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Type III secretion system genes *hrcJ* and *hrpE* affect virulence, hypersensitive response and biofilm formation of group II strains of *Acidovorax citrulli*

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Bacterial fruit blotch (BFB) caused by *Acidovorax citrulli* is a worldwide threat to watermelon and melon production. The type III secretion system (T3SS) plays an important role in the virulence of *A. citrulli* in its host plants and to induce hypersensitive response (HR) in its non-host plants. Little is known, however, about the contribution of the T3SS to biofilm formation in *A. citrulli*. We mutated two T3SS-related genes *hrcJ* and *hrpE*, respectively, and compared the mutants with their wild-type strain Aac-5 of *A. citrulli*, and their complementation strains on virulence, HR, and biofilm formation. Both mutants significantly reduced virulence in watermelon and melon seedlings and their ability to induce HR in tobacco leaves. Such reduction phenotypes were significantly recovered to the wild-type level, when the mutant strains were complemented with the wild-type *hrcJ* and *hrpE* genes. Interestingly, the two T3SS-related gene mutants also displayed enhanced ability to form biofilm, suggesting a different role of biofilm in the virulence of the group II stains of *A. citrulli*.

KEYWORDS

***Acidovorax citrulli*, group II strains, type III secretion system, biofilm formation, virulence, hypersensitive response**

Introduction

Bacterial pathogens share common strategies to infect and colonize their hosts. One such strategy is to deliver type III effector proteins into host cells *via* the type III secretion system (T3SS) (Abramovitch and Martin, 2004) to overcome the defense response of their host. The T3SS is encoded by the *hrp* (HR and pathogenicity) cluster, which contains over 20 genes including *hrp* and *hrc* (HR and conserved) genes that encode a type III secretion tunnel, as well as *avr* (avirulence) and *hop* (Hrp-dependent out

protein) genes that encode effector proteins (Staskawicz et al., 2001). In the *hrp* cluster, *hrcJ* is a key factor of virulence on a host and of HR on a non-host in *Xanthomonas oryzae* pv. *oryzicola* (Zhao et al., 2010). The HrcJ apparatus is located across the inner and outer membrane, likely held by its hydrophobic domain at the C-terminal and lipid moiety at the N-terminal. It is important for secretion of harpins, which are virulence factors that target the extracelluar space of plant tissues (Choi et al., 2013). The *hrpE* encodes a major component of a surface appendage named the Hrp pilus (Weber and Koebnik, 2005), which is involved in secretion of effector proteins. Bacterial pathogen *X. oryzae* pv. *oryzae* was unable to elicit HR on non-hosts and showed reduced virulence on its host when either *hrcJ* or *hrpE* was absent (Cho et al., 2008).

Bacterial fruit blotch is one of the most devastating diseases infecting watermelon and other melons, and poses a serious threat to cucurbit production worldwide (Yan et al., 2012). It is caused by the gram-negative bacterium *Acidovorax citrulli* that has been divided into two major groups: group I strains are more pathogenic to melon, while group II strains are highly aggressive on watermelon (Walcott et al., 2004; Yan et al., 2012). Additionally, group II strain W1 is reportedly unable to form biofilm, while group I strain M6 was observed to be able (Bahar et al., 2009). Recently, whole genome of representative strains belonging to Group II (AAC00-1, accession number NC_008752.1) and Group I [pslb65 (Wang et al., 2015a) and tw6 (Wang et al., 2015b)] were sequenced. Genomic analysis of the AAC00-1 strain isolated from the U. S. revealed that it contains a ∼30 kb *hrp* cluster and genes coding for putative type III-secreted effectors.

Like other gram-negative plant-pathogenic bacteria, *A. citrulli* may also rely on the T3SS to translocate virulence proteins from the bacterial cell into the cytoplasm of the host plant cell (Johnson et al., 2011). Virulence of the *A. citrulli* strain AAC00-1 on watermelon was abolished when the *hrcC* gene was mutated (Johnson et al., 2011). The *hrcV* mutants generated in the background of group I strain M6 and group II strain W1 were unable to induce HR on non-host plant (tomato) and showed impaired virulence on their host plant (melon), suggesting the importance of T3SS in *A. citrulli* (Bahar and Burdman, 2010). Biofilm formation is required for causing disease symptoms of *X. citri* on lemon leaves (Rigano et al., 2007), and T3SS is necessary for the biofilm formation of *X. citri* (Zimaro et al., 2014). Biofilm formation is also critical for virulence in group I strain M6 of *A. citrulli*, since its mutants unable to form biofilm showed significantly impaired virulence on watermelon (Bahar et al., 2009). Little is known, however, about whether the T3SS affects biofilm formation in group II strains of *A. citrulli*. To investigate the role of the T3SS on biofilm formation in the group II strains of *A. citrulli*, we constructed the T3SS-related *hrcJ* and *hrpE* gene mutants and their complementation strains, and compared to their wild-type strain Aac-5, a group II strain isolated from watermelon in China.

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *A. citrulli* strains were grown in King's B broth (KB) (Walcott et al., 2000) or on KA plate (KB containing 15 g/L agar) with appropriate antibiotics at 28°C. *Escherichia coli* strains were grown in Luria Bertani (MacLean et al., 2006) broth or plate with appropriate antibiotics at 37°C. Antibiotics used in this study were rifampicin (Rif), ampicillin (Ap), gentamicin (Gm), chloramphenicol (Cm) 20 $\mu g \cdot ml^{-1}$ and kanamycin (Km) at concentrations of 100 $\mu g\ ml^{-1}$ for ampicillin, 20 $\mu g \cdot ml^{-1}$ for chloramphenicol and 50 $\mu g\ ml^{-1}$ for the other antibiotics.

Construction of the *hrcJ* and *hrpE* mutants and the complemented mutant strains

The *hrcJ* and *hrpE* genes in the wild-type strain Aac-5 were inactivated by homologous integration as described by Windgassen et al. (2000), respectively, using the suicide vector pK18mobsacB (Schäfer et al., 1994). Primers for PCR amplification of the two genes were designed using the free online program Primer 3.0 (http://www.simgene.com/Primer3) (Table 2). Each reaction mixture contained 0.5 μl of DNA template, 6.25 μl of 2×PCR Mix (TaKaRa, Dlian, China) and 0.5 μl of each primer for a total reaction volume of 12.5 μl. The PCR conditions were 94°C for 3 min, 30 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 90 s, followed by 72°C for 5 min. The 1,791-bp fragment of Aac-5 amplified by the hrcJ-up-F and hrcJ-dn-R primers (Table 2) contained an 864-bp coding region of the *hrcJ* gene, as well as 455- and 472-bp upstream and downstream sequences of the gene. The 1,962-bp fragment of Aac-5 amplified by the hrpE-up-F and hrpE-dn-R primers (Table 2) contained an 834-bp coding region of the *hrpE* gene, as well as 515- and 613-bp upstream and downstream sequences of the gene. After confirmation by sequencing, the fragments were digested by *Eco*RI and *Hind*III, as well as by *Eco*RI and *Sma*I, respectively, and cloned into pK18mobsacB to create plasmids pK18-*hrcJ* and pK18-*hrpE* (Table 1). The two plasmids were digested with *BamH*I and *Nde*I, and the *hrcJ* and *hrpE* gene regions were replaced with a *Gm* gene cassette (855 bp), respectively, to create plasmid pK18-*hrcJ*Gm and pK18-*hrpE*Gm (Table 1). The pK18-*hrcJ*Gm and pK18-*hrpE*Gm were introduced from *E. coli* DH5α into Aac-5, respectively, by triparental conjugation using pRK600 as a helper plasmid. Transconjugants were screened

TABLE 1 Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Acidovorax citrulli		
Aac-5	Wild-type, group II, isolated from watermelon in China, Rif^{Ra}, Amp^{R}	This study
Δ*hrcJ*	Aac-5 derived *hrcJ* deletion mutant. An 864-bp *hrcJ* was replaced with an 855-bp Gm cassette, Rif^{R}, Gm^{R}	This study
Δ*hrpE*	Aac-5 derived *hrpE* mutant. An 834-bp *hrpE* was replaced with an 855-bp Gm cassette, Rif^{R}, Gm^{R}	This study
Δ*hrcJ*-comp	*hrcJ* complementation strain. Δ*hrcJ* containing pBBR1MCS-2-hrcJ, Rif^{R}, Gm^{R}, Km^{R}	This study
Δ*hrpE*-comp	*hrpE* complementation strain. Δ*hrpE* containing pBBR1MCS-2-hrpE, Rif^{R}, Gm^{R}, Km^{R}	This study
Escherichia coli		
DH5α	*Φ*80 *lacZ* Δm15, *rec*A1, containing pRK600 plasmid	TakaRa (Dalian, China)
pRK600	Helper strain in triparental matings, Cm^{R}	(Zhao et al., 2010)
Plasmids		
pBRR1MCS-2	Broad host range complementation vector, Km^{R}	This study
pK18mobsacB	Cloning and suicide vector, sacB+, Km^{R},	This study
pK18-hrcJ	pK18mobsacB containing *hrcJ*, Km^{R}	This study
pK18-hrpE	pK18mobsacB containing *hrpE*, Km^{R}	This study
pK18-ΔhrcJGm	pK18mobsacB containing truncated *hrcJ* with an 864-bp *hrcJ* replaced by an 855-bp Gm cassette, Km^{R}, Gm^{R}	This study
pK18-ΔhrpEGm	pK18mobsacB containing truncated *hrpE* with an 834-bp *hrpE* replaced by an 855-bp Gm cassette, Km^{R}, Gm^{R}	This study
pBR1MCS-2-hrcJ	pBRR1MCS-2 containing a full-length *hrcJ* gene, Km^{R}	This study
pBR1MCS-2-hrpE	pBRR1MCS-2 containing a full-length *hrpE* gene, Km^{R}	This study

[a]Rif ^{R}, Amp^{R}, Gm^{R}, Km^{R}, and Cm^{R} indicate resistance to rifampicin, ampicillin, gentamycin, kanamycin and chloramphenicol, respectively.

TABLE 2 PCR primers used in this study.

Primers	Sequence (5′-3′, restriction enzyme sites are underlined)	Product of PCR
hrcJ-up-F	CG GAATTC GCGGTAGCCCACCCAGGA (*EcoR* I)	1791 bp
hrcJ-dn-R	C AAGCTT CGCTCACCAATCCCTTCG (*Hind* III)	
hrpE-up-F	CG GAATTC CGGCGGGTGCGAAGATG (*EcoR* I)	1962 bp
hrpE-dn-R	CCC AAGCTT GCTGGCCTGTGAACTG (*Sma* I)	
Gm-F	TCC CCCGGG GACGCACACCGTGGAAA (*Sma* I)	855 bp
Gm-R	GC TCTAGA GCGGCGTTGTGACAATTT (*Xba* I)	
hrcJ-F	GCTCTAGATCATCCCGCGAGTCCC	864 bp
hrcJ-R	CCCAAGCTTATGACGCATGACACGC	
hrpE-F	CGGGATCCTCATGCATCGTCATCC	834 bp
hrpE-R	CAAGCTTATGCTGATCTGGTCTTCT	

on KB supplemented with 10% sucrose and antibiotics (Rif, Ap and Gm) and confirmed by PCR using the hrcJ-F/hrcJ-R and hrpE-F/hrpE-R primers, respectively. To confirm the presence of the *Gm* cassette in the transconjugants, Southern blotting was performed with primers Gm-F/Gm-R using marker BM5000 (Biomed, 5,000 bp, 3,000 bp, 2,000 bp, 1,000 bp, 750 bp, 500 bp, 250 bp, 100 bp) as the probe. The confirmed *hrcJ* and *hrpE* mutant strains, Δ*hrcJ* and Δ*hrpE* (Table 1), was used for subsequent studies.

To generate complementation strains, the *hrcJ* and *hrpE* genes (864 bp and 834 bp, respectively) in Aac-5 were amplified using primers hrcJ-F/hrcJ-R and hrpE-F/hrpE-R, respectively (Table 2). The gene fragments were cloned separately into pBBR1MCS-2 to generate pBR1MCS-2-*hrcJ* and pBR1MCS-2-*hrpE* (Table 1), which were transferred into the mutant strains Δ*hrcJ* and Δ*hrpE* by triparental conjugation, respectively. Transconjugants named *hrcJ*-comp and *hrpE*-comp were identified through screening on KB [amended with Rif, Km and Gm (Table 1)]. All obtained plasmids and *A. cirulli* strains were confirmed by PCR and DNA sequencing.

HR assays

To prepare bacterial inocula, *A. citrulli* strains were grown in KB for 24 h at 28°C, and their OD_{600} was adjusted to 0.3 (~10^{8} CFU ml^{-1}). Tobacco plants (*Nicotiana benthamiana*) were grown at room temperature (25°C) with a 12-h photoperiod and

used when they were 6-week-old. HR assays were performed based on the method of Johnson et al. (2011) by infiltrating a fully expended tobacco leaf with 100 μl of the bacterial inoculum into the leaf area among the midrib and the lateral veins. Three leaves from different tobacco seedlings were infiltrated for each strain. Sterile water was used as a negative control. Infiltrated tobacco leaves were kept at 100% humidity at room temperature. The HR-associated cell death was recorded 24 h after inoculation.

Virulence assays

The bacterial inocula was prepared as described above. For inoculation, two hundred milliliters of each inoculum were sprayed to all leaves of ten 3 to 4-week-old melons (*Cucumis melo* cultivar IVF, provided by Insitute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China) and watermelon (*Citrullus lanatus* cultivar Jingxin#6, provided by Beijing Academy of Agriculture and Forestry Sciences, Beijing, China) seedlings. Inoculated seedlings were kept in the greenhouse at 25–30°C and 80% relative humidity. Disease index (DI) was recorded 8 days after inoculation according to the method of Tian et al. (2015) with a modified disease severity scale. The disease severity in each seedling was rated as follows: 0, no symptoms; 1, 3, and 5, necrotic lesions on 25, 50 and 75% of the leaves, respectively; 7, necrotic lesions on ∼100% of the leaves; and 9, complete death of the seedlings. The DI for each treatment was calculated based on the following formula:

$$\bullet DI = \sum (A \times B) \times 100 / \sum C \times 9$$

where A is the disease severity rating (0, 1, 3, 5, 7, or 9), B is the number of seedlings associated with each disease severity rating, and C is the total number of seedlings used for each strain in an experiment. The experiment was repeated three times.

Measurement for biofilm formation

Biofilm formation was measured based on a previous method (Aschtgen et al., 2008). *A. citrulli* strains were grown overnight in KB and adjusted to OD_{600} of 1.0 with sterile water. Each cell suspension was diluted by a factor of 100 with KB (without any antibiotics) in a glass flask and incubated at 28°C for 7 days without agitation. The cell suspensions were then poured out slowly and the glass tubes were rinsed gently three times with sterile distilled water. The biofilm formed on the inner wall of the glass tubes was fixed by heating at 80°C in an oven (Memmert, Schwabach, Germany) for 50 min, stained with 1% crystal violet for 2 h, and washed three times with sterile distilled water. The stained biofilm was then dissolved in 3 ml of absolute ethanol for 12 h, and measured quantitatively at OD_{570} using a spectrophotometer (Biophotometer, Eppendorf, Hambug, Germany). The experiment was repeated three times.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test in the Excel 2010 software (Microsoft Inc., Seattle, WA, USA). Differences were considered statistically significant if $P < 0.05$.

Results

Confirmation of the *hrcJ* and *hrpE* mutants and complementation strains

PCR amplification of the *hrcJ* and *hrpE* mutant strain Δ*hrcJ* and Δ*hrpE* with the hrcJ-up-F/hrcJ-dn-R and hrpE-up-F/hrpE-dn-R primers and the subsequent sequencing of the PCR products confirmed that strains Δ*hrcJ* and Δ*hrpE* contained truncated *hrcJ* and *hrpE* genes replaced by the Gm cassette, respectively (Figures 1A,B). The presence of the Gm cassette (855 bp) in Δ*hrcJ* and Δ*hrpE* was further confirmed by Southern blot and was absent from the wild-type strain Aac-5 (Figures 2A,B). The Δ*hrcJ* and Δ*hrpE* strains were stable after continuous culturing for 20 generations in KB medium. The fact that the *hrcJ* and *hrpE* complementation strain Δ*hrcJ*-comp and Δ*hrpE*-comp were Km^R suggested the successful transfer of the plasmid pBR1MCS-2-*hrcJ* and pBR1MCS-2-*hrpE* into the Δ*hrcJ* and Δ*hrpE* strains, respectively. The presence of pBR1MCS-2-*hrcJ* and pBR1MCS-2-*hrpE* in Δ*hrcJ*-comp and Δ*hrpE*-comp was further confirmed by PCR using hrcJ-F/hrcJ-R and hrpE-F/hrpE-R primers, respectively, since only one PCR band was amplified as expected from each complementation strain. An 864-bp fragment from Δ*hrcJ*-comp and a 834-bp band from Δ*hrpE*-comp were amplified from pBR1MCS-2-*hrcJ* and pBR1MCS-2-*hrpE*, respectively, while no bands were amplified from the mutant strains Δ*hrcJ* and Δ*hrpE* (Figures 1A,B).

Effect of *hrcJ* and *hrpE* on HR in tobacco

Twenty-four hours after infiltrating tobacco leaves with *A. citrulli* strains, typical and obvious HR necrotic lesions were observed where the infiltration was done with the wild-type strain Aac-5 (Figures 3A3,B3). Much like in the water control (Figures 3A1,B1), no such lesions were observed in areas of tobacco leaves infiltrated with the *hrcJ* and *hrpE* mutant strains Δ*hrcJ* and Δ*hrpE*, respectively (Figures 3A2,B2). When the Δ*hrcJ* and Δ*hrpE* mutant strains were complemented, however, similar necrotic HR lesions were observed in areas of tobacco

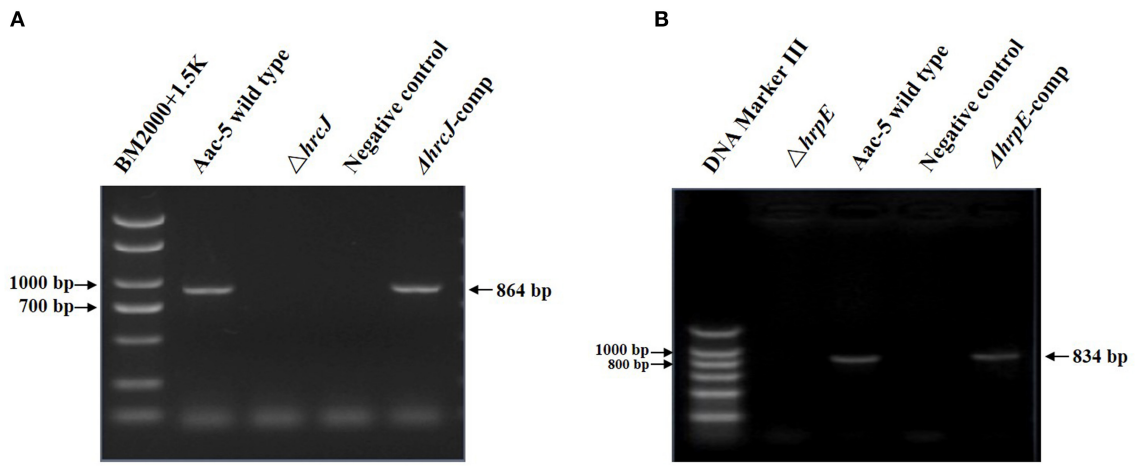

FIGURE 1
PCR results for confirmation of the *hrcJ* **(A)** and *hrpE* **(B)** mutants and complementation strains. **(A)** An 864-bp band of *hrcJ* gene was present in the lane of Aac-5 wild type and Δ*hrcJ*-comp, while was absent in the lane of Δ*hrcJ* and negative control (ddH_2O as template). **(B)** An 834-bp band of *hrpE* gene was present in the lane of Aac-5 wild type and Δ*hrpE*-comp, while was absent in the lane of Δ*hrpE* and negative control.

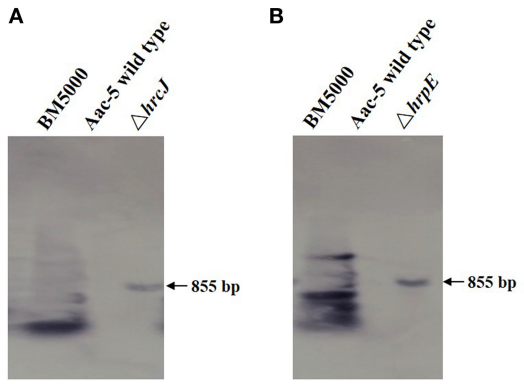

FIGURE 2
Southern blot for confirmation the presence of Gm in mutant strains of *A. citrulli*. The Δ*hrcJ* **(A)** and Δ*hrpE* **(B)** strain showed an 855-bp band on gel, while the wild type strain showed no band, indicating the mutant strains harbored a Gm cassette.

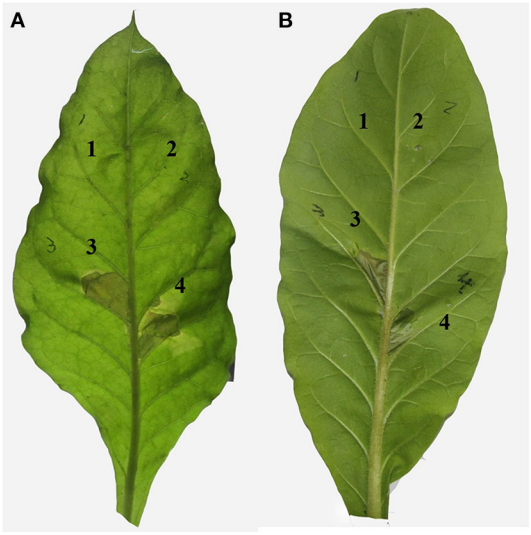

FIGURE 3
Effect of *hrcJ* **(A)** and *hrpE* **(B)** on hypersensitive response in tobacco (*Nicotiana benthamiana*) leaves infiltrated with water (1), *hrcJ* or *hrpE* mutant strain Δ*hrcJ* or Δ*hrpE* (2), wild-type strain Aac-5 (3), and the complementation strain Δ*hrcJ*-comp or Δ*hrpE*-comp (4).

leaves infiltrated with the complementation strains *hrcJ* -comp and *hrpE* -comp, as compared to those with the wild-type strain Aac-5 of *A. citrulli* (Figures 3A4,B4).

Effect of *hrcJ* and *hrpE* on virulence of *A. Citrulli* in watermelon and melon seedlings

Eight days after inoculation, more than 60% of leaves on watermelon seedlings inoculated with the wild-type strain Aac-5 developed necrotic lesions with a mean DI of 62.67% (Figures 4A,C). Even though this group II strain was not as virulent on melon as it was on watermelon, it still caused an average DI of 23.11% on melon (Figures 4B,D). When the *hrcJ* and *hrpE* genes were mutated, DIs caused by the mutant strains Δ*hrpE* and Δ*hrcJ* were 28.44 and 27.11% on watermelon

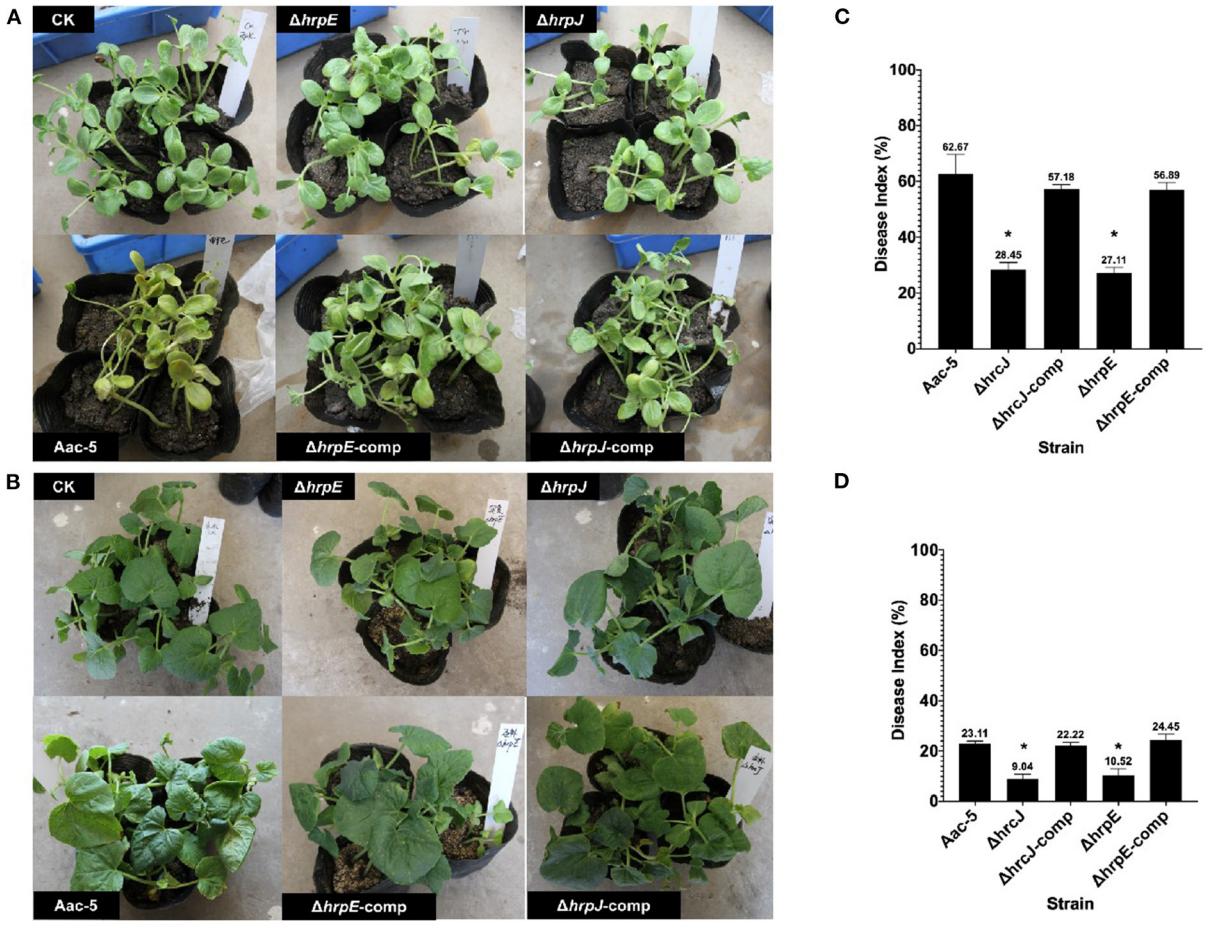

FIGURE 4
Virulence of *A. citrulli* strains on watermelon **(A)** and melon **(B)** seedlings 8 days after spray inoculation. Aac-5, wild-type strain; Δ*hrcJ*, *hrcJ* mutant strain; Δ*hrcJ*-comp, Δ*hrcJ* complementation strain; Δ*hrpE*, *hrpE* mutant strain; Δ*hrpE*-comp, Δ*hrpE* complementation strain. **(C,D)** Statistical analysis of the virulence of Aac-5 strains. Disease index for each *A. citrulli* strain was calculated from three experiments based on a 0 to 9 disease severity reading of each seedling in each experiment. Disease index with * indicates a significant difference as compared to that of the wild-type strain ($P < 0.05$).

(Figure 4C), and 9.04% and 10.52% on melon (Figure 4D), respectively. When the Δ*hrpE* and Δ*hrcJ* mutant strains were complemented, however, the complementation strains caused statistically similar DIs of 57.19 and 56.89% on watermelon, and 22.22 and 24.44% on melon, respectively, as compared to their wild-type strain Aac-5 (Figures 4C,D).

Effect of *hrcJ* and *hrpE* on biofilm formation

Seven days after incubation, the absorbance measurement at OD_{570} for the stained biofilm dissolved with ethanol was 0.04 for the wild-type strain Aac-5, but significantly higher for the Δ*hrcJ* and Δ*hrpE* mutant strains at 0.123 and 0.093, respectively ($P < 0.05$) (Figure 5). When the mutant strains were complemented, however, OD_{570} was measured to be 0.048 and 0.046, similar to the wild-type level but significantly lower that the two mutant strains ($P > 0.05$) (Figure 5).

Discussion

Pathogenicity assays on *X. oryzae* pv. *oryzae* have demonstrated that all *hrp-hrc* genes were very critical and their absence in the bacterium has led to loss of disease symptoms in the susceptible rice cultivar (Cho et al., 2008). Like *X. oryzae*, *A. citrulli* also requires a functional T3SS to cause disease on host plants and induce HR on non-host plants, since a *hrcC* deletion mutant of AAC00-1 abolished its pathogenicity on watermelon seedling tissues (Johnson et al., 2011), and the *hrcV* mutants of M6 (group I) and W1 (group II) strains failed to cause HR on tobacco and showed reduced virulence

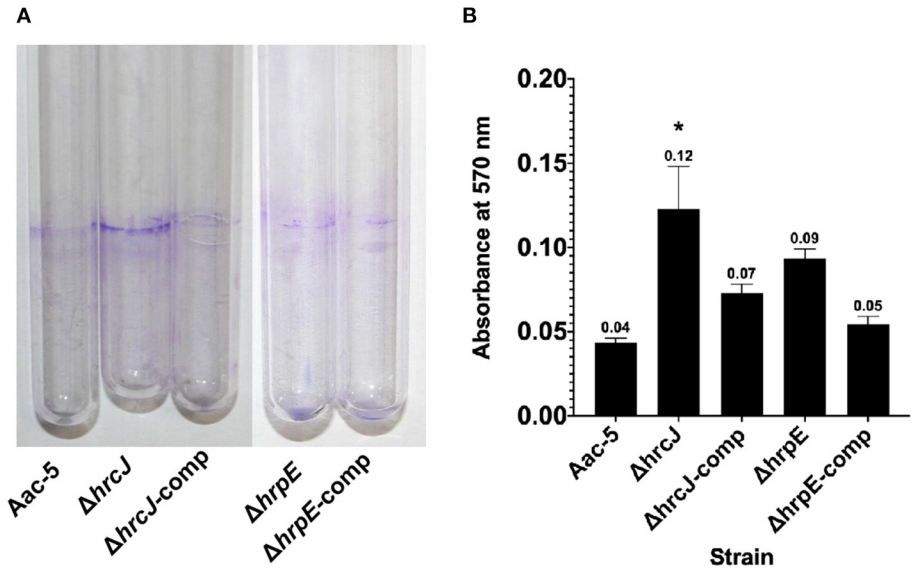

FIGURE 5
Effect of *hrcJ* and *hrpE* on biofilm formation of *A. citrulli* strains. **(A)** After 7 days incubation, the *A. citrulli* strains formed biofim on the inner wall of the cultural tubes, and then were visualized by staining with crystal violet. **(B)** Biofilm formation was quantified by measuring absorbance at 570 nm of the crystal violet stained biofilm dissolved with ethanol. The OD_{570} for each strain is the average of three experiments. The bars represent standard errors of the means. Asterisk indicates significant difference at $P < 0.05$ by the Student's *t*-test.

when inoculated to melon seeds (Bahar and Burdman, 2010). Genome sequencing of the group II strain AAC00-1 of *A. citrulli* revealed that it contains a *hrp* gene cluster coding for core proteins of the T3SS (Bahar et al., 2009). Besides the *hrcC* and *hrcV* genes, however, the function of other genes of T3SS in *A. citrulli* remains unknown. In our study, we investigated the biological functions of two of the T3SS genes, *hrcJ* and *hrpE*, in the ability of *A. citrulli* strain Aac-5, a group II strain from China, on biofilm formation, HR in tobacco and virulence in watermelon and melon seedlings. Aac-5 strain was isolated in Taiwan province of China, and was previously identified as a group II strain based on the Pulsed Field Gel Electrophoresis and Multilocus sequence typing (Yan et al., 2012). To achieve this goal, we therefore used Aac-5 as background to construct *hrcJ* and *hrpE* mutants and their complementation strains, and compared them to their wild-type strain Aac-5. The mutant strains Δ*hrcJ* and Δ*hrpE* were greatly reduced in virulence in watermelon and melon seedlings and lost ability to cause HR in tobacco leaves. Such reduction in virulence/loss of HR, however, was recovered/regained to the wild-type level, when the mutants were complemented. Our results revealed that in addition to the *hrcC* gene as reported previously (Johnson et al., 2011), both *hrcJ* and *hrpE* genes in the T3SS are required for virulence of *A. citrulli* in host plants and for HR in non-host plants.

The *hrp* clusters are most characterized in four plant pathogenic bacteria *Erwinia amylovora*, *Pseudomonas syringae*, *Ralstonia solanacearum* and *X. campestris* (Wengelnik and Bonas, 1996; Deng and Huang, 1999; Gijsegem et al., 2002; Rantakari et al., 2007). The clusters can be divided into two groups based on sequences, operon structures and regulation (Alfano and Collmer, 1997). Group I clusters include those of *E. amylovora* and *P. syringae*, and group II clusters include those of *R. solanacearum* and *X. campestris*. The most critical difference between the two cluster groups is that they are regulated by different regulators. Group I *hrp* operons are activated by HrpL and group II ones by HrpB (Alfano and Collmer, 1997). Based on the genome analysis of *A. citrulli* strain AAC00-1, the *hrp* operon contains a *hrpB* gene, suggesting that the *hrp* cluster in AAC00-1 belongs to group II *hrp* clustes. When the *hrpB* gene in *X. citri* was deleted, the bacterium greatly reduced its ability to form biofilm (Zimaro et al., 2014). In *A. citrulli*, the contribution of Type IV pili (TFP) to biofilm formation was revealed. The TFP, a form of surface appendage, is involved in many processes in bacteria including DNA transfer, twitching motility, and adherence to surfaces. The Type IV pili deficient mutants of the group I strain M6 of *A. citrulli* also greatly reduced biofilm formation as compared to its wild-type strain (Bahar et al., 2009). Whether the T3SS contributes to biofilm formation in *A. citrulli* strains, however, had been unknown before our study. The wild-type group II strain Aac-5 (Rantakari et al., 2007) we used in our study displayed low ability to form biofilm, compared to group I strain M6 of *A. citrulli* (Bahar et al., 2009). Interestingly, when the two T3SS-related genes *hrcJ* and *hrpE* were mutated, the mutant strains greatly increased their ability to form biofilm, but the increase was reduced to the wild-type level when the mutants were complemented, suggesting

that *hrcJ* and *hrpE* negatively control biofilm formation in Aac-5. This is different from the positive role played by the Type IV pili in the group I strain M6 of *A. citrulli*, suggesting that different genes may control biofilm formation differently in *A. citrulli*, or that different strains of *A. citrulli* may use different strategies to attach to their *in vitro* environment or host plant surfaces.

In conclusion, our study showed that the T3SS genes *hrcJ* and *hrpE* positively control HR and virulence, but negatively contribute to biofilm formation in the group II strain Aac-5 of *A. citrulli*. This also suggests that biofilm formation in the group II strains of *A. citrulli* may not contribute, at least not directly, to virulence. Future research is needed to determine the biological function of biofilm, if any, in *A. citrulli*, especially in group II strains, and different functions biofilm may play in different groups of strains in *A. citrulli*.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

Conceptualization: TW, QH, and WG. Methodology: XA and YY. Writing—original draft preparation: WG. Writing—review and editing: QH. Funding acquisition: TZ. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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