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The type VI protein secretion system contributes to biofilm formation and seed-to-seedling transmission of *Acidovorax citrulli* on melon

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SUMMARY

The type VI protein secretion system (T6SS) is essential for the virulence of several Gram-negative bacteria. In this study, we identified a T6SS gene cluster in *Acidovorax citrulli*, a plant-pathogenic bacterium that causes bacterial fruit blotch (BFB) of cucurbits. One T6SS cluster, of approximately 25 kb in length and comprising 17 genes, was found in the *A. citrulli* AAC00-1 genome. Seventeen *A. citrulli* mutants were generated, each with a deletion of a single T6SS core gene. There were significant differences in BFB seed-to-seedling transmission between wild-type *A. citrulli* strain, xjl12, and Δ*vasD*, Δ*impK*, Δ*impJ* and Δ*impF* mutants (71.71%, 9.83%, 8.41%, 7.15% and 5.99% BFB disease index, respectively). In addition, we observed that these four mutants were reduced in melon seed colonization and biofilm formation; however, they were not affected in virulence when infiltrated into melon seedling tissues. There were no significant differences in BFB seed-to-seedling transmission, melon tissue colonization and biofilm formation between xjl12 and the other 13 T6SS mutants. Overall, our results indicate that T6SS plays a role in seed-to-seedling transmission of BFB on melon.

Keywords: *Acidovorax citrulli*, biofilm formation, seed-to-seedling transmission, T6SS.

INTRODUCTION

Acidovorax citrulli is the causal agent of bacterial fruit blotch (BFB) (Schaad *et al*., 1978, 2008; Willems *et al*., 1992), a serious threat to cucurbit (mainly watermelon and melon) production worldwide. The bacterium is seed borne (Hopkins and Thompson, 2002), and seed transmission and infested/infected seeds represent the most important primary inoculum sources for BFB outbreaks. Hence, for effective BFB management, it is critical to limit seed-to-seedling transmission of *A. citrulli*, which requires an accurate understanding of the host–pathogen interactions underlying this phenomenon. In an effort to better understand *A. citrulli* host–pathogen interactions, Bahar *et al*. (2009) demonstrated that type IV pili and polar flagella play an important role in virulence, twitching motility and biofilm formation. Johnson *et al*. (2009) showed that the *A. citrulli* type III secretion system (T3SS) and, in particular, the *hrcC* gene that encodes the type III pilus protein, are important for pathogenicity, but are not required for the colonization of germinating seeds during the early stages of seed-to-seedling transmission of BFB. The role of T3SS in *A. citrulli* pathogenicity and the ability to induce a hypersensitive response (HR) was also confirmed by the characterization of *hrcV* mutants (Bahar and Burdman, 2010). In contrast, Johnson *et al*. (2009) reported that the type II secretion system (T2SS) contributed to watermelon seed colonization during seed-to-seedling transmission of BFB.

Secreted effector proteins play a central role in the interaction between bacterial phytopathogens and their host plants (Kostakioti *et al*., 2005; Mougous *et al*., 2006). To date, six different secretion systems have been found in Gram-negative pathogenic bacteria (Mougous *et al*., 2006; Pukatzki *et al*., 2006). Currently, the mechanism of the type VI secretion system (T6SS) and the functions of the secreted effectors are poorly understood. In previous studies, T6SS has been shown to enhance the adaptability of bacteria to environmental conditions (Weber *et al*., 2009), to mediate pathogenicity to host cells (Pukatzki *et al*., 2006; Suarez *et al*., 2008; Zheng and Leung, 2007) and to affect other bacterial functions, including biofilm formation (Aschtgen *et al*., 2010), modulation of quorum sensing and stress response in *Vibrio anguillarum* (Weber *et al*., 2009). These functions promote the establishment of commensalistic or mutualistic relationships between bacteria and eukaryotes (Bernard *et al*., 2010; Chow and Mazmanian, 2010; Jani and Cotter, 2010). In addition, Huddleston

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(2011) reported that the T6SS effector proteins, Tse1 and Tse3, degrade the peptidoglycan of other bacteria, conferring a competitive advantage to *Pseudomonas aeruginosa*.

Studies of human and animal pathogens have contributed most to our current understanding of the structure and function of T6SS. However, T6SSs are also prevalent in the genomes of plant pathogens and other plant-associated bacteria, such as *Agrobacterium tumefaciens*, *Pectobacterium atrosepticum* and *Pseudomonas syringae* (Mattinen *et al*., 2007; Records and Gross, 2010; Wu *et al*., 2008). Wang (2008) showed that the deletion of both T6SS gene clusters in *P. syringae* pv. *tomato* DC3000 reduced the bacterium's ability to colonize *Nicotiana benthamiana* leaves and induce bacterial speck symptoms on tomato leaves. When either the T6SS-II or T6SS-III gene cluster was deleted separately, the disease severity and bacterial colonization of plant tissues were reduced. In contrast, deletion of the valine–glycine repeat protein G1 (*vgrG1*) or *vgrG2*, two core T6SS genes, had no effect on disease development on tomato or *N. benthamiana*.

In another study, microarray analysis showed that the T6SS of *Ag. tumefaciens* was induced by mildly acidic conditions, such as those encountered in plant tissues and in the rhizosphere (Yuan *et al*., 2008). It was shown that deletion of haemolysin co-regulated protein (*hcp*), a core T6SS gene, from *Ag. tumefaciens* resulted in reduced tumorigenesis on potato tuber slices (Wu *et al*., 2008). Similarly, Mattinen *et al*. (2007) showed that the T6SS of *Pe. atrosepticum* was induced by potato tuber extracts. Using transcriptome profiling, Liu *et al*. (2008) showed that the T6SS of *Pe. atrosepticum* was regulated by quorum sensing.

Bingle *et al*. (2008) identified the putative cluster of T6SS genes in the *A. citrulli* genome. In their report, the *A. citrulli* T6SS comprised one gene cluster, but lacked a *vgrG* homologue, which raised doubts about the functionality of the system. To date, no experimental evidence has been provided to support the role of T6SS in *A. citrulli* pathogenicity or in seed-to-seedling transmission of the BFB pathogen. In this study, a 17-gene cluster encoding signature T6SS proteins in *A. citrulli* was identified in the AAC00-1 genome sequence. Seventeen core T6SS genes were deleted individually to generate mutants in the *A. citrulli* xjl12 background. Using these mutants, the objective of this work was to investigate the role of the *A. citrulli* T6SS in the seed-to-seedling transmission of BFB in melon.

RESULTS

Acidovorax citrulli has a T6SS cluster

Based on bioinformatics analysis of genome sequences for *P. aeruginosa*, *Vibrio cholerae*, *Edwardsiella tarda*, *Salmonella typhimurium* and *Rhizobium leguminosarum*, one T6SS cluster was found in the AAC00-1 genome. This 25-kb cluster comprised 17 core genes (GenBank Accession Number: NC_008752) (Fig. 1) including homologues of *hcp* (Aave_1465) to *clpB* (Aave_1482) (Table 1).

Effect of T6SS on seed-to-seedling transmission of BFB

Melon seeds (n = 1000) were inoculated with ~1×10^6 colony-forming units (CFU)/mL of *A. citrulli* xjl12 and individual T6SS protein mutants and planted in sterile test cups (three seeds per cup). BFB seed-to-seedling transmission percentage and disease severity were observed daily for 12 days after planting. Wild-type (WT) strain xjl12 caused a mean BFB index of 71.71% by 12 days after planting, whereas mutants Δ*vasD*, Δ*impJ*, Δ*impK* and Δ*impF* caused disease indices of 9.83%, 8.41%, 7.15% and 5.99%, respectively (Fig. 2). In all cases, the differences between the WT strain xjl12 and strains Δ*vasD*, Δ*impJ*, Δ*impK* and Δ*impF* were statistically significant ($P < 0.05$). The complemented strains Δ*vasD*comp, Δ*impJ*comp, Δ*impK*comp and Δ*impF*comp induced mean BFB indices of 66.39%, 64.47%, 67.19% and 59.19%, respectively, by 12 days after planting. There were no significant ($P > 0.05$) differences in the BFB index between WT strain xjl12 and the complemented strains, including Δ*vasD*comp, Δ*impJ*comp and Δ*impK*comp. The disease index of the complemented strain Δ*impF*comp was only partially rescued. The differences in disease indices between the WT strain and the other 13 T6SS mutants were not significant ($P > 0.05$).

Effect of T6SS on *A. citrulli* colonization of melon seeds during germination

The reduction in melon seed-to-seedling transmission of BFB by the *A. citrulli* single T6SS gene mutants Δ*vasD*, Δ*impJ*, Δ*impK* and Δ*impF* prompted the investigation of the role of T6SS in *A. citrulli* colonization of melon seeds during germination. The role of T6SS

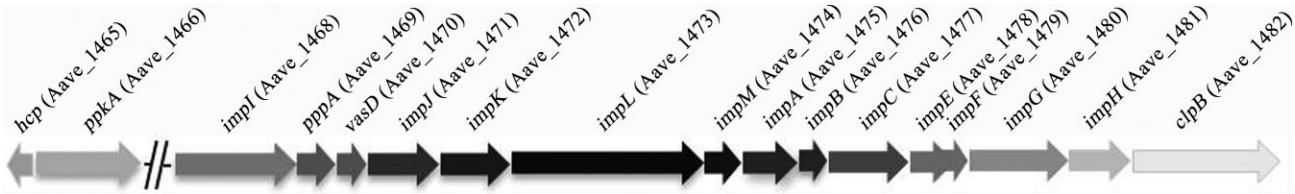

Fig. 1 Arrangement of *Acidovorax citrulli* AAC00-1 type VI secretion system gene cluster indicating the relative position of gene homologues.

Table 1 Type VI secretion system genes in the *Acidovorax citrulli* AAC00-1 genome and homologues in *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Edwarsilella tarda* and *Rhizobium leguminosarum*.

Protein name	AAC00-1 locus tag	*Pseudomonas aeruginosa*	Identity/ homology (%)	*Vibrio cholerae*	Identity/ homology (%)	*Edwardsiella tarda*	Identity/ homology (%)	*Salmonella typhimurium*	Identity/ homology (%)	*Rhizobium leguminosarum*	Identity/ homology (%)	Conserved domain
Hcp	Aave_1465	PA0085/Hcp1	67/162	VCA0117/VasH	29/140	EvpC	47/162	SciK	64/160	Hcp	47/162	DUF796
								SciM	64/160			
PpkA	Aave_1466	PA0074/PpkA		—		—		—		—		PKc
ImpI	Aave_1468	PA0081/Fha1	90/306	VCA0112/VasC	27/84	—		—		ImpI	0	FHA
PppA	Aave_1469	PA0075/PppA	70/208	—		—		—		—		PP2Cc
VasD	Aave_1470	PA0080/Lip1	48/113	VCA0113/VasD	34/128	EvpL	0	SciN	43/111	—		COG3521
ImpJ	Aave_1471	PA0079/HsiJ1	208/443	VCA0114/VasE	178/444	EvpM	116/477	SciO	166/446	ImpJ	192/447	DUF876
ImpK	Aave_1472	PA0078/DotU1	174/382	VCA0115/VasF	72/224	EvpN	35/108	SciP	123/346	ImpK	141/412	DUF2077
ImpL	Aave_1473	PA0077/IcmF1	445/1142	VCA020/VasK	312/1218	EvpO	106/366	SciS	408/1241	ImpL	382/1193	ImcF;DUF1215
ImpM	Aave_1474	PA0076/PppB	44/139	—		—		—		ImpM	41/91	DUF2094
ImpA	Aave_1475	PA0082/HsiA1	120/359	VCA0119/VasJ	15/60	EvpK	17/51	SciA	104/341	ImpA	80/362	ImpA family
ImpB	Aave_1476	PA0083/HsiB1	119/158	VCA0107	0	EvpA	66/157	SciH	117/170	ImpB	64/152	DUF770
ImpC	Aave_1477	PA0084/HsiC1	382/498	VCA0108	184/430	EvpB	232/441	SciI	378/503	ImpC	243/490	COG3517/DUF877
ImpE	Aave_1478	PA0086/HsiE1	113/249	—		—		SciE	93/241	ImpE	65/236	ImpE
ImpF	Aave_1479	PA0087/HsiF1	71/161	VCA0109	0	EvpE	43/152	SciD	62/153	ImpF	21/54	DUF1316
ImpG	Aave_1480	PA0088/HsiG1	306/625	VCA0110/VasA	179/638	EvpF	219/629	SciV	273/628	ImpG	214/624	DUF879
ImpH	Aave_1481	PA0089/HsiH1	146/301	VCA0111/VasB	81/277	EvpG	120/329	SciB	114/290	ImpH	97/322	DUF1305
ClpB	Aave_1482	PA0090/ClpV1	186/463	VCA0116/VasG	277/684	EvpH	290/659	SciG	270/503	ClpB	305/725	ClpN, AAA ATPase

in *A. citrulli* colonization of melon seeds during the early stages of germination was determined by measuring the bacterial populations on artificially inoculated seeds during the initial 96 h of seed germination. Individual seeds were infiltrated with *A. citrulli* xjl12 (WT) or individual T6SS mutants, and bacterial populations per seed were estimated at 24-h intervals for 96 h. The populations of all strains increased on germinating melon seeds by 96 h after planting (Fig. 3). By 48 h after planting, the mean populations of xjl12, Δ*vasD*, Δ*impJ*, Δ*impK* and Δ*impF* were ~8.91×10^6, ~3.80×10^4, ~1.35×10^4, ~5.25×10^5 and ~4.57×10^4 CFU/g of seed, respectively. By 96 h after planting, the mean populations of xjl12, Δ*vasD*, Δ*impJ*, Δ*impK* and Δ*impF* were ~4.27×10^9, ~4.17×10^5, ~1.29×10^5, ~3.24×10^6 and ~1.05×10^5 CFU/g of seed, respectively. Based on the analysis of AUPDC (area under population dynamics curve) data, there were significant differences between the abilities of Δ*vasD*, Δ*impJ*, Δ*impK* and Δ*impF* to colonize seed relative to the WT strain ($P < 0.05$). There was no significant difference in the colonization of melon seed between xjl12, Δ*vasD*comp, Δ*impJ*comp and Δ*impK*comp ($P > 0.05$). However, populations of Δ*impF*comp remained at ~2.51×10^6 CFU/g of seed for 48 h and ~1.74×10^8 CFU/g of seed for 96 h. This suggested that the colonization of melon seed by Δ*impF*comp was only partially rescued.

T6SS is involved in *A. citrulli* biofilm formation

The quantification of the biofilm confirmed that *A. citrulli* xjl12 (WT) produced significantly ($P < 0.05$) more biofilm than the T6SS mutants Δ*vasD*, Δ*impJ*, Δ*impK* and Δ*impF*. Optical density (OD) values for stained biofilms for xjl12 (WT), Δ*vasD*, Δ*impJ*, Δ*impK* and Δ*impF* were 2.20, 0.29, 0.10, 1.07 and 0.12, respectively (Fig. 4). In addition, biofilm formation by Δ*impK* was significantly ($P < 0.05$) higher than that of Δ*impJ* and Δ*impF* (Fig. 4). There was no significant difference in biofilm formation between the WT strain xjl12, Δ*vasD*comp, Δ*impJ*comp and Δ*impK*comp ($P > 0.05$). In contrast, complementation of strain Δ*impF* only partially rescued WT biofilm formation. There were no significant ($P > 0.05$) differences in biofilm formation between the other single T6SS gene mutants and the WT strain.

DISCUSSION

Bacterial pathogens use a range of different secretion systems to deliver virulence factors into host cells. Recently, a novel secretion system was discovered in several Gram-negative bacterial pathogens and was designated as T6SS (Das and Chaudhuri, 2003). T6SSs have been shown to play a role in the virulence of a range of animal and plant pathogens, including *V. cholerae*, *P. aeruginosa*, *Ed. tarda*, *Salmonella enterica* serovar *gallinarum*, avian pathogenic *Escherichia coli*, *Ag. tumefaciens*, *Pe. atrosepticum*, *Xanthomanas oryzae* and *P. syringae* (Aschtgen

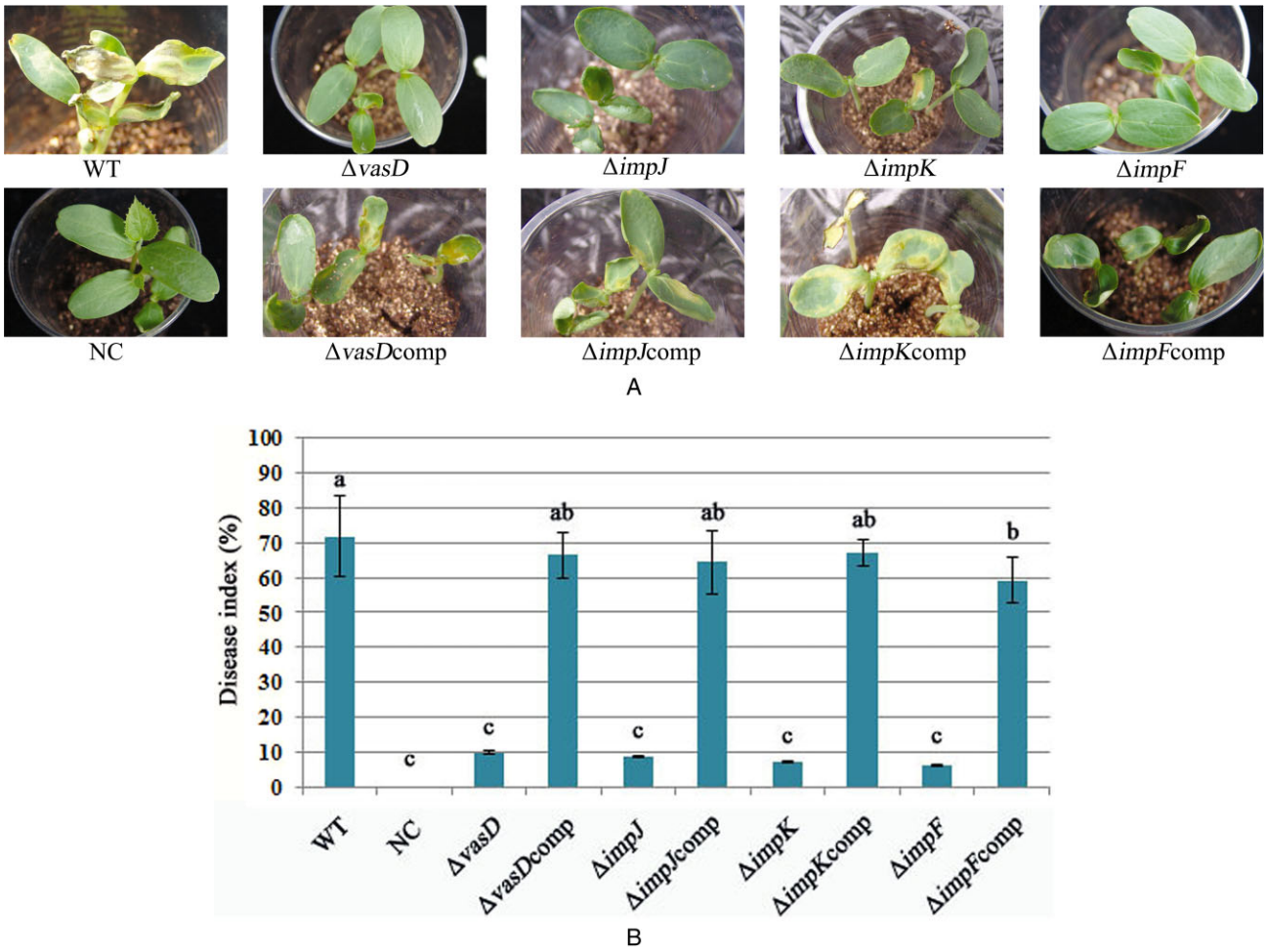

Fig. 2 Effects of type VI secretion system (T6SS) on seed-to-seedling transmission of *Acidovorax citrulli*. Melon seeds (cv. Huanghou, $n = 1000$) were inoculated with ~1×10^6 colony-forming units (CFU)/mL of wild-type or T6SS mutants, and three seeds were planted per sterile test cup. Seedlings were evaluated 12 days after planting and disease severity was evaluated based on a 0–5 scale. The experiment was repeated three times. WT, wild-type strain of *A. citrulli*, xjl12; NC, negative control, double-distilled H_2O (ddH_2O); Δ*vasD*, *vasD* gene deletion mutant of *A. citrulli*; Δ*vasD*comp, complemented strain of Δ*vasD*; Δ*impJ*, *impJ* gene deletion mutant of *A. citrulli*; Δ*impJ*comp, complemented strain of Δ*impJ*; Δ*impK*, *impK* gene deletion mutant of *A. citrulli*; Δ*impK*comp, complemented strain of Δ*impK*; Δ*impF*, *impF* gene deletion mutant of *A. citrulli*; Δ*impF*comp, complemented strain of Δ*impF*. Vertical bars represent standard errors of the means. Different letters above the data bars indicate a significant difference between the wild-type strain and T6SS mutants, complemented strains or negative control ($P < 0.05$, *t*-test).

et al., 2010; Blondel *et al*., 2010; Filloux *et al*., 2008; Hsu *et al*., 2009; Mattinen *et al*., 2007; Records and Gross, 2010; Shrivastava and Mande, 2008; Tseng *et al*., 2009; Wu *et al*., 2008; Zheng and Leung, 2007). However, the exact mechanism of T6SS and the contribution of T6SS effectors to virulence remain to be elucidated. T6SSs are typically encoded by clusters of 12 to more than 20 genes; however, the minimal number of genes needed to produce a functional apparatus is 13 (Boyer *et al*., 2009). In *V. cholerae* and *P. aeruginosa*, the T6SS exports haemolysin co-regulated proteins (Hcp) and valine–glycine repeat (Vgr) proteins (Mougous *et al*., 2006; Pukatzki *et al*., 2006). These proteins have been proposed to act as effectors associated with cytotoxicity in some *in vitro* models (Mougous *et al*., 2006; Pukatzki *et al*., 2006). Hcp1, which forms hexameric rings that assemble into nanotubes *in vitro*, plays an important role in the pathogenicity of *P. aeruginosa* (Mougous *et al*., 2006). In the current study, however, deletion of *hcp* did not reduce *A. citrulli* virulence or the seed-to-seedling transmission of BFB on melon. The *A. citrulli* mutant xjl12Δ*hcp* colonized melon cotyledons and induced BFB symptoms at WT levels. It is possible that the Hcp protein requires an inducible signal for it to be active, as observed with T6SS in *Ag. tumefaciens* (Wu *et al*., 2012). More detailed studies are needed to prove that *hcp* is secreted by *A. citrulli*.

In the current study, a 17-gene cluster encoding signature T6SS proteins in *A. citrulli* was identified in the AAC00-1 genome. Seventeen core T6SS genes were deleted individually to generate

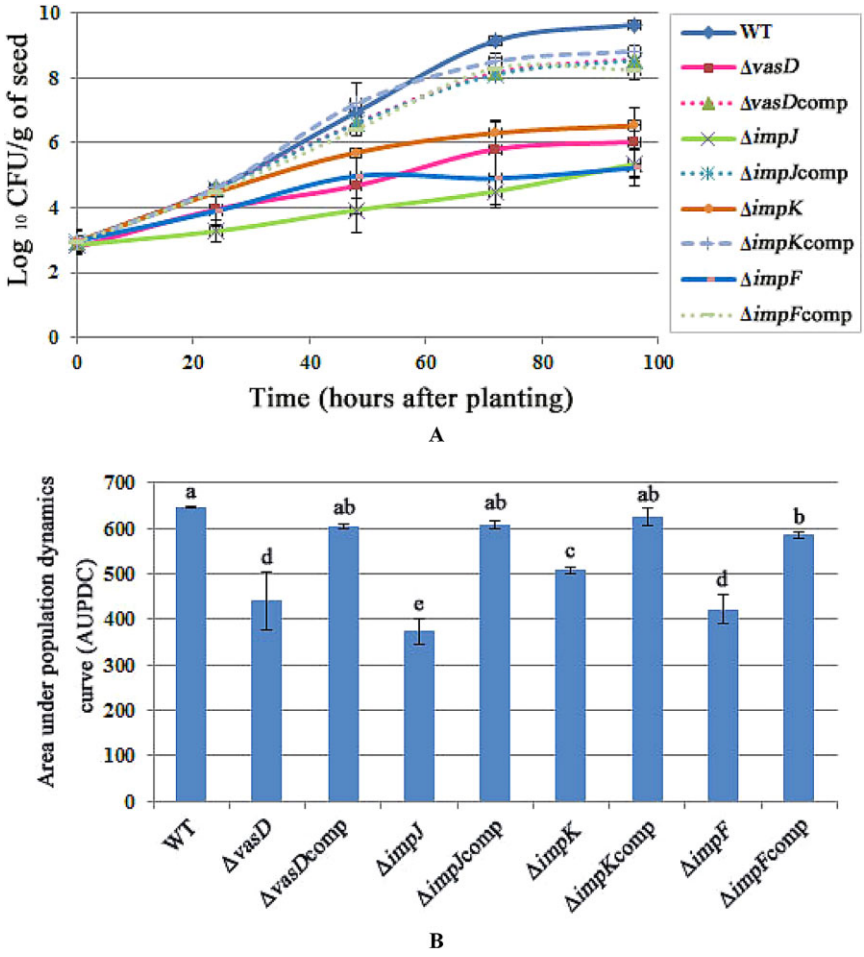

Fig. 3 (A) Effect of type VI secretion system (T6SS) on colonization of germinating melon seeds by *Acidovorax citrulli*. (B) Bar chart of area under population dynamics curve (AUPDC) calculated for seed infiltrated with 10^3 colony-forming units (CFU) of wild-type or T6SS mutants. WT, wild-type strain of *A. citrulli*, xjl12; Δ*vasD*, *vasD* gene deletion mutant of *A. citrulli*; Δ*vasD*comp, complemented strain of Δ*vasD*; Δ*impJ*, *impJ* gene deletion mutant of *A. citrulli*; Δ*impJ*comp, complemented strain of Δ*impJ*; Δ*impK*, *impK* gene deletion mutant of *A. citrulli*; Δ*impK*comp, complemented strain of Δ*impK*; Δ*impF*, *impF* gene deletion mutant of *A. citrulli*; Δ*impF*comp, complemented strain of Δ*impF*. Vertical bars represent standard errors of the means. Different letters above the data bars indicate a significant difference between the wild-type strain and mutants, complemented strains or negative control ($P < 0.05$, *t*-test).

mutants in an *A. citrulli* xjl12 background. Of these mutants, four (Δ*vasD*, Δ*impK*, Δ*impJ* and Δ*impF*) were impaired in seed-to-seedling transmission of BFB in melon and in biofilm formation. Our results showed that the *sciN*-like *vasD* (Aave_1470) gene is required for biofilm formation of *A. citrulli* on glass surfaces. Aschtgen *et al*. (2008) showed that SciN is an outer membrane lipoprotein that is exposed to the periplasmic space of enteroaggregative *E. coli*, as revealed by the inhibition of its processing by globomycin and *in vivo* labelling with [^{3}H]palmitic acid. Lipoproteins have been identified in some secretion systems, including T2SS, T3SS and T4SS, as having a large number of subunits (Allaoui *et al*., 1992; D'Enfert and Pugsley, 1989; Fernandez *et al*., 1996; Schuch and Maurelli, 2001; Shevchik and Condemine, 1998). In all of these secretion systems, lipoproteins are essential components and have been shown to be involved in secretion machine assembly. Aschtgen *et al*. (2008) also demonstrated that SciN is critical for biofilm formation. In addition, mutations in the T6SS gene cluster have been found to be involved in biofilm formation for *V. parahaemolyticus* and *P. aeruginosa* (Enos-Berlage *et al*., 2005; Sauer *et al*., 2002; Southey-Pillig *et al*., 2005).

Another *A. citrulli* T6SS protein, ImpJ (Aave_1471), is homologous (40%) to the *V. cholera* TssK-like VCA0114 protein, which has been identified as a trimeric cytoplasmic protein that interacts with components of phage-like and membrane anchoring complexes of the T6SS (Zoued *et al*., 2013). VCA0114 has also been shown to be critical for a functional T6SS (Zheng *et al*., 2011). The current study suggests that ImpJ is important for *A. citrulli* biofilm formation and seed-to-seedling transmission, even though its specific functions are unknown.

A *V. cholerae* T6SS protein identified as VCA0109 has sequence homology to T4 gp25, which forms part of bacteriophage tail baseplates (Leiman *et al*., 2009). In addition, the VCA0109-like protein, HsiF1, has been shown recently to localize to the cytoplasm of *P. aeruginosa* (Lossi *et al*., 2011). Furthermore, VCA0109 has been shown to be essential for sheath biogenesis in *V. cholerae* (Basler *et al*., 2012). Although detailed characterization studies must be conducted, our results show that the VCA0109-like ImpF (Aave_1479) is required for *A. citrulli* biofilm formation on glass surfaces and for seed-to-seedling transmission. Interestingly, to our knowledge, this is the first report to associate T6SS with seed-to-seedling transmission of bacterial plant

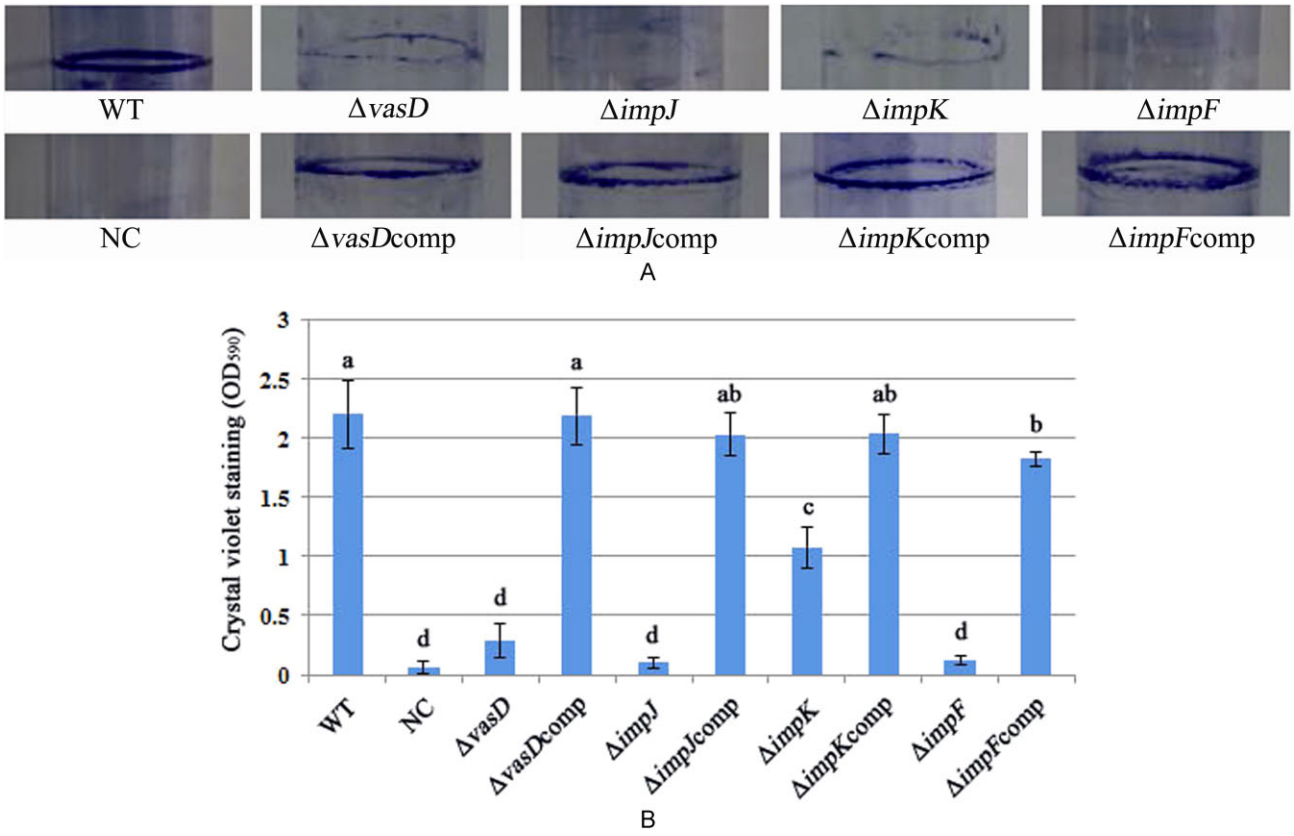

Fig. 4 Effect of type VI secretion system (T6SS) on *Acidovorax citrulli* biofilm formation. (A) Images of biofilms formed in glass tubes after staining with methyl violet. (B) Biofilm was quantified after washing the methyl violet stain with ethanol and measuring the optical density at 590 nm. The experiment was repeated three times. WT, wild-type strain of *A. citrulli*, xjl12; NC, negative control, double-distilled H_2O (ddH_2O); Δ*vasD*, *vasD* gene deletion mutant of *A. citrulli*; Δ*vasD*comp, complemented strain of Δ*vasD*; Δ*impJ*, *impJ* gene deletion mutant of *A. citrulli*; Δ*impJ*comp, complemented strain of Δ*impJ*; Δ*impK*, *impK* gene deletion mutant of *A. citrulli*; Δ*impK*comp, complemented strain of Δ*impK*; Δ*impF*, *impF* gene deletion mutant of *A. citrulli*; Δ*impF*comp, complemented strain of Δ*impF*. Vertical bars represent standard errors of the means. Different letters above the data bars indicate a significant difference between the wild-type strain and T6SS mutants, complemented T6SS mutant strains or negative control ($P < 0.05$, *t*-test).

pathogens. In this study, complementation of strain Δ*impF* only partially rescued the WT strain, which might be due to the expression vector contains *impF* extrachromosomally and *impF* forms part of bacteriophage tail baseplates. Future detailed characterization of *A. citrulli* T6SS should provide important information to understand how it is assembled and how it functions, particularly with regard to how it contributes to seed-to-seedling transmission of BFB.

We observed that the *A. citrulli* AAC00-1 T6SS cluster lacks a *vgrG* homologue. However, by bioinformatics analysis, 12 genes encoding putative VgrG proteins (*Aave_0481*, *Aave_3347*, *Aave_0497*, *Aave_2840*, *Aave_3486*, *Aave_2127*, *Aave_4009*, *Aave_2047*, *Aave_2735*, *Aave_0241*, *Aave_3752* and *Aave_3783*) were observed (Table S2, see Supporting Information) throughout the genome. VgrG proteins are structurally similar to T4 bacteriophage tail spike proteins, gp27 and gp5 (Pukatzki *et al*., 2007). The existence of multiple copies of putative *vgrG* genes in the AAC00-1 genome is interesting, as it is unclear why a bacterium might have so many copies of this gene. Many bacteria have several copies of *vgrG* (Hachani *et al*., 2011; Smits *et al*., 2010). However, the presence of these putative *vgrG* homologues may explain why *A. citrulli* has a functional T6SS, even though the T6SS cluster does not include *vgrG*. The functionality of these homologues will be explored in future studies.

In many pathogens, biofilm formation is crucial for disease development (Merz *et al*., 1999). Type IV pili and polar flagella have been shown to be involved in biofilm formation of *A. citrulli* using microfluidic flow chambers (Bahar *et al*., 2009). Preliminary observations indicating that *A. citrulli* grows as an epiphytic, non-pathogen on seeds during the initial stages of seed germination prompted us to investigate the role of T6SS in the putative switch to pathogenic growth on emerging melon seedlings. Based on our observations that BFB seed-to-seedling transmission and biofilm formation are reduced for *A. citrulli* T6SS mutants Δ*vasD*, Δ*impK*, Δ*impJ* and Δ*impF*, it is possible that biofilm formation might be critical for *A. citrulli* colonization of melon seed tissues during the

early stages of germination. A similar phenomenon has been reported for the avian pathogenic *E. coli* (De Pace *et al*., 2011) based on the observation that an *icmF* mutant displayed decreased adherence to and invasion of epithelial cells, as well as decreased intra-macrophage survival. In addition, the *icmF* mutant was defective in biofilm formation on abiotic surfaces (De Pace *et al*., 2011). It is possible that this reduced ability to form a biofilm leads to a decrease in the disease index in the seed-to-seedling BFB transmission assay. Overall, our results provide evidence that *A. citrulli* has a functional T6SS for biofilm formation and seed-to-seedling transmission of BFB on melon. To our knowledge, this is the first report of the effect of T6SS on seed-to-seedling transmission of a phytopathogenic bacterium.

EXPERIMENTAL PROCEDURES

Bacterial cultures, media and inoculum preparation

The bacterial strains and plasmids used in this study are described in Table 2. *Acidovorax citrulli* was grown at 28 °C on Luria–Bertani (LB) agar or broth (Sambrook *et al*., 1989) and *E. coli* was grown on LB agar or broth at 37 °C. Minimal medium (MMX) (Daniels *et al*., 1984) was routinely used as minimal medium and, when required, the culture media were supplemented with the following antibiotics: kanamycin (Km), 50 mg/mL; rifampicin (Rif), 100 mg/mL; gentamicin (Gm), 50 mg/mL.

Generation of T6SS mutants of *A. citrulli*

Strain descriptions are given in Table 2. Deletion mutants of T6SS genes were generated by the *sacB*-based allele replacement method, as described previously (Johnson *et al*., 2011; Zou *et al*., 2011). Deletion mutants were confirmed by polymerase chain reaction (PCR) amplification using primers flanking the genes of interest. Primer sequences used for mutant construction are listed in Table S1 (see Supporting Information). The resulting constructs were introduced into *E. coli* S17-1(λpir) for use in biparental mating with *A. citrulli* strain xjl12. For complementation, the coding regions of these genes were amplified by PCR using the primers listed in Table S1, and cloned into the expression vector pUFR034. The resulting constructs were transferred into the corresponding mutants by biparental mating. BLASTN, BLASTX, BLASTP sequence homologies and conserved protein domain analyses were performed using the National Center for Biotechnology Information (NCBI) BLAST. Protein sequences were analysed using the NCBI database.

Effect of T6SS on seed-to-seedling transmission of BFB

To determine the effect of T6SS on seed-to-seedling transmission of BFB, melon seeds (cv. Huanghou, n = 1000 seeds) were inoculated by

Table 2 Strains and plasmids used in this study. All complemented strains were generated in the *Acidovorax citrulli* strain xjl12 background.

Bacteria, plasmids	Relevant characteristics*	Source
Acidovorax citrulli		
xjl12	Wild-type, Rif^R	Laboratory collection
Δ*hcp*	*hcp* knockout mutant	This study
Δ*ppkA*	*ppkA* knockout mutant	This study
Δ*impI*	*impI* knockout mutant	This study
Δ*pppA*	*pppA* knockout mutant	This study
Δ*vasD*	*vasD* knockout mutant	This study
Δ*vasD*comp	Δ*vasD* complemented with intact *vasD* gene, Km^R	This study
Δ*impJ*	*impJ* knockout mutant	This study
Δ*impJ*comp	Δ*impJ* complemented with intact *impJ* gene, Km^R	This study
Δ*impK*	*impK* knockout mutant	This study
Δ*impK*comp	Δ*impK* complemented with intact *impK* gene, Km^R	This study
Δ*impL*	*impL* knockout mutant	This study
Δ*impM*	*impM* knockout mutant	This study
Δ*impA*	*impA* knockout mutant	This study
Δ*impB*	*impB* knockout mutant	This study
Δ*impC*	*impC* knockout mutant	This study
Δ*impE*	*impE* knockout mutant	This study
Δ*impF*	*impF* knockout mutant	This study
Δ*impF*comp	Δ*impF* complemented with intact *impF* gene, Km^R	This study
Δ*impG*	*impG* knockout mutant	This study
Δ*impH*	*impH* knockout mutant	This study
Δ*clpB*	*clpB* knockout mutant	This study
Escherichia coli		
DH5α(λpir)	*Φ80 lacZΔM15,Δ(lacZYA-argF)U169. recA1, endA1.thi-1*	TaKaRa, Dalian, China
S17-1(λpir)	*Λpir pro hsdR, recA*	Simon *et al*. (1983)
Plasmids		
pMD19-T	'TA' cloning vector	TaKaRa
pEX18GM	Suicide vector with a *sacB* gene, Gm^R	Hoang *et al*. (1998)
pUFR034	Broad-host-range cloning vector, *IncW*, Km^R, *Mob*$^+$, *Mob(p)*, *lacZ*alpha,	DeFeyter *et al*. (1990)

*Rif^R, Gm^R and Km^R indicate resistance to rifamycin, gentamicin and kanamycin, respectively.

immersion in 200 mL of a cell suspension containing ~1×10^6 CFU/mL of *A. citrulli* strain xjl12 and each T6SS deletion mutant for 2 h. Seeds treated in a similar manner with sterile double-distilled H_2O served as a negative control. After inoculation, seeds were air dried at room temperature for 24 h and then planted, three seeds per sterile test cup (Wuhao, Nanjing, China), in soil-less potting medium (Xingnong, Zhenjiang, China) saturated with deionized water. Seeds were incubated at 100% relative humidity and 28 °C for 12 days. The proportion of seedlings displaying BFB symptoms was recorded daily and the experiment was repeated three times. Each seedling was also evaluated for BFB severity daily based on the disease index, as described previously (Araujo *et al*., 2005), with the following modifications. The disease severity scale ranged from '0' to '5': 0, no symptoms; 1, necrotic lesions on approximately 25% of the cotyledons; 2, necrotic lesions on approximately 50% of the cotyledons; 3, necrotic lesions on approximately 75% of the cotyledons; 4, necrotic lesions on approximately 100% of the cotyledons; and 5, total death of the seedling. The disease index (DI) was calculated each day based on the following formula:

$$DI = \sum (A \times B) \times 100 / \sum B \times 5$$

where A is the disease class (0, 1, 2, 3, 4 or 5) and B is the number of plants showing that disease class per treatment.

Effect of T6SS on *A. citrulli* colonization of germinating melon seeds

The role of T6SS in *A. citrulli* seed colonization during the early stages of seed germination was determined by infiltrating melon seeds (cv. Huanghou) with ~1×10^3 CFU of each T6SS mutant strain or double-distilled H_2O as a negative control, as described previously (Walcott *et al*., 2006). After inoculation, melon seeds (n = 25) were incubated in transparent plastic boxes (Wuhao) on moist blotter paper (Whatman, England, UK) at 28 °C with 100% relative humidity. Three seeds were collected at 0, 24, 48, 72 and 96 h after planting, and bacterial populations were estimated by macerating each seed separately in 900 μL of double-distilled H_2O in a sterile microcentrifuge tube; 100 μL aliquots of appropriate 10-fold serial dilutions of each seed homogenate were spread onto LB agar plates with appropriate antibiotics and incubated for 48–96 h at 28 °C. Subsequently, the *A. citrulli* colonies were counted. The experiment was repeated five times and plots of $\log_{10}$ bacterial CFU/seed against time were used to calculate AUPDC values (Bjarko and Line, 1988) as follows:

$$AUPDC = \sum_{i=1}^{n} [(Yi + 1 + Yi)/2][Xi + 1 - Xi]$$

where Y_i is the $\log_{10}$ bacterial population at the ith observation, X_i is the time in hours at the ith observation and n is the total number of observations. These data were then used to determine the significance of the effects of T6SS on *A. citrulli* colonization of germinating melon seeds.

Effect of T6SS on *A. citrulli* biofilm formation

Biofilm assays were performed on T6SS mutants of *A. citrulli* using glass tubes, as described by Aschtgen *et al*. (2010), with the following modifications. Briefly, cell suspensions (overnight broth cultures adjusted to OD_{590} = 1.0) of *A. citrulli* strain xjl12 and each T6SS mutant were diluted 1:99 in LB broth in glass tubes and incubated at 28 °C for 48 h without agitation. Cultures were then poured out and tubes were rinsed three times with sterile double-distilled H_2O. Following fixation at 80 °C for 20 min, biofilms were stained with 1% crystal violet for 45 min and then washed three times with double-distilled H_2O. For quantification, biofilms were dissolved in 3 mL of 95% ethanol for 2 h, and OD_{590} of the stained suspension was measured using a spectrophotometer (Biophotometer, Eppendorf, Hambug, Germany).

Statistical analyses

All analyses were conducted using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). The *t*-test (P = 0.05) was used to determine significant differences in disease severity, bacterial growth (AUPDC) and biofilm formation among WT and T6SS mutants of *A. citrulli*.

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SUPPORTING INFORMATION

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

Table S1 Oligonucleotide primers used in this study.

Table S2 Valine–glycine repeat G (VgrG) proteins in the *Acidovorax citrulli* AAC00-1 genome and homologues in *Vibrio cholerae*, *Edwardsiella tarda* and *Rhizobium leguminosarum*.