZ*, *xopAO1*, *xopR* and *xopE4*) T3Es in *Xam* CIO151 (Arrieta-Ortiz *et al.*, 2013). We used pK18*mobsacB* to produce unmarked deletions of T3Es in this strain. Subsequently, the ability of these KO mutant strains to produce symptoms in leaves of cassava (cultivar MCOL2215) was evaluated. Leaves were inoculated by the perforation and drop method (Restrepo *et al.*, 2000). Characteristic *Xam* lesions, evidenced by chlorosis and necrosis surrounding the inoculation area, were detected in all strains. We measured the lesion area caused by each strain at 15 days post-infiltration (dpi) (Fig. 1A,B). The wild-type strain caused chlorosis, water-soaking and necrotic symptoms typical of CBB. The disease symptoms caused by strains with KOs in genes *xopR*, *xopQ*, *xopE4*, *xopN* and *xopV* were similar to those caused by the wild-type strain (Fig. 1A,B), suggesting that these genes are either dispensable for maximal virulence of this strain or that there is functional redundancy with other T3Es. However, KO strains for the T3Es *xopAO1*, *xopX*, *xopZ* and *avrBs2* in *Xam* CIO151 showed a reduction in virulence compared with the wild-type strain, suggesting that these T3Es are important for maximal symptom development.
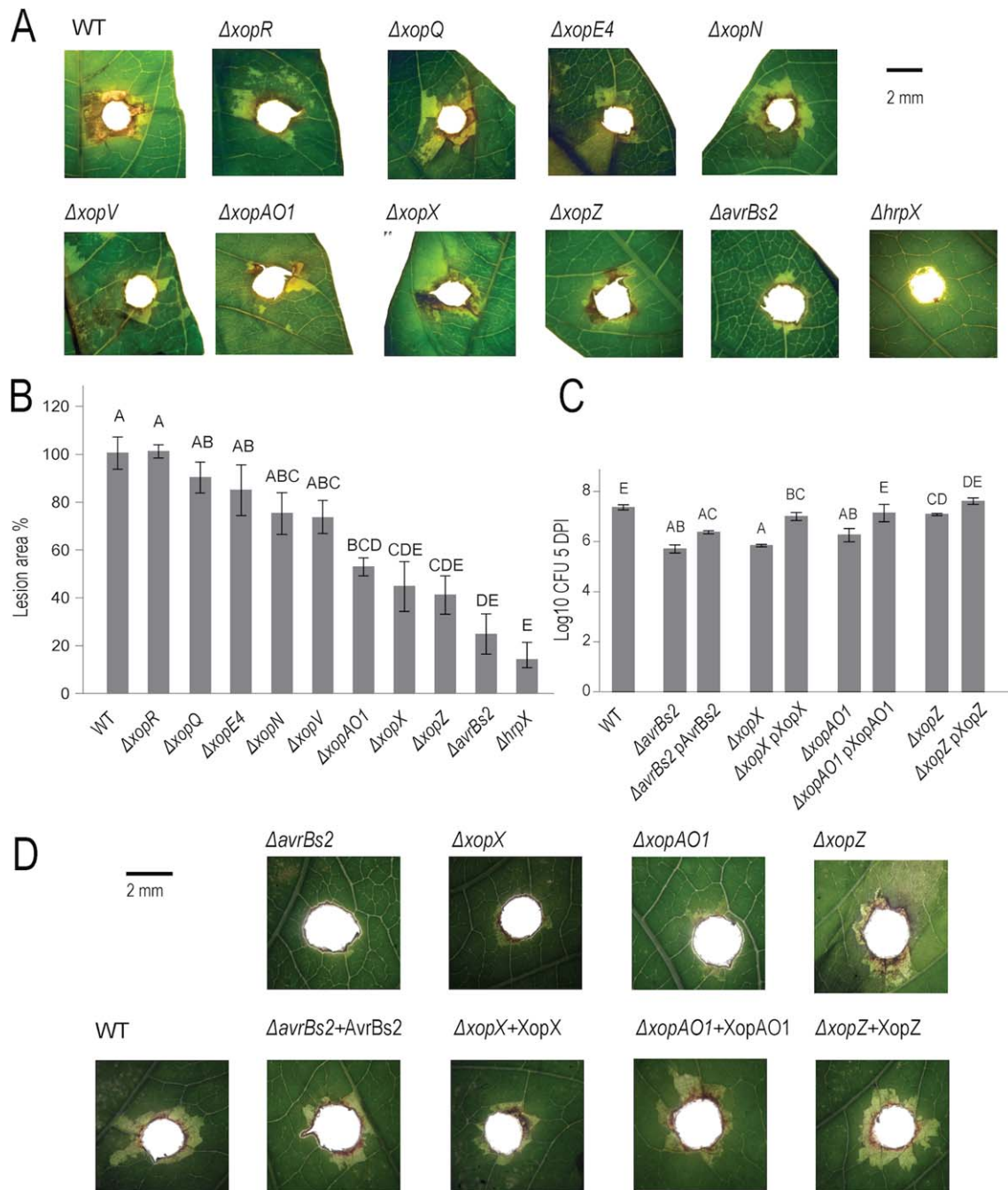
Fig. 1 Role of type III effectors (T3Es) from *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) strain CIO151 in virulence on susceptible cassava plants. (A) Lesions caused by different knockout (KO) mutants of T3Es in cassava leaves. (B) Quantification of the lesions caused by different KO mutants of *Xam* CIO151. Values represent the lesion area in per cent with respect to the wild-type CIO151. Δ*hrpX* is a type III secretion mutant (used as negative control). (C) Bacterial growth *in planta* in colony-forming units (CFU)/0.5 cm^2 of plant tissue at 5 days post-infiltration (dpi) (log$_{10}$ CFU at 5 dpi). (D) Lesions caused by KO mutants in individual T3Es which showed a role in virulence and were transformed with the corresponding T3E gene. Leaf fragments of all strains that showed a difference in (A) and the corresponding complementation strains using vector pBBR1-MCS5. Error bars for (B) and (C) correspond to ±1 standard deviation (SD). Different letters in (B) and (C) show significant differences when analysed with Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).

To determine whether these changes in symptom development were reflected in the ability of the pathogen to grow *in planta*, the KO strains were inoculated by leaf infiltration of a bacterial suspension and bacterial growth was measured at 0 and 5 dpi in cassava MCOL2215 by dilution plating. Strains with KOs in *xopX* and *avrBs2* reached more than one log unit lower populations
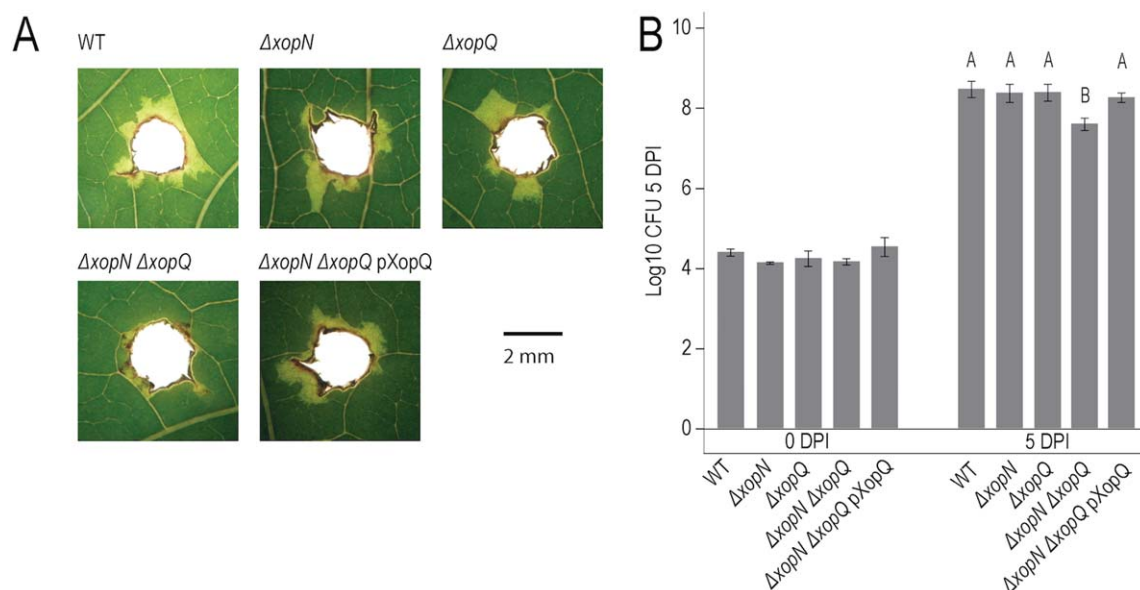
Fig. 2 Redundant function between type III effectors (T3Es) from *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) strain CIO151. (A) Lesions caused by single and double knockout (KO) mutants for T3E in *Xam*. (B) Bacterial growth of double KO Δ*xopN* Δ*xopQ* vs. single KO in *Xam* strain CIO151 represented as $\log_{10}$ of bacterial growth *in planta* at 5 days post-infiltration (dpi). Error bars are ± 1 standard deviation (SD) in an experiment with three replicates. Strains with a different letter were statistically significantly different based on Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).

than those observed for the wild-type strain (Fig. 1C). A smaller but statistically significant difference was observed for strains with KO in *xopAO1* and *xopZ* when compared with the wild-type strain CIO151 [Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$)]. These data suggest that these four T3Es are required for full growth of *Xam* in a susceptible cassava cultivar.

To confirm that the changes in the virulence of the KO strains were caused by the deletion in the targeted T3E gene, we transformed each KO strain with the broad-host-range vector pBBR1MCS5 (Kovach *et al.*, 1995) containing the corresponding wild-type T3E gene under the *lac* promoter. A statistically significant growth increase was observed in the transformed *xopX* and *xopAO1* strains with respect to the KO strains (Fig. 1C). The *xopZ* KO strain was complemented in its ability to cause symptoms (Fig. S1A, D, see Supporting Information), and a small but not statistically significant difference was observed in the growth of the pathogen *in planta* (Fig. 1C). However, the *avrBs2* KO strain was only partially complemented in its ability to cause symptoms and to grow *in planta* (Figs 1C, D and S1B). This could be a result of the use of a strong promoter or high copy number, which, in other systems, has been reported to affect the functionality of effectors (Guttman and Greenberg, 2001). We therefore attempted complementation with the pCU18-mini-Tn*7*T-Gm system (Choi *et al.*, 2005), which inserts the gene in a neutral chromosomal location, under its native promoter and with one copy per genome. The introduction of *avrBs2* and *xopX* using this system fully rescued the lesser ability of the mutants to grow *in planta* (Fig. S2, see Supporting Information).

In other bacteria, T3Es can have redundant functions, where one could mask the phenotype of a T3E KO strain (Kvitko *et al.*, 2007, 2009). To determine redundancy among T3Es in *Xam*, we generated double mutants in T3E genes whose single mutants exhibited no detectable virulence phenotype, and therefore no apparent role in virulence. These double KO mutants were inoculated onto cassava and the lesion areas were measured (Fig. S3, see Supporting Information). Strains with double KO in Δ*xopX* Δ*xopZ* or Δ*xopQ* Δ*xopZ* did not show significant differences when compared with the corresponding single KO mutants, suggesting that these gene pairs do not show redundancy in the virulence of *Xam* strain CIO151, although we cannot exclude the possibility that they show redundancy with other untested effectors. The double KO mutant Δ*xopN* Δ*xopQ* was less aggressive than its single counterparts (Fig. S3). In addition, *in planta* bacterial growth was reduced at 5 dpi in the double KO mutant Δ*xopN* Δ*xopQ* (Fig. 2B) with respect to *Xam* CIO151 and individual KO strains Δ*xopN* or Δ*xopQ*. Finally, we observed complementation of double KO mutant Δ*xopN* Δ*xopQ* when transforming a plasmid copy of XopQ (Fig. 2A,B), which confirms that these two T3Es are functionally redundant. Tukey's HSD test ($\alpha = 0.05$) was carried out to determine significant differences, and the experiment was performed twice with similar results.

Three *Xam* effectors are involved in the suppression of PTI

Many T3Es have been shown to suppress PTI. We therefore tested whether different *Xam* T3Es had the ability to suppress PTI by
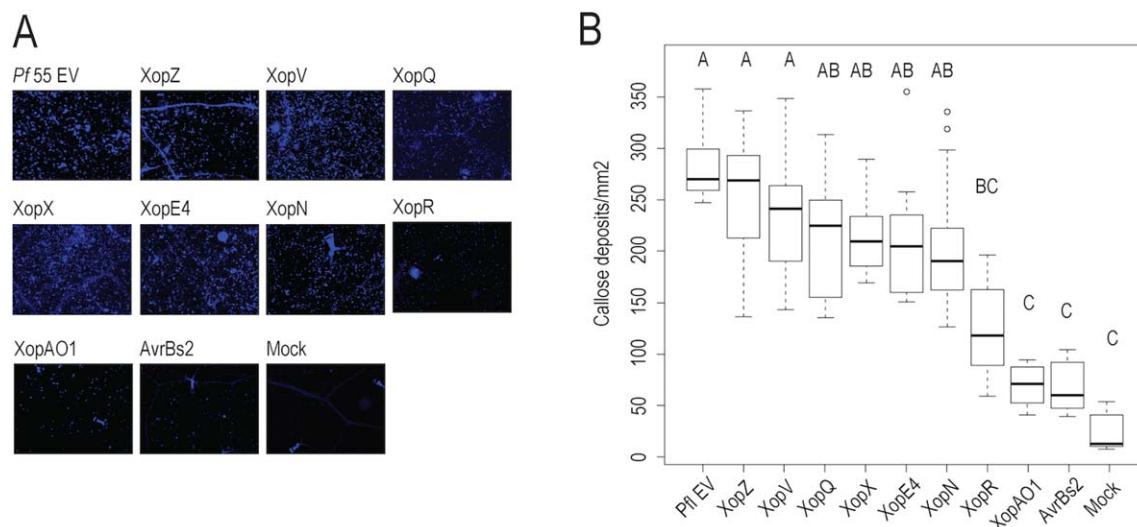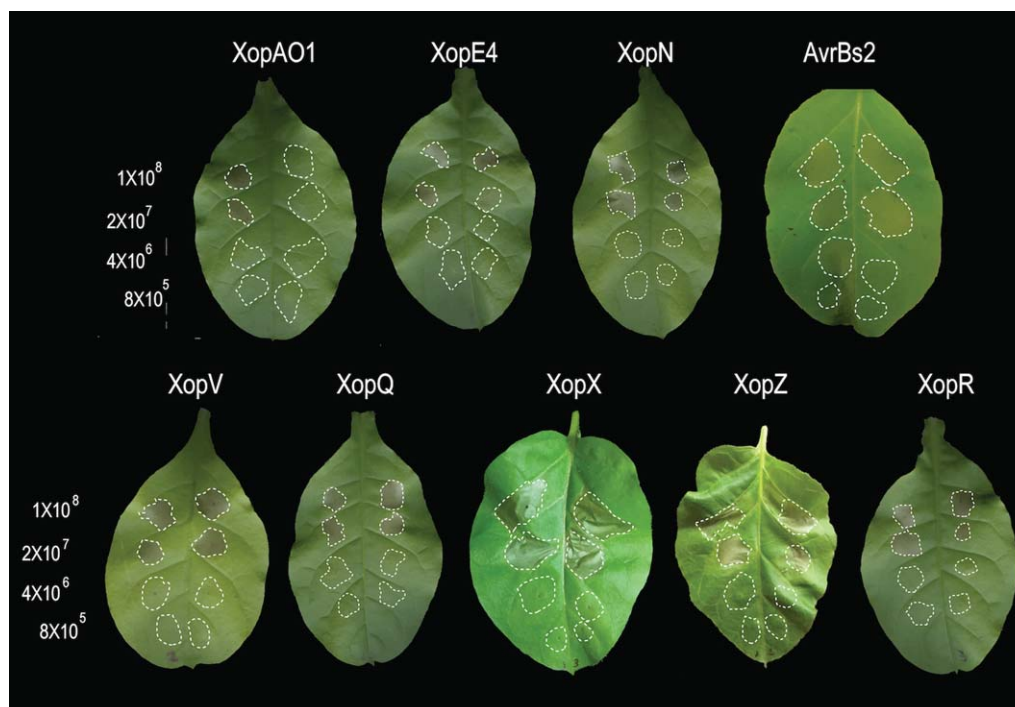
Fig. 3 Ability of type III effectors (T3Es) from *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) strain CIO151 to suppress pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). (A) Representative images of callose deposition in *Arabidopsis thaliana* ecotype Col-0 leaves infiltrated with *Pseudomonas fluorescens* 55 (*Pf*55)(pLN1965) carrying an empty vector (pML123) or constructs that expressed different T3Es. (B) Average number of callose deposits per square millimetre. Error bars correspond to ±1 standard deviation (SD) in an experiment with 10 replicates. A non-parametric Kruskal–Wallis test ($P < 0.05$) was performed to determine significant differences. Different letters correspond to statistically significantly different treatments based on Dunnett's test ($\alpha = 0.05$).

measuring their ability to suppress PAMP-induced callose deposition. To this end, we used *Pseudomonas fluorescens* 55 (*Pf*55), a non-pathogenic bacterium capable of eliciting PTI in *Arabidopsis* Col-0 plants (Guo *et al.*, 2009). *Pf*55 harbours a functional *Pseudomonas syringae* pv. *syringae* T3SS in the cosmid pLN1965 (Guo *et al.*, 2009). T3Es from *Xam* CIO151 were cloned into the plasmid pLN615 and expressed using *Pf*55(pLN1965) (Guo *et al.*, 2009). The ability of each *Xam* T3E to suppress callose deposition was tested by comparing the amount of callose deposits induced by *Pf*55(pLN1965) with *Pf*55(pLN1965) strains also expressing individual *Xam* T3Es (Fig. 3A). *Pf*55(pLN1965) induced an average of 300 callose deposits/mm^2 (Fig. 3A, B). *Pf*55(pLN1965) expressing XopZ, XopX, XopQ, XopE4 or XopV elicited the formation of similar amounts of callose deposits in Col-0, suggesting that these T3Es are not able to suppress PTI under the assayed conditions. Interestingly, *Pf*55(pLN1965) also expressing XopR, AvrBs2 or XopAO1 showed a statistically significant reduction in callose deposits compared with *Pf*55(pLN1965) (Fig. 3A, B). These results suggest that three of the nine *Xam* CIO151 effectors tested in this study have the ability to suppress PTI in this heterologous system. XopN was a variable suppressor of callose deposition in that it showed reduction in certain replicates, but not in others. There was no statistically significant difference between *Pf*55 expressing XopN and the strain harbouring the empty vector, and therefore XopN was not considered as a suppressor of PTI.

Only XopE4 and XopAO1 from *Xam* CIO151 have the ability to suppress ETI

We tested whether these *Xam* T3Es were able to suppress ETI. Again, the heterologous *Pf*55 system was used to measure the ability of T3Es from *Xam* CIO151 to suppress an HR in *Nicotiana tabacum* cv. Xanthi. We used the methodology described by Guo *et al.* (2009) with modifications (see Experimental procedures). *Pf*55(pHIR11) expressing a functional *P. syringae* type III system and the HopA1 T3E elicited a strong HR at 48 h post-inoculation at two cell densities [1×10^8 and 2×10^7 colony-forming units (CFU)/mL]. *Xam* T3Es AvrBs2, XopN, XopQ, XopV, XopR, XopX and XopZ were unable to suppress the HopA1-induced HR when these T3Es were individually expressed in *Pf*55(pHIR11) (Fig. 4), suggesting that these effectors do not act as ETI suppressors under the conditions tested in this study. Co-inoculations with XopE4 showed a variable HR at lower bacterial cell densities (2×10^7 CFU/mL), suggesting that it is a weak suppressor of ETI. Leaf tissue inoculated with *Pf*55(pLN1965) expressing XopAO1 showed no HR even at the highest cell density used. This suggests that XopAO1 is a strong suppressor of HopA1-induced HR in tobacco (Fig. 4). These results suggest that XopE4 and XopAO1 are able, at different strengths, to suppress HopA1-induced ETI in *N. tabacum* cv. Xanthi.

Guo *et al.* (2009) suggested a classification for T3Es that are capable of suppressing HopA1-dependent HR in tobacco at different cell densities. In this classification, T3Es that suppress HR at

Fig. 4 Hypersensitive response (HR) suppression ability of different type III effectors (T3Es) from *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) strain CIO151. *Nicotiana tabacum* cv. Xanthi plants were infiltrated on the left side of the leaves with *Pseudomonas fluorescens* 55 (*Pf*55) pHIR11 strains carrying an empty vector (pML123) and, on the right side, with *Pf*55 pHIR11 mixed with *Pf*55 strains expressing different T3Es. All infiltrations were performed at cell densities of 1×10^8, 2×10^7, 4×10^6 and 8×10^5 cells/mL (from the top). Leaves were evaluated at 48 h post-inoculation. The experiment was repeated three times with five biological replicates, each time with similar results.

high cell densities (1×10^8 cells/mL) are classified as class I; class II T3Es suppress HR at 2×10^7 cells/mL, and class III suppressors are variable in their ability to suppress HopA1-dependent HR at 2×10^7 cells/mL. Class IV T3Es are unable to suppress HR at 2×10^7 cells/mL. In our results, XopE4 would be considered as a class III suppressor, whereas XopAO1 would be classified as a class I suppressor. Because AvrBs2, XopN, XopQ, XopV, XopR, XopX and XopZ T3Es were unable to suppress HopA1-induced HR, they belong to class IV (Fig. 4).

DISCUSSION

Cassava bacterial blight, caused by *Xam*, is a disease occurring worldwide in cassava-growing regions. Different mechanisms have been proposed to control CBB, including cultural practices, sanitary methods and biological control (Lozano, 1986, Fanou and Wydra, 2014). However, the use of resistant varieties seems to be the most efficient method to control CBB (Verdier *et al.*, 2004). In order to deploy durable resistance, it is important to understand the population genetics of the pathogen (Restrepo *et al.*, 2004; Trujillo *et al.*, 2014a,b) and the virulence factors that are crucial for the pathogen to cause disease (Lopez and Bernal, 2012). Ideally, for plant breeding approaches, resistance should be targeted against T3Es that are broadly conserved in the pathogen

and that are crucial for bacterial fitness (Boyd *et al.*, 2013). Here, we have determined the importance of nine different *Xam* T3Es in virulence and suppression of plant defence (Table 1). We found four T3Es individually important for full virulence (XopZ, XopX, XopAO1 and AvrBs2) and a redundant role in virulence for XopN and XopQ. We identified three T3Es that suppressed PTI (XopR, AvrBs2 and XopAO1) and two T3Es (XopE4 and XopAO1) that suppressed ETI. XopV did not show any role in virulence or suppression of PTI or ETI. However, a redundant function of this T3E might be uncovered by generating double mutants with other effectors and by testing other possible roles in the interaction with plants. These findings will not only help in our understanding of *Xam* biology, but will also be the basis for a more effective deployment of cassava resistant varieties targeting T3Es important for disease development.

In this study, single, markerless KO strains were generated for nine T3Es. Our findings demonstrate the importance of four genes in full virulence: XopZ, XopX, AvrBs2 and XopAO1 (Fig. 1A, B). The importance of XopX and AvrBs2 in virulence in *Xam* is supported by the recent report from Mutka *et al.* (2016), which was published during the revision of our manuscript. Our results are also in agreement with previous reports for other pathosystems. For example, XopZ is important in virulence for *Xanthomonas*

Table 1 Summary of type III effectors tested in this study.

Name	Alternative name*	Homologue*	iANT code	Domains	Role in virulence‡‡	PTI suppression‡‡	ETI suppression‡‡
AvrBs2		XCV0052‡, XAC0076†	xanmn_chr02_0062	Glycerophosphoryl diester phosphodiesterase (Zhao *et al.*, 2011)	Yes	Yes	No
XopR		XCV0285‡, XAC0277†	xanmn_chr02_0199	CC-like motif (Akimoto-Tomiyama *et al.*, 2012)	No	Yes	No
XopX		XCV0572‡	xanmn_chr03_0215	No	Yes	No	No
XopV		XCV0657‡, XAC0601†	xanmn_chr03_0302	No	No	No	No
XopZ	HopAS1	XCV2059‡, XAC2009†	xanmn_chr06_0393	SMC domain**	Yes	No	No
XopAO1§		*Xg* and *Xap*¶	xanmn_chr06_5019	Poly(ADP-ribosyl) polymerase**	Yes	Yes	Yes
XopN	HopAU	XCV2944‡, XAC2786†	xanmn_chr09_5006	No	Yes§§	No	No
XopQ	HopQ	XCV4438‡, XAC4333†	xanmn_chr15_5505	No	Yes§§	No	No
XopE4	HopX (AvrPphE)		xanmn_pla04_0019	Myristoylation†† (Thieme *et al.*, 2007)	No	No	Yes

*Data taken from Xanthomonas.org.
†Homologue in *Xanthomonas axonopodis* pv. citri *Xac*.
‡Homologue in *Xanthomonas euvesicatoria* (*Xeu*).
§There is another copy of XopAO, named XopAO2, in *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) strain CIO151.
¶Present in *Xanthomonas gardneri* (*Xp*) and *Xanthomonas arboricola* pv. *pruni* (*Xap*) MAFF 301420.
**Not evaluated experimentally.
††Myristoylation signal predicted by http://mendel.imp.ac.at/myristate/SUPLpredictor.htm.
‡‡Data obtained in this work.
§§Role in virulence with redundant effect.

oryzae pv. *oryzae* (*Xoo*) PXO99, as a KO in both copies of this gene showed a reduction in virulence and lesion length in rice (Song and Yang, 2010). The *xopX* KO in *Xanthomonas euvesicatoria* (*Xeu*) showed reduced growth and symptom development in *Capsicum annuum* (Metz *et al.*, 2005) and *Solanum lycopersicum* (Stork *et al.*, 2015), compared with the wild-type. In addition, AvrBs2 has been reported to be important for virulence in pepper and rice (Kearney and Staskawicz, 1990; Li *et al.*, 2015). AvrBs2 is considered to be a core T3E in *Xanthomonas* spp. and has been widely proposed as a key target for the development of resistant varieties in tomato (Horvath *et al.*, 2012), mainly because Bs2 resistance has been maintained over many years of field trials (Dangl *et al.*, 2013). Finally, *Xam* T3E XopAO1 has a high identity (61%) to AvrRpm1. AvrRpm1 is important for virulence in *Pseudomonas syringae* pv. *maculicola* M2 on susceptible *Arabidopsis* accessions (Ritter and Dangl, 1995). The effector XopAO1 is relatively new in the genus *Xanthomonas*, as it has only been described in *Xam* (Arrieta-Ortiz *et al.*, 2013), *Xanthomonas gardneri* (Potnis *et al.*, 2011) and *Xanthomonas arboricola* pv. *pruni* MAFF 301420 (GI:573458294). Deletion of this gene results in a reduction in the ability to cause symptoms and to grow *in planta* in *Xam* CIO151.

Previous work has described a functional redundancy between T3Es, and this redundancy has been demonstrated only by poly-T3E deletions (Cunnac *et al.*, 2009; Kvitko *et al.*, 2007, 2009). In *Pseudomonas syringae* pv. *phaseolicola*, only one of 15 single effector mutants showed a reduction in virulence (Macho *et al.*, 2012). Although single KOs of some T3Es in *Pseudomonas syringae* pv. *tomato* result in a reduction in virulence, stronger phenotypes were observed when poly-mutants were generated (Cunnac *et al.*, 2011; Kvitko *et al.*, 2009). In *Xam*, four of nine effectors showed an important role in virulence, which suggests a lower redundancy among T3Es. To test for redundant functions among T3Es in *Xam*, we produced strains with double deletions in different combinations of these genes (Fig. S3). Interestingly, we found a redundant function in virulence in the double Δ*xopN* Δ*xopQ* mutant. XopN has been reported to be an important virulence factor in other *Xanthomonas* spp. (Cheong *et al.*, 2013; Jiang *et al.*, 2008). It interacts with a 14-3-3 (TFT1) protein and with the Tomato Atypical Receptor-Like Kinase 1 (TARK1) in *S. lycopersicum* (Kim *et al.*, 2009). It is interesting that XopN from *Xam* CIO151 and from *Xeu* strain 85-10 are closest in distance by hierarchical grouping when compared with homologues from other *Xanthomonas* spp. (Fig. S4, see Supporting Information). Moreover, the protein residues (L64, L65 and S688) required for binding to TFT1 and TARK1 in *Xeu* XopN (Taylor *et al.*, 2012) are conserved in XopN from *Xam*. In a similar manner, XopQ interacts with the 14-3-3 isoform SlTFT4 from *S. lycopersicum* (Teper *et al.*, 2014) and HopQ, a protein homologous to XopQ present in *P. syringae*, interacts with 14-3-3 proteins TFT1 and TFT5

(Li W *et al.*, 2013). 14-3-3s constitute a protein family with phosphobinding domains (Chevalier *et al.*, 2009), involved in diverse metabolic processes (Cotelle and Leonhardt, 2015; Denison *et al.*, 2011), including plant defence (Lozano-Duran and Robatzek, 2015). A BLAST search for tomato 14-3-3 proteins in the cassava genome (https://phytozome.jgi.doe.gov) retrieved 21 results. The best reciprocal hit with TFT1 was Manes.18G060900.1, with 86.7% identity and 97.6% similarity. It is possible that TFT1 is the target of both XopN and XopQ from *Xam*, explaining the redundancy in virulence observed in our experiments. Interestingly, *xopN* is a core effector gene in *Xam*, conserved in 65 genomes sequenced with different geographical and temporal origins (Bart *et al.*, 2012). *xopQ*, however, although present in most genomes, is a pseudogene in 8% of the genomes examined. This might have occurred because of the presence of *xopN*, which could fulfil the same function in a more efficient manner. Alternatively, but not exclusively, XopQ might be recognized by NLRs present in cassava populations, therefore exerting a negative selection pressure on strains expressing it.

The main virulence role demonstrated for T3Es in plant-pathogenic bacteria is the suppression of plant immunity (White *et al.*, 2009). We have identified XopAO1, XopR and AvrBs2 from *Xam* as suppressors of PTI, measured by the suppression of callose deposition. We have also identified XopE4 and XopAO1 as suppressors of ETI, measured by the suppression of HopA1-elicited HR. These results are in agreement with previous reports in other pathosystems. For example, XopR from *Xoo* has previously been shown to be important in the suppression of PTI in *Arabidopsis* (Akimoto-Tomiyama *et al.*, 2012), and AvrBs2 from *Xanthomonas oryzae* pv. oryzicola suppresses the expression of genes related to PTI in rice (Li *et al.*, 2015). However, this is the first report of XopAO1 in *Xanthomonas* as a PTI suppressor, and it would be interesting to determine whether this is the case in *X. gardneri* or *X. arboricola* pv. *pruni*. We hypothesize that the major virulence role of XopAO1 and AvrBs2 from *Xam* is a result of their ability to suppress ETI and/or PTI. However, their targets and activities in cassava cells are unknown. T3Es suppressing both PTI and ETI have been reported previously (Guo *et al.*, 2009; Schulze *et al.*, 2012). This could be a result of T3Es targeting shared components in both layers of plant immunity (Thomma *et al.*, 2011). Finally, the plant immune system is considered to be a co-evolutionary process in which PTI appears as a first defence layer and ETI as a second defence layer (Jones and Dangl, 2006). Therefore, it is most common to find T3Es that suppress PTI, e.g. XopAO1, XopR and AvrBs2. However, there are only a few cases of T3Es suppressing ETI without affecting PTI. One is HopD1 (Block *et al.*, 2014), which has been proposed as 'new' or more recently acquired in the co-evolutionary process because of its ability to exclusively suppress ETI. XopE4 from *Xam* falls in the same category, and it is therefore possible that it is a more

recently acquired effector in the co-evolution between *Xanthomonas* and plants. XopE4 has an N-terminal myristoylation motif (Thieme *et al.*, 2007). It would be interesting to determine whether this motif is required for its ability to suppress HR.

Our results show that several *Xam* T3Es do not have a detectable role in PTI or ETI suppression, at least under the conditions tested in this report. These are XopZ, XopN, XopQ, XopV and XopX. Previous reports have shown that XopN from *Xeu* reduces callose deposition in *Arabidopsis* (Kim *et al.*, 2009); however, a different system to test callose suppression was used (*P. syringae* pv. *tomato* DC3000 ΔCEL). This strain still expresses a few T3Es that might help to suppress PTI. It is possible that, with these suppressors, a weak suppressor like *Xam* XopN (Fig. 3) could become detectable. Conversely, some effectors of *Xam* may be poorly translocated by the T3SS of *P. syringae* pv. *syringae* 61 used here. A previous report has confirmed the secretion of TALEs from *Xanthomonas* by the *P. syringae* T3SS inserted in *Pf*55 (Fujikawa *et al.*, 2006), suggesting that divergence in the two systems does not preclude the secretion of *Xanthomonas* effectors. In addition, a few effectors had a measurable outcome on plant tissues both in the present study and that of Fujikawa *et al.* (2006), which is indicative of a functional translocation of *Xanthomonas* effectors by this heterologous system. However, further confirmation of the secretion of effectors that were negative for both PTI and ETI suppression should be performed in order to rule out this possibility.

Another apparent discrepancy with previous studies was observed with XopZ. XopZ of *Xoo* PXO99 causes a reduction in callose deposition in *Nicotiana benthamiana* (Song and Yang, 2010); however, this difference could be caused by the use of different host plants or the divergence between these two XopZ homologues (81.4% identity). It has been proposed that effectors co-evolve with their targets (Win *et al.*, 2007). Hence, it is possible that the inability of several *Xam* T3Es to suppress HR in tobacco is because these proteins cannot effectively act on their targets in this plant as a result of sequence and, possibly, structural divergence from those present in cassava. Sequences of *Xam* effectors show some divergence from those reported in other *Xanthomonas* species, although it would be difficult to quantify the effects that this divergence might have in their functionality in a heterologous system.

XopAO1 is an important T3E for *Xam* virulence in cassava and for the suppression of PTI and ETI. This effector has only been found in *X. gardneri*, *X. arboricola* pv. *pruni* and *Xam* so far, and it has a high identity with AvrRpm1 (Potnis *et al.*, 2011). AvrRpm1 possesses a catalytic triad (H63, Y122, N185) at the poly(ADP-ribosyl) polymerase (PARP) domain, which is important for its role in virulence and activation of the resistance protein RPM1 (Cherkis *et al.*, 2012). A multiple sequence alignment using diverse AvrRpm1 and XopAO1 amino acid sequences shows that they share a catalytic triad in the PARP domain, suggesting a functional

homology (data not shown). In plants, PARPs are involved in different responses in biotic and abiotic stress, and the maintenance of homeostasis (Briggs and Bent, 2011). The PARP catalytic domain binds NAD^+ and shares homology with mono-ADP ribosylating toxins, such as exotoxinA and diphtheria toxin (Cherkis *et al.*, 2012; Gibson and Kraus, 2012). XopAO1 was able to suppress a HopA1-dependent HR at 1×10^8 CFU/mL in a consistent manner. Accordingly, we classified this T3E as a class I suppressor. Similarly, *P. syringae* AvrRpm1 was classified in previous reports as a class I suppressor (Guo *et al.*, 2009). Both XopAO1 and AvrRpm1 are able to suppress HR elicited by HopA1. HR suppression by AvrRpm1 is non-specific (Reuber and Ausubel, 1996); therefore, it is probable that AvrRpm1 and XopAO1 act as general suppressors of HR, probably targeting conserved components of NLR signalling.

AvrBs2 has been shown to be important in bacterial virulence and PTI suppression in *Xam*. However, *avrBs2* is not considered to be a core T3E gene in *Xam* (Bart *et al.*, 2012) because of a premature stop codon in one strain out of 65. Nonetheless, in all 65 *Xam* strains sequenced, *avrBs2* shares a high nucleotide identity (>99%). Therefore, among the effectors studied in this report, AvrBs2 may represent a good target for the generation of resistant cassava varieties, because it is conserved and is important for bacterial virulence. Moreover, AvrBs2 is a conserved effector in *Xanthomonas* spp. (Hajri *et al.*, 2009) (i.e. it is present in almost all pathovars; Roux *et al.*, 2015). Therefore, transforming Bs2 from solanaceous plants into cassava may lead to durable resistance, if other components required for this resistance are present in cassava. Hierarchical clustering shows that AvrBs2 of *Xam* strain CIO151 is more closely related to AvrBs2 proteins of *Xanthomonas translucens* and *Xanthomonas axonopodis* pv. *glycines* than to phylogenetically closer strains, such as *Xeu* strain 85-10 (Fig. S5, see Supporting Information). Moreover, the catalytic sites E304, D306 and H319 for the glycerophosphoryl diester phosphodiesterase domain (GDE) (Zhao *et al.*, 2011) is fully conserved among different AvrBs2 homologues in *Xanthomonas* spp., including *Xam* strains (CIO151, *Xam*668, CFBP 1851 and IBSBF 2539). However, two residues involved in the activation of Bs2 (Gassmann *et al.*, 2000) are not fully conserved in AvrBs2 from *Xam* strains: all sequenced Xam strains show mutations R403Q and A410T, which have been reported in *Xeu* to disrupt AvrBs2 recognition by Bs2. This, and the premature stop codon present in one of the surveyed strains (Bart *et al.*, 2012), could suggest a mechanism to avoid recognition by potential *R* genes in cassava. Therefore, it would be desirable to evaluate the efficiency of Bs2 resistance in cassava. However, because it is well known that AvrBs2 variants arise in nature which render the Bs2–AvrBs2 reaction compatible, it would be important to pyramid this with other sources of resistance to ensure durability in the field.

Among the nine evaluated T3Es, XopV did not appear to have an important role in virulence, PTI or ETI suppression. Interestingly, XopV is a core *Xam* T3E present in all 65 sequenced strains (Bart *et al.*, 2012), which suggests that this T3E is important for bacterial fitness. This observation underscores the importance of experimental testing of bacterial fitness to determine which effectors are desirable as targets for the generation of resistant cassava varieties. It is possible that XopV has a redundant function in virulence with other T3Es from *Xam* CIO151, as observed for XopN and XopQ. It would therefore be important to test redundancy in virulence with other T3Es or other virulence factors. In addition, it would be desirable to test for ETI suppression using other elicitors (Teper *et al.*, 2015). However, it is also possible that T3Es function in roles other than the suppression of plant immunity, which were not explored in our study. These may include the modulation of nutrient metabolism, hormone signalling or pathogen dissemination between plants (Macho, 2016).

Our work highlights the importance of eight T3Es in cassava virulence and suppression of plant immunity. This new knowledge in T3E biology will shed light on efforts to develop new durable resistance to CBB directed towards T3Es with roles in plant immunity. Efforts should be focused on the identification and use of NLRs activated by T3Es that are important for bacterial fitness (Boyd *et al.*, 2013; Dangl *et al.*, 2013). A recent study has identified 1061 proteins as the repertoire of immune-related proteins (IRPs) in the cassava genome, including TIR-NB-ARC-LRR (Toll/interleukin-1 receptor, nucleotide-binding domain, Apaf-1, R protein and CED-4), NB-ARC-LRR or NB-ARC (Soto *et al.*, 2015). It would be interesting to define whether some of these proteins recognize, directly or indirectly, AvrBs2, XopZ, XopAO1 or XopX, and whether this might be the basis for quantitative resistance observed for some cultivars. Although it would be desirable to test the importance of other effectors in fitness under other conditions (field cultivation, for example), the knowledge generated on T3Es in *Xam* could be used to assess the cassava genetic diversity to search for cassava cultivars or wild species with the ability to recognize these T3Es. These corresponding genes could be subsequently introgressed into commercial varieties in order to develop durable resistance to CBB in cassava.

EXPERIMENTAL PROCEDURES

Bacterial strains and plant growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1 (see Supporting Information). *Escherichia coli* was grown in Luria–Bertani (LB) medium; *Xam* was grown in LPGA solid medium (yeast extract, 5 g/L; bacto peptone, 5 g/L; glucose, 5 g/L; agar-agar, 15 g/L) and Phi (Φ) broth (yeast extract, 1 g/L; bacto peptone, 10 g/L; casamino acids, 1 g/L). *Pseudomonas fluorescens* 55 (*Pf*55) was grown in King's B (KB) medium. Antibiotics were added to the media, when pertinent, in the following concentrations: rifampicin, 100 μg/mL; kanamycin, 50 μg/mL; gentamicin,

15 μg/mL; tetracycline, 15 μg/mL; nalidixic acid, 100 μg/mL. Sucrose at 5% was used for selection of double recombinants. Virulence assays were performed on 2-month-old cassava plants, cultivar MCOL2215, grown in a glasshouse at 20–30 °C under a 12-h photoperiod. Callose suppression assays were performed on 8-week-old *Arabidopsis thaliana* Col-0 ecotype grown at 20–22 °C under a 9-h photoperiod. Assays for the suppression of HR were performed on 10-week-old *N. tabacum* cv. Xanthi plants grown at 24 °C under a 16-h photoperiod.

Cloning procedures

Deletions in T3E genes in *Xam* strain CIO151 were made using double crossing over in the suicide plasmid pK18*mobsacB* (Dodds and Rathjen, 2010; Kvitko *et al.*, 2009). Briefly, a 1-kb flanking region on both sides of each T3E gene was cloned in the suicide vector pK18*mobsacB* (Table S2, see Supporting Information). *Xam* was conjugated using triparental mating and the exconjugants were selected by crossing over events, as described previously (Kvitko *et al.*, 2009). *Xam* transformants were confirmed using polymerase chain reaction (PCR) screening with primers flanking the deletion site and then sequencing to validate the T3E gene deletion.

Sequences of the T3E genes were extracted from the iANT database (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/xanmn/xanmn.cgi). T3E gene sequences were amplified under standard PCR conditions using *Pfx* polymerase and were cloned directionally in pENTR/SD/D-TOPO, following the manufacturer's instructions. Clones were confirmed by PCR, restriction digestions and sequencing. T3Es were subcloned into the destination vector pLN615 for callose deposition and HR assays (Table S1) using a Gateway LR reaction. Sequences of destination vector inserts were confirmed and then transformed in *Pf*55(pLN1965). T3E genes were cloned into the broad-host-range vector pBBR1MCS5 under the control of the *lac* promoter for complementation assays. The resulting constructs were transformed by electroporation into *Xam* mutant strains. In cases in which complementation was partial, T3E genes were amplified with their native promoter using primers 150 bp upstream of the start codon and 100 bp downstream of the stop codon. The T3Es were then cloned into the plasmid pUC18-mini-Tn*7*T-Gm (Choi and Schweizer, 2006) and transformed into bacterial mutants.

Virulence assays

As a first measure of virulence, *Xam* T3E mutants were inoculated to record the ability to cause symptoms in cassava cultivar MCOL2215 under glasshouse conditions, as described previously (Restrepo *et al.*, 2000). Cassava leaves of five plants were inoculated by placing a 10-μL drop of a bacterial suspension of 1×10^8 CFU/mL [optical density at 600 nm (OD_{600}) = 0.1] in a 2-mm-diameter hole. Leaf photographs were taken in a Carl Zeiss (Oberkochen, Germany) Stemi™ DV4 Series Stereomicroscope with transmitted light. To quantify the lesion area, typical *Xam* symptoms, such as necrosis and chlorosis around the inoculation point, were considered. Lesions from eight leaves per treatment were measured at 15 dpi using the software Fiji (Schindelin *et al.*, 2012) and the data were plotted. Tukey's HSD tests ($\alpha = 0.05$) were performed. Experiments were performed three times showing similar results.

A second measure of virulence was the bacterial growth in cassava cultivar MCOL2215. For this, bacterial suspensions were infiltrated with 1×10^7 CFU/mL in 10 mM $MgCl_2$ ($OD_{600} = 0.01$), as described previously (Bart *et al.*, 2012). The leaves of three different plants were infiltrated and bacterial growth was tested at 0 and 5 dpi. A 0.5-cm^2 leaf fragment was ground in 10 mM $MgCl_2$ and serial dilutions were plated on LPGA to estimate the bacterial count. The bacterial titre at day 0 did not differ among treatments. Tukey's HSD test ($\alpha = 0.05$) was performed. All reported experiments were performed three times showing similar results.

Assay for the suppression of callose deposition

Callose deposition was assessed using previously described methods (Guo *et al.*, 2009; Jamir *et al.*, 2004). Briefly, each T3E gene from *Xam* CIO151 was cloned into the broad-host-range vector pLN615, a derivative from pML123 (Labes *et al.*, 1990). The resulting clone was transformed by electroporation into *Pf*55(pLN1965). *Arabidopsis* leaves were inoculated with *Pf*55(pLN1965) expressing *Xam* T3Es at $OD_{600} = 0.01$ (1×10^6 CFU), and the leaves were collected at 16 h post-infiltration (hpi). We used *Pf*55(pLN1965) transformed with pML123 empty vector as a positive control for the induction of callose deposition. Leaves were cleared with a lactophenol alcoholic solution (1 vol of distilled water, lactic acid, phenol and glycerol and 2 vol of ethanol) (Adam and Somerville, 1996). Samples were subsequently boiled at 95 °C for 10 min and rinsed in a 50% ethanol and water solution (McDowell *et al.*, 2011). Cleared leaves were stained with 0.01% (w/v) aniline blue in a 150 mM K_2HPO_4 solution, pH 9.5, for 30 min. Leaf sections were captured in 10× fluorescence microscopy (Nikon Ti, Minato, Tokyo, Japan), and callose deposits were measured using the software Fiji (Schindelin *et al.*, 2012). Graphic-derived data were manually curated. Ten fields were observed per infiltrated leaf and three leaves were observed per treatment. Experiments were repeated three times. Because data did not show a normal distribution, Kruskal–Wallis and Dunnett tests ($P < 0.05$) were performed to determine significant differences.

Assay for the suppression of HR

We used a modified HR assay from Guo *et al.* (2009). Briefly, we used *Pf*55 expressing the pHIR11 cosmid (Huang *et al.*, 1988) harbouring T3SS from *P. syringae* and the effector HopA1 from *P. syringae* pv. *syringae* 61, which elicits an HR in *N. tabacum* cv. Xanthi. *Pf*55(pHIR11) and *Pf*55(pLN1965) expressing individual T3Es were resuspended at 2×10^8 CFU/mL in 5 mM MES (morpholineethanesulfonic acid), pH 5.6. Equal amounts of both strains were mixed and three five-fold serial dilutions were performed (1×10^8, 2×10^7, 4×10^6 and 8×10^5 CFU/mL). Each dilution was infiltrated into *N. tabacum* cv. Xanthi leaves including the control vector: *Pf*55(pHIR11, pML123) co-inoculated with *Pf*55(pLN1965, pML123). Each T3E was inoculated in five biological replicates, with three experiments at different times to assess the ability to suppress HopA1-induced HR. The HR was recorded 48 h after infiltration.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

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

Fig. S1 (A) Lesion area (mm^2) of *xopZ* knockout (KO) and complemented strains. (B) Lesion area (mm^2) of mutant and complemented strains of *xopX*, *xopAO1* and *avrBs2*. Error bars correspond to ± 1 standard error (SE) in an experiment with five replicates. Strains with a different letter are statistically significantly different based on Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).

Fig. S2 Bacterial growth at 5 days post-infiltration (dpi) of knockout (KO) strains in *avrBs2* and *xopX* and corresponding strains complemented with the Tn7 system. Values represent $\log_{10}$ of colony-forming units (CFU) and error bars are ± 1 standard deviation (SD) in an experiment with three replicates. Strains with different letters were statistically significantly different based on Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).

Fig. S3 Lesion area (mm^2) of different single, double and triple knockouts (KOs).

Fig. S4 Alignment and hierarchical clustering of XopN proteins. Amino acid sequences were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/protein/) and iANT (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/xanmn/xanmn.cgi), and were aligned using MUSCLE with default parameters. Numbers at the top right indicate amino acid positions. Hierarchical clustering of full-length amino acid sequences of type III effectors (T3Es) was conducted with MEGA 7.1 (Kumar *et al.*, 2016) using the neighbour-joining

method (Saitou and Nei, 1987), assuming a Poisson substitution model and uniform rates among sites. Pairwise deletion was used to handle sequence gaps, and 1000 bootstrap replicates were performed. Sequences analysed for XopN are: gi|549145689, gi|374351730, gi|78036930, xanmn_chr09_5006, gi|409033098, gi|353460308 and gi|345294984.

Fig. S5 Hierarchical clustering of AvrBs2 proteins and consensus positions between amino acid sequences. Amino acid sequences were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/protein/) and iANT (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/xanmn/xanmn.cgi), and were aligned using MUSCLE with default parameters. Amino acid positions are numbered at the top based on *Xanthomonas euvesicatoria* strain 85-10. Hierarchical clustering of full-length amino acid sequences of type III effectors (T3Es) was conducted with MEGA 7.1 (Kumar *et al.*, 2016) using the neighbour-joining method (Saitou and Nei, 1987), assuming a Poisson substitution model and uniform rates among sites. Pairwise deletion was used to handle sequence gaps, and 1000 bootstrap replicates were performed. Different colours represent different groups of AvrBs2. Sequences analysed for AvrBs2 are: gi|565804782, gi|564594007, gi|325546470, gi|325535564, gi|573459684, gi|410719447, gi|21110996, gi|917793134, gi|582989392, xanmn_chr02_0062, gi|422794743, gi|440370775, gi|917801111, gi|917796707, gi|353460121, gi|188518881, gi|549144016, gi|325542792, gi|346647736, gi|4206159, gi|78034038, gi|292603865, gi|644957664, gi|372554024 and gi|780547033.

Table S1 Bacterial strains and plasmids used in this study.

Table S2 Primers used in this study.