Involvement of hrpX and hrpG in the Virulence of Acidovorax citrulli Strain Aac5, Causal Agent of Bacterial Fruit Blotch in Cucurbits

Xiaoxiao Zhang, Mei Zhao, Jianpei Yan, Linlin Yang, Yuwen Yang, Wei Guan, Ron Walcott and Tingchang Zhao

Acidovorax citrulli causes bacterial fruit blotch, a disease that poses a global threat to watermelon and melon production. Despite its economic importance, relatively little is known about the molecular mechanisms of pathogenicity and virulence of A. citrulli. Like other plant-pathogenic bacteria, A. citrulli relies on a type III secretion system (T3SS) for pathogenicity. On the basis of sequence and operon arrangement analyses, A. citrulli was found to have a class II hrp gene cluster similar to those of Xanthomonas and Ralstonia spp. In the class II hrp cluster, hrpG and hrpX play key roles in the regulation of T3SS effectors. However, little is known about the regulation of the T3SS in A. citrulli. This study aimed to investigate the roles of hrpG and hrpX in A. citrulli pathogenicity. We found that hrpG or hrpX deletion mutants of the A. citrulli group II strain Aac5 had reduced pathogenicity on watermelon seedlings, failed to induce a hypersensitive response in tobacco, and elicited higher levels of reactive oxygen species in Nicotiana benthamiana than the wild-type strain. Additionally, we demonstrated that HrpG activates HrpX in A. citrulli. Moreover, transcription and translation of the type 3-secreted effector (T3E) gene Aac5_2166 were suppressed in hrpG and hrpX mutants. Notably, hrpG and hrpX appeared to modulate biofilm formation. These results suggest that hrpG and hrpX are essential for pathogenicity, regulation of T3Es, and biofilm formation in A. citrulli.

Keywords: hrpG, hrpX, Acidovorax citrulli, type III secretion system, hypersensitive response, reactive oxygen species, pathogenicity, applications of biological

INTRODUCTION

Bacterial fruit blotch (BFB), caused by the gram-negative bacterium Acidovorax citrulli (Schaad et al., 1978, 2008; Willems et al., 1992), is a globally occurring destructive disease of cucurbit crop species (Burdman and Walcott, 2012). Multiple studies have evaluated methods to mitigate the impact of BFB, including pathogen exclusion by quarantine, seed health testing, seed treatment, and field applications of biological control agents (Dutta et al., 2014; Jiang et al., 2015; Tian et al., 2016). Unfortunately, the success of these approaches has been limited, and BFB still poses a serious threat to commercial watermelon and melon production worldwide (Burdman and Walcott, 2012).
Despite the economic importance of BFB, little is known about the molecular mechanisms of *A. citrulli* pathogenesis (Burdman and Walcott, 2012). Recent studies have shown that important pathogenicity and virulence determinants of *A. citrulli* include the type II (T2SS) (Johnson, 2010), type III (T3SS) (Johnson et al., 2011), and type VI (T6SS) secretion systems (Tian et al., 2015); type IV pili (T4P) (Bahar et al., 2009a), polar flagella (Bahar et al., 2011), and quorum sensing (QS) (Wang T. et al., 2016). Most gram-negative, biotrophic, phytopathogenic bacteria rely on a functional T3SS to promote disease or trigger a hypersensitive response (HR) in susceptible and resistant plants, respectively (Büttner and Bonas, 2002). Importantly, gram-negative phytopathogenic bacteria secrete type 3-secreted effectors (T3Es) directly into plant host cells via the T3SS (Block et al., 2008; Van Engelenburg and Palmer, 2010; Nomura et al., 2011; Jiang et al., 2013; Xin et al., 2016). These T3Es may function to overcome pathogen-associated molecular pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Cui et al., 2013) or promote effector-triggered susceptibility (Jehle et al., 2013). The genes encoding the components of the T3SS are named *hrp* genes (Kim et al., 2003; Tampakaki et al., 2010). The *hrp* genes are located in large clusters, generally 20–25 kb in size (Büttner and Bonas, 2002). On the basis of gene organization, sequence, and regulation, *hrp* clusters can be divided into two classes: class I contains clusters of *Pseudomonas syringae* and enteric plant-pathogenic bacteria, whereas class II contains the *hrp* genes of *Xanthomonas* spp. and *Ralstonia solanacearum* (Rogdanove et al., 1996; Büttner and Bonas, 2002). Analysis of the genome sequence of the *A. citrulli* group II strain AAC001 revealed the presence of a *hrp*-T3SS (Burdman and Walcott, 2012). Moreover, sequence and cluster organization analyses showed that the *A. citrulli* *hrp* cluster belongs to class II (Burdman and Walcott, 2012). The *hrp* genes are induced in plant leaves and in T3SS-inducing, nutrient-poor medium. Generally, T3SS is suppressed in rich medium (Schulte and Bonas, 1992; Clarke et al., 2010). Gram-negative bacterial plant pathogens of the genera *Erwinia*, *Pseudomonas*, *Ralstonia*, and *Xanthomonas* require a fully active T3SS for pathogenicity, and the recent establishment of an inducing medium has facilitated the study of *hrp* gene expression and regulation in *in vitro* experiments (Yuan and He, 1996; Rossier et al., 1999; Murata et al., 2006; Ancona et al., 2015).

In *Xanthomonas* spp., the expression of *hrp* genes is controlled by two regulators: HrpG and HrpX. HrpG is an OmpR family regulator that activates the expression of *hrpX* in *X. campestris* pv. *vesicatoria* (Wengelnik et al., 1996b) and in *X. campestris* pv. *campestris* (Huang et al., 2009). HrpX, an AraC-type transcriptional activator, controls the expression of other *hrp* genes and some effector genes (Wengelnik et al., 1996a). The HrpG regulon regulates the expression of *hrp* genes, as shown by cDNA-amplified fragment length polymorphism analysis (Noël et al., 2001). Regulated genes include the *hrp* gene cluster, effector genes, and genes encoding proteases. Thirty HrpG-induced and five HrpG-repressed genes have been identified in *X. campestris* pv. *vesicatoria*. Phosphorylated HrpG is predicted to activate the expression of *hrpX* (Büttner and Bonas, 2010; Tampakaki et al., 2010). HrpX binds to a conserved cis-regulatory element called the plant-inducible promoter (PIP) (TTCGC-N15-TTCCG) or a less conserved PIP-like motif (TTCGC-N8-TTCTG) in promoter regions associated with *Xanthomonas* virulence (Fenselau and Bonas, 1995). However, the mechanism differs from that of the HrpL/HrpS-dependent systems in *Erwinia carotovora* and *Pseudomonas* spp., which belong to class I *hrp* clusters (Tampakaki et al., 2010). Recently, Guo et al. (2011) showed that *hrpG* and *hrpX* in *Xanthomonas axonopodis* pv. *citri* are involved in the regulation of multiple physiological functions, such as biofilm formation, by genome-wide microarray analyses. Thus, *hrp* gene regulation may be involved in a wide array of functions.

*A. citrulli* relies on a functional T3SS for pathogenicity (Ren et al., 2009; Burdman and Walcott, 2012; Eckstein-Levi et al., 2014). Unfortunately, not much is known about the T3SS and T3Es in *A. citrulli* as compared to other plant-pathogenic bacteria (Burdman and Walcott, 2012) because of the lack of a T3SS-inducing medium for *A. citrulli* until recently (Chen, 2016). Recent research has focused mainly on the characterization of genetically distinct groups of *A. citrulli* strains and on host preference (Silva et al., 2016; Zivanovic and Walcott, 2017). In addition, the arsenal of T3S effectors in *A. citrulli* strains were reported to be related to host preference on melon fruits (Yan et al., 2017). More specifically, group I *A. citrulli* strains can cause water-soaked lesion on detached immature watermelon rinds, while group II strains cannot. Previously, we reported that the *A. citrulli* group II strain Aac5, which was isolated from watermelon in Taiwan also relies on the T3SS for pathogenicity. As expected, Aac5 lost the ability to infect watermelon when the homologous hrcN gene was deleted (Yan et al., 2015). In addition, biofilm formation was crucial for Aac5 virulence and may be related to the T3SS (Wang T. et al., 2016). Based on sequence analysis of *A. citrulli* strain AAC001-1, *hrpX* and *hrpG* homologs were identified. We hypothesized that the *hrpG* and *hrpX* homologs in *A. citrulli* strain Aac5 may function as T3SS regulators. Hence, the objective of this study was to characterize the roles of these homologs in *hrp* gene regulation and pathogenicity.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions**

The strains and plasmids used in this study are listed in Table 1. All *A. citrulli* strains were grown on King's B (KB) or T3SS-inducing medium (Chen, 2016) (10 g/L Bacto Peptone, 5 g/L yeast extract, 5 g/L NaCl, 10 mM MgCl₂, pH 5.8, in sterilized distilled water [SDW]) at 28°C. *Escherichia coli* strains were grown on LB medium at 37°C (Sambrook and Russel, 2001). Liquid cultures of the strains were grown in sterilized test tubes containing KB, LB, or T3SS-inducing broth continuously agitated at 200 rpm on a rotary shaker (DDHZ-300; Taicang Experimental Instrument
TABLE 1 | Bacterial strains and plasmids used in this study.

| Strains and plasmids | Characteristics | Reference |
|----------------------|----------------|-----------|
| **Acidovorax citrulli STRAINS** | | |
| Aac5 | Wild-type group II strain; AmpR | Yan et al., 2013 |
| ΔhrpG | hrpG markerless mutation of Aac5; AmpR | This study |
| ΔhrpX | hrpX markerless mutation of Aac5; AmpR | This study |
| ΔhrpG-comp | ΔhrpG containing pBBRNolac-4FLAG carrying hrpG with its native promoter; AmpR; KmR | This study |
| ΔhrpX-comp | ΔhrpX containing pBBRNolac-4FLAG carrying hrpX with its native promoter; AmpR; KmR | This study |
| WT-EV | Wild-type strain Aac5 transformed with pBBRNolac-4FLAG | This study |
| ΔhrpG-EV | ΔhrpG transformed with pBBRNolac-4FLAG | This study |
| ΔhrpX-EV | ΔhrpX transformed with pBBRNolac-4FLAG | This study |
| **Escherichia coli** | | |
| DH5α | supE44 ΔlacU169 (Φ80lacZ ΔM15) | TIANGEN |
| | lacIq recA1 thi-1 relA1 | |
| **Pseudomonas syringae STRAIN** | | |
| DC3000 | Wild-type strain; RifR | Lab collection |
| Plasmids | | |
| pK18mobsacB | Suicide vector with sacB gene; KmR | Kovach et al., 1995 |
| pK18hrpG | Suicide vector containing upstream and downstream fragments of hrpG gene on pK18mobsacB; KmR | This study |
| pK18hrpX | Suicide vector containing upstream and downstream fragments of hrpX gene on pK18mobsacB; KmR | This study |
| pBBR1MCS-2 | pBBR1MCS-2 containing pBluescript II KS-λacZ; KmR | Kovach et al., 1995 |
| pBBRNolac-4FLAG | lac promoter was deleted from pBBR1MCS-2 and C-terminal 4×FLAG tag was inserted; need native promoter to drive only. | This study |
| pBBR2166 | pBBRNolac-4FLAG containing Aac5_2166 gene with its native promoter | This study |
| pBBRNolacGUS | lac promoter was deleted from pBBR1MCS-2 and GUS reporter gene was inserted | This study |
| pBBRNolac2166GUS | pBBRNolacGUS containing Aac5_2166 gene with its native promoter | This study |
| pBBRBhpX | pBBRBhpG containing hrpX gene with its native promoter; KmR | This study |
| PK600 | Helper strain in tri-parental mating; CmR | Lab collection |
| pYBA1132 | plant expression vector containing 3SS promoter, KmR | Lab collection |
| pYBA1132-2166 | pYBA1132 containing Aac5_2166 gene | This study |
| PJY-mini-TN7-TUS | For cloning the GUS reporter, AmpR | Zhang et al., 2013 |

*Ampr, Kmr, and RifR indicate resistance to ampicillin, kanamycin, rifampicin, and chloramphenicol, respectively.*

Factory, Jiangsu, China). When required, the growth media were supplemented with the following antibiotics: ampicillin (Amp), 100 μg/mL; chloromycetin, 3.4 μg/mL; and kanamycin (Km), 50 μg/mL. For assays, bacterial concentrations were estimated by optical density (OD₆₀₀) using a spectrophotometer (Evolution 300 UV/VIS; Thermo Scientific, Waltham, MA, USA).

Molecular Manipulations
Molecular manipulations were carried out using standard procedures (Sambrook and Russel, 2001). Constructs were ligated using ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China). Polymerase chain reaction (PCR) primers used in this study (Supplementary Table 1) were synthesized by BGI Laboratories (BGI, Shenzhen, China). KOD-Plus neo (Toyobo, Shanghai, China) and 2× Taq Plus PCR MasterMix (Tiangen, Beijing, China) were used for PCR amplification.

**Construction of hrpG and hrpX Gene Deletion Mutants and Complementation**

Markerless hrpG and hrpX mutants of *A. citrulli* strain Aac5 were generated through allele exchange (Ren et al., 2014). Primers were designed on the basis of *A. citrulli* AAC00-1 sequences for *Aave_0445* and *Aave_0444*, which encode *hrpG* and *hrpX* homologs, respectively, and their flanking regions. The flanking regions were cloned from Aac5 and were aligned to those of to AAC00-1 using DNAMAN version 5.2.2 (Lynnon Biosoft, Quebec, Canada), and the sequence similarity was 100%. For the *hrpG* gene (*Aave_0445*), the upstream and downstream flanking regions were amplified by PCR using the primer pairs hrpG-1F/hrpG-1R and hrpG-2F/hrpG-2R (Supplementary Table 1),
respectively. The two fragments were concatenated by overlap PCR (Zhang et al., 2013) and subsequently ligated into the suicide plasmid, pk18mobsacB, to generate pk18hrpG. pk18hrpG was transformed into A. citrulli strain Aac5 by tri-parental mating with an E. coli strain carrying the helper plasmid, pRK600. Single crossover colonies were selected on KB plates containing Amp and Km. Individual transformants were then sub-cultured continuously in KB liquid medium containing Amp alone to promote double crossover homologous recombination. The hrpG deletion mutant, ΔhrpG, which was resistant to Amp but sensitive to Km, was confirmed by PCR using the primer pairs hrpG-TF/hrpG-TR, hrpG-1F/hrp-2R, km-F/km-R, and WFB1/WFB2 (Supplementary Table 1).

To construct a complementation vector, the complete hrpG gene (1,074 bp) including its native promoter, was amplified with primers HBhrpG-F/HBhrpG-R (Supplementary Table 1) and inserted into the shuttle vector, pBBRNolac-4FLAG, to create pBBRHBhrpG. The pBBRNolac-4FLAG was constructed by deleting the lac promoter of pBBR1MCS-2 using a KOD-Plus-Mutagenesis Kit (Toyobo, Shanghai, China), and inserting a 4× FLAG tag into the vector with C-terminal fusion expression, so that only the native promoter drove target gene expression. The complementation vector pBBRHBhrpG was introduced into the ΔhrpG mutant to generate the complemented strain ΔhrpG-comp. HrpG expression was verified in the complemented strain by western blotting (Sambrook and Russel, 2001). Similar methods were used to generate ΔhrpX and ΔhrpX-comp mutant strains from A. citrulli Aac5.

Roles of hrpG and hrpX in A. citrulli Virulence and Hypersensitive Response Induction

To determine the roles of hrpG and hrpX in seed-to-seedling BFB transmission, seed transmission assays were performed as described previously (Bahar et al., 2009b), with slight modifications. The WT, ΔhrpG, and ΔhrpX Aac5 strains, were cultured in liquid KB medium and adjusted to 5 × 10^6 CFU/mL with sterilized distilled water. Watermelon seeds (cv. Ruighong, n = 24) were soaked in a bacterial cell suspension (~5 × 10^6 colony-forming units [CFU]/mL) at room temperature for 2 h with continuous agitation at 60 rpm using a rotary shaker (Ecotron; Infors HT, Bottmingen, Switzerland). The inoculated seeds were then air-dried for 24 h and sown in plastic pots (Rongxiangruihui, Beijing, China, six seeds per pot) filled with a potting mix (vermiculite, soil, and peat at a 1:1:1 ratio). The pots were placed in a growth chamber for 12 days under the following conditions: 25–30°C; 65% mean relative humidity; and 16 h light/8 h darkness cycle during the experiments. BFB severity was visually assessed 12 days after seedling emergence, as described previously (Bahar et al., 2009b).

To determine the roles of hrpG and hrpX in A. citrulli virulence on watermelon seedlings, infiltration assays were performed as described previously (Ren et al., 2014). Briefly, cotyledons of 2-week-old watermelon seedlings (cv. Ruighong, n = 6) were syringe-infiltrated with bacterial suspensions (2 × 10^4 CFU/mL) and incubated at room temperature. Cotyledons were photographed at 24, 48, 72, and 96 h post inoculation (hpi) using an EOS 70D camera (Canon, Beijing, China). As a negative control, plants were injected with 10 mM MgCl₂.

To evaluate the effects of hrpG and hrpX deletion on HR induction, HR assays were performed by injecting a bacterial cell suspension into the leaves of 3-week-old tobacco (Nicotiana tabacum), as described previously (Ren et al., 2014). Leaves were visually examined for HR (tissue collapse) 24 h after infiltration. Electrolyte leakage was quantified for detecting HR on 3-week-old tobacco leaves injected with wild-type A. citrulli Aac5, ΔhrpG, and ΔhrpX, and the respective complementation strains, as described previously (Stork et al., 2015). In brief, the strains were inoculated into tobacco leaves, and four leaf disks from each treatment were harvested after 24 h, washed, and floated on 5 ml of distilled water for 4 h with gentle shaking prior to measuring the conductivity of the bath water. As a negative control (mock treatment), 10 mM MgCl₂ was used.

Assay for Reactive Oxygen Species (ROS)

ROS were measured by chemiluminescence as described previously (Liu et al., 2015), with slight modifications. N. benthamiana leaves were inoculated with cell suspensions of A. citrulli Aac5, ΔhrpG, and ΔhrpX, and respective complementation strains (~10^6 CFU/mL). P. syringae pv. tomato DC3000 was used as a negative control. At 24 hpi, leaf disks (0.4 cm in diameter) were excised and placed into wells of 96-well plates pre-loaded with 100 μL SDW. Subsequently, 100 μL of 0.5 mM L-012 (Wako, Guangzhou, China) in 10 mM MOPS-KOH buffer (pH 7.4) were added to each well. The intensity of ROS generation was determined as described by Liu et al. (2015). N. benthamiana leaf disks infiltrated with P. syringae pv. tomato DC3000 were used as a negative control.

Evaluation of Biofilm Formation

To determine the roles of hrpG and hrpX in biofilm formation, assays were performed using A. citrulli wild-type Aac5, ΔhrpG, and ΔhrpX, and respective complementation strains, as previously described (Bahar et al., 2009a), with slight modifications. Briefly, 24-well plates (Corning, NY, USA) were pre-loaded with liquid T3SS-inducing medium and inoculated with a 1:1,000 dilution of overnight cultures of A. citrulli Aac5, ΔhrpG, and ΔhrpX, and the respective complementation strains. The plates were incubated in a tilted position at 28°C for 48 h, without agitation. Then, 0.1% crystal violet was added to each well for 30 min, after which the wells were washed with distilled water. Biofilm formation for each strain was compared quantitatively by solubilizing the stained biofilms with 100% ethanol and measuring the OD₅₉₀ of the stained-cell suspensions with a spectrophotometer.

Quantitative Reverse-Transcription (qRT-)PCR

Total RNA was extracted from wild-type A. citrulli Aac5, ΔhrpG, and ΔhrpX cultured in T3SS-inducing liquid medium up to OD₆₀₀ = 0.45, using TRIzol reagent (Invitrogen, Waltham, MA, USA). Contaminant DNA was digested and cDNA was synthesized using ReverTra Ace qPCR RT MasterMix with gDNA.
β-Glucuronidase (GUS) Reporter Activity Assay For Detecting Promoter Activity

To construct a GUS reporter vector, the GUS gene was inserted into pBBRNoLaGUS (without 4×FLAG-tag) to generate the pBBRNoLaGUS vector using restriction enzymes XbaI and HindIII. The native promoter sequence of the putative T3S effector Aac5_2166 (GenBank accession MG879253) was cloned from genomic DNA of A. citrulli Aac5 by PCR, and the product was ligated into pBBRNoLaGUS to generate pBBR2166GUS. pBBR2166GUS and pBBRNoLaGUS (negative control vector) were transformed into Aac5, ΔhrpG, and ΔhrpX to generate WT-2166GUS, hrpG-2166GUS, hrpX-2166GUS, and WT-GUS strains (negative control strain). Promoter activity was detected as previously described (Zhang et al., 2013).

Assay of HrpG and HrpX Protein Production by A. citrulli Strains in T3SS-Inducing Media

HrpX and HrpG protein expression was quantified by western blotting. A. citrulli strains (OD_{600} = 1.0) were pre-incubated for ~12–18 h in KB broth and washed twice. Twenty microliters of the bacterial suspension were transferred into 10 mL of T3SS-inducing medium and incubated at 28 °C until the OD_{600} reached 0.5. To extract intracellular protein, 4 mL of the bacterial suspension as cell lysate was treated with Protease Inhibitor Cocktail (Bimake) and heated for 10 min. 200 µL of 4× Laemmli Sample Buffer (Bio-Rad, Beijing, China) and 4°C for 3 min, and the cells were resuspended in 200 µL of 4× Laemmli Sample Buffer (Bio-Rad, Beijing, China) and heated for 10 min.

To extract secreted protein, the bacterial cell supernatants were treated with Protease Inhibitor Cocktail (Bimake) and separated by centrifugation, as previously described (Liu et al., 2017). Proteins were precipitated with 10% trichloroacetic acid (Sigma, Shanghai, China), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5% gels, and transferred to PVDF membranes (Millipore, Shanghai, China). The PVDF membranes were blocked with Tris-buffered saline plus Tween-20 (Sigma, Shanghai, China) containing 5% skim milk powder for 1 h at room temperature. After incubation with primary and secondary antibodies, the PVDF membranes were used for protein detection with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Shanghai, China) by LAS 4000 (GE Biotech, Marlborough, MA, USA). The sources and dilutions of antibodies were as follows: anti-FLAG antibodies (anti-DDDDK-tag mAb-HRP-DirecT; 1:2,500 dilution; MBL, Beijing, China), anti-HrpG antibodies (1:1,000 dilution; Abclonal, Wuhan, China), anti-HrpX antibodies (1:1,000 dilution; Abclonal), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (1:1,000 dilution; Abclonal). The secondary antibodies were anti-rabbit antibodies (MBL) used at a 1:5,000 dilution. Protein quantification was carried out using ImageJ software (NIH, Bethesda, MD).

Statistical Analysis

All experiments were conducted three times. Six biological replicates were used in each biofilm assay, electrolyte leakage quantification assay, and promoter activity. Twelve biological replicates were used for ROS detection. Twenty-four leaves as biological replicates were used for assessing BFB severity. For the other assays, three biological replicates were evaluated. Data were analyzed by one-way analysis of variance (ANOVA) and Tukey’s honest significant difference (HSD) tests. For qRT-PCR, data were analyzed by the independent-samples t-test. Statistical analyses were conducted using SPSS version 17.0 (SPSS, Chicago, IL, USA) and GraphPad PRISM 5.0 software (GraphPad Software, La Jolla, CA, USA). Differences with p < 0.05 were considered significant.

RESULTS

Sequence Analysis of hrpG and hrpX Homologs in A. citrulli Strain Aac5

The group II A. citrulli strain Aac5 has been routinely used in studies on pathogenicity (Wang T. et al., 2016). BLAST searches using the AAC00-1 genome (GenBank accession NC_008752) identified Aave_0445 and Aave_0444, which encode response regulator receiver protein (A1TJB4), which showed 100% amino acid sequence identity with HrpG (Q5EF45), and transcriptional regulator, AraC family (A1TJB3), which showed 100% amino acid sequence identity with HrpX (Q5EF44) in UniProt, respectively. The predicted HrpG (Q5EF45) and HrpX (Q5EF44) in UniProt were also from A. citrulli. To verify whether homologous genes coding HrpG and HrpX exist in strain Aac5, we cloned the sequences from Aac5 and found that they were 100% identical to the sequences of Aave_0445 and Aave_0444 from AAC00-1, named hrpG (GenBank accession no.: MG879251) and hrpX (MG879252), respectively. Amino acid sequence alignment to AAC00-1 using DNASAN version 5.2.2 (Figures 1A,B) revealed 100% identity. PCR with the primer pair hrpG-TF/hrpG-TR yielded a 417-bp amplicon when using DNA template from wild-type Aac5, but not when the DNA template was from the ΔhrpG mutant. DNA from wild-type Aac5 and ΔhrpG mutant yielded 2,249- and 3,027-bp PCR amplicons, respectively, with the primer pair hrpG-1F/hrp-2R, and no amplicons were generated with the primer pair km-F/km-R. To confirm the identity of the wild-type Aac5 and ΔhrpG mutant, a 360-bp amplicon was produced with the diagnostic primer pair WFB1/WFB2. The hrpG gene deletion mutant was confirmed using the same approach.

Roles of hrpG and hrpX in A. citrulli Biofilm Formation

Since biofilm formation is involved in A. citrulli virulence and pathogenicity (Bahar et al., 2009a; Tian et al., 2015; Wang T.
et al., 2016), we investigated the roles of hrpG and hrpX in biofilm formation. To rule out interference by plasmid introduction, we additionally transformed the empty vector, pBBRNolac-4FLAG, into wild-type A. citrulli Aac5 (WT-EV), ΔhrpG (ΔhrpG-EV), and ΔhrpX (ΔhrpX-EV). Biofilm formation was quantified by crystal violet staining, which showed that ΔhrpG-EV and ΔhrpX-EV exhibited significantly enhanced biofilm formation as compared with Aac5. The ΔhrpG-comp strain produced significantly less biofilm than ΔhrpX-EV, but significantly more than the wild-type strain (Figure 2). ΔhrpX-comp produced significantly more biofilm than the wild-type strain.

hrpG and hrpX Mutants of A. citrulli Fail to Induce HR in N. tabacum

To determine whether hrpG and hrpX are involved in HR induction by A. citrulli, we examined HR induction by the mutant and wild-type strains in N. tabacum, a non-host plant typically used to test HR induction (Ren et al., 2009). The strains ΔhrpG-comp and ΔhrpX-comp induced an HR in N. tabacum similar to that induced by the WT strain after 24 h. In contrast, ΔhrpG-EV and ΔhrpX-EV did not elicit an HR (Figure 3). These observations suggested that Aac5 requires hrpG and hrpX to induce an HR in N. tabacum.

To substantiate these results, we used electrolyte leakage analysis. As expected, ΔhrpG-comp and ΔhrpX-comp induced electrolyte leakage to levels similar to those induced by WT-EV. In contrast, ΔhrpG-EV and ΔhrpX-EV induced significantly lower levels of electrolyte leakage, similar to that induced by mock treatment and 3-fold lower than the level induced by WT-EV (Figure 4). Overall, these findings revealed that Aac5 requires hrpG and hrpX to induce an HR in N. tabacum.

hrpG and hrpX Negatively Regulate PTI-Associated ROS Generation

As hrpG and hrpX were found to be essential for HR induction by A. citrulli, we investigated whether PTI-associated ROS was altered during the A. citrulli–host interaction. Thus, we examined the ROS levels induced by the A. citrulli strains in N. benthamiana leaves using chemiluminescence (Liu et al., 2015). ΔhrpG and ΔhrpX elicited significantly higher levels of ROS (∼12-fold and ∼8-fold, respectively) than the WT strain, which induced ROS to a level similar to that of DC3000 (Figure 5). These results indicated that hrpG and hrpX act as negative regulators of PTI-associated ROS generation in the interplay between A. citrulli and N. benthamiana.

hrpG and hrpX Contribute to A. citrulli Virulence on Watermelon Seedlings

Based on the above observations, we hypothesized that hrpG and hrpX are critical for A. citrulli virulence. We tested this hypothesis by conducting seed transmission and seedling infiltration assays in watermelon. The mean BFB severity index upon inoculation of ΔhrpG and ΔhrpX was similar to that of the WT strain after 24 h. In contrast, ΔhrpG-EV and ΔhrpX-EV did not elicit an HR (Figure 3). These observations suggested that Aac5 requires hrpG and hrpX to induce an HR in N. tabacum.

To substantiate these results, we used electrolyte leakage analysis. As expected, ΔhrpG-comp and ΔhrpX-comp induced electrolyte leakage to levels similar to those induced by WT-EV. In contrast, ΔhrpG-EV and ΔhrpX-EV induced significantly lower levels of electrolyte leakage, similar to that induced by mock treatment and 3-fold lower than the level induced by WT-EV (Figure 4). Overall, these findings revealed that Aac5 requires hrpG and hrpX to induce an HR in N. tabacum.
Interaction Between HrpG and HrpX

To evaluate the interaction between HrpG and HrpX, we assessed their translation levels by western blot analysis using anti-HrpX and anti-HrpG antibodies. Total protein was extracted from A. citrulli wild-type strain (WT), HrpG, HrpX, and ΔhrpG, ΔhrpX mutants transformed with empty vector, pBBRNolac-4FLAG; ΔhrpG-EV, ΔhrpX-EV, ΔhrpG mutant transformed with empty vector, pBBRNolac-4FLAG; ΔhrpG-comp, ΔhrpX mutant transformed with the empty vector pBBRNolac-4FLAG; ΔhrpG-comp, ΔhrpX-comp complemented with vector, pBBRHBhpG (native promoter); ΔhrpX-comp, ΔhrpX-comp complemented with the vector, pBBRHBhpX (native promoter). Each column shows the mean and standard deviation. The experiment was conducted three times. Averages and standard deviations from one of three experiments with similar results are shown. Different letters above bars indicate statistically significant differences as determined by one-way analysis of variance (ANOVA) and Tukey’s honest significant difference (HSD), p < 0.05.

hrpG and hrpX Regulate the Expression of T3Es

Based on the above results, we hypothesized that the expression of T3Es in A. citrulli is regulated by HrpG and HrpX. To test this hypothesis, we used qRT-PCR to compare the transcript levels of T3Es in ΔhrpG, ΔhrpX, and the WT strain in T3SS-inducing medium. We transiently expressed Aave_2166 homolog, encoding the YopJ homolog (Eckshtain-Levi et al., 2014; Traore, 2014), as a putative effector inducible programmed cell death in N. benthamiana (Supplementary Image 2) in WT, ΔhrpG, and ΔhrpX. Since the gene cloned from Aac5 has 100% sequence identity with the sequence of Aave_2166, it was named Aac5_2166 (GenBank accession MG879253). We then measured the transcript levels of Aac5_2166 in ΔhrpG, ΔhrpX, and the WT strain. The mRNA level of Aac5_2166 was significantly lower in ΔhrpG and ΔhrpX (∼3-fold and ∼2-fold, respectively) than in the WT strain. These results indicated that hrpG and hrpX regulate the expression of Aac5_2166 (Figure 8A).

To verify these results, we analyzed the promoter activity of Aac5_2166. We constructed a reporter vector, pBBRNolacGUS to measure promoter activity. In pBBRNolacGUS, the constitutive lac promoter was deleted and the reporter gene was inserted, which is commonly used to analyze promoter activity in bacteria (Zhang et al., 2013; Wang L. et al., 2016). The results showed that the promoter activities of Aac5_2166 in ΔhrpG, ΔhrpX, and ΔhrpG-ΔhrpX-2166GUS strains were significantly lower (∼4-fold and ∼6-fold lower, respectively) than in WT-2166GUS, but similar to that in mock-treated WT-GUS. These data indicated that hrpG and hrpX regulate Aac5_2166 promoter activity (Figure 8B).

We speculated that the secretion of T3Es might be impaired in the ΔhrpG and ΔhrpX mutants. To test this hypothesis, we cloned Aac5_2166 and its native promoter and inserted them into pBBRNolac-4FLAG to concatenate a C-terminal fusion 4 × FLAG-tag. This construct was transformed into A. citrulli Aac5 and ΔhrpG to generate WT-2166 and ΔhrpG-2166. No signal was detected in the cell lysates and supernatants of ΔhrpG-2166, while a strong signal was detected for the WT-2166...
cell lysates and supernatants (Supplementary Image 3). These results indicated that HrpG regulates the expression of Aac5_2166 and that Aac5_2166 could not be synthesized in the hrpG mutant.

DISCUSSION

The mechanisms by which pathogenic bacteria regulate T3Es are critical for successful infection (Tampakaki et al., 2010) and have been well characterized in some plant-pathogenic bacteria. For example, hrpG and hrpX regulate T3Es in Xanthomonas spp. (Xue et al., 2014). Moreover, hrpG, as an OmpR family regulator, is typically regulated as a core gene of the T3SS by phosphorylation. OmpR family regulators, as response regulator (RR) proteins, belong to the two-component systems that consist of a histidine kinase and an RR protein, which serve as a basic stimulus-response coupling mechanism to allow organisms to sense and respond to changes in environmental conditions (Tampakaki et al., 2010; Li et al., 2014). The histidine kinase transfers a phosphoryl group to the RR in a reaction catalyzed by the RR. This phosphotransfer to the RR results in the activation of a downstream effector domain that elicits a specific response (Stock et al., 2000; Laub and Goulian, 2007). In the HrpG–HrpX pathway, HrpG activates HrpX, an AraC-type transcriptional activator, to regulate T3E expression. Unfortunately, the mechanisms that regulate the T3SS in A. citrulli are poorly understood. However, based on the similarity of the hrp clusters of A. citrulli and Xanthomonas spp. (Tampakaki et al., 2010; Burdman and Walcott, 2012), we hypothesized that a similar mechanism exists in A. citrulli. This mechanism likely involves genes homologous to those encoding the core proteins that regulate the T3SS in Xanthomonas spp. Genome sequence analyses identified two genes from the AAC00-1 genome (GenBank accession NC_008752) that
were homologs of hrpG and hrpX, designated Aave_0445 and Aave_0444. We cloned the homologs of these genes from *A. citrulli* strain Aac5 (Wang T. et al., 2016).

In the past two decades, researchers have shown that hrpG and hrpX regulate the expression of T3SS (Wengelnik et al., 1996b; Noël et al., 2001; Huang et al., 2009). However, these molecules also have other functions. Guo et al. (2011) showed that hrpG and hrpX play global roles in coordinating different virulence traits of *X. axonopodis pv. citri*, including traits involved in biofilm formation. Wang T. et al. (2016) showed that in Aac5, the transcription of hrpE, a core gene of T3SS, consistently is altered when biofilm formation changes, indicating that biofilm formation may be regulated by T3SS. The results of the current study confirm that hrpG and hrpX regulate biofilm formation in *A. citrulli* strain Aac5.

During the early stages of infection, plant-pathogenic bacteria colonize the host with the aid of biofilm formation. When hrpG and hrpX were deleted in *A. citrulli*, we observed significant changes in biofilm formation and virulence. As expected, the hrpG and hrpX mutants lost the ability to induce an HR in *N. tabacum*, which was confirmed by electrolyte leakage assays. Furthermore, pathogenicity on watermelon seedlings was lost in the mutants. Pathogenic bacteria must overcome the plant's immune systems, including PTI and ETI, to achieve successful infection (Zhang et al., 2007). Physiological markers for PTI typically include ROS generation (Boller and Felix, 2009), and the ETI pathway commonly results in localized, rapid PCD (Stork et al., 2015). The T3SS is essential for inducing a defense response in the host (Boller and Felix, 2009). Bacterial resistance mechanisms involve the secretion of T3Es, which come into contact with host-cell receptors and result in suppression of the host immune system (Dickman and Fluhr, 2013; Jiang et al., 2013). We observed that ΔhrpG and ΔhrpX elicited high-level ROS production, indicating that proteins encoded by these genes affect PTI. Additionally, expression of the putative T3E gene *Aac5_2166*, which encodes a YopJ homolog, was downregulated at the transcriptional level—via alterations in promoter activity—and at the translational level in the hrpG and hrpX mutants. Combined with the observation that the mutants lost the ability to induce an HR in *N. tabacum*, we concluded that the reduction in virulence in the hrpG and hrpX mutants was because they could not secrete T3Es and therefore, could not suppress PTI and ETI. This suggests that hrpG and hrpX play key roles in the regulation of T3Es.

Our findings demonstrate that *A. citrulli* strain Aac5 requires hrpG and hrpX to regulate virulence factors. hrpG and hrpX contribute to the expression of the T3SS and are involved in biofilm formation in this strain. Importantly, our findings

---

**FIGURE 7** The interaction of hrpG and hrpX in *Acidovorax citrulli*. (A) Western blotting analysis showed that HrpG activated HrpX. The WT, ΔhrpG, and ΔhrpX strains were cultured in liquid T3SS-inducing medium up to OD_600 = 0.5. Total cell extracts were analyzed by SDS-PAGE and immunoblotting, using specific antibodies. The experiment was performed three times. (B) Represents real-time quantitative RT-PCR to determine hrpX mRNA levels. hrpX expression was analyzed through RT-qPCR in wild-type strains Aac5 and the hrpG mutant strain, using specific primers. Bacteria were grown in liquid T3SS-inducing medium to OD_600 = 0.45 and harvested to extract total RNA. (C) Represents real-time quantitative RT-PCR to determine hrpG mRNA levels. hrpX expression was assayed through RT-qPCR in wild-type strain Aac5 and the hrpX mutant, using specific primers. All the data shown are means ± standard deviation of duplicate samples from one representative experiment and are reported as fold induction relative to expression of WT. The *rpoB* gene was used as a reference gene. Each column shows the mean and standard deviation. The experiment was performed three times, with similar results obtained each time. Asterisks above bars indicate significant differences as determined by t-test, p < 0.05.
provide, for the first time, strong evidence that hrpG and hrpX play key roles in A. citrulli Aac5 pathogenicity. Though strain Aac5 belongs to the group II strains of A. citrulli (Yan et al., 2013; Wang et al., 2016), on the basis of a genome sequence (GenBank accession JYHM00000000), it seems that the T3SS system structures of group I and group II strains are homologous. Moreover, key A. citrulli T3Es may be identified in hrpG and hrpX mutants using transcriptome analysis, which will improve our understanding of the molecular mechanisms of A. citrulli pathogenicity.

**AUTHOR CONTRIBUTIONS**

XZ and TZ designed the research and wrote the paper. XZ, JY, and LY executed the experiments. XZ, MZ, YY, and WG performed the data analyses. XZ, RW, TZ, and MZ critically reviewed the manuscript. All authors read and approved the final manuscript.

**ACKNOWLEDGMENTS**

We would like to thank Prof. Wenxian Sun at China Agricultural University for providing the vector PJY-mini-TN7T-GUS carrying GUS reporter gene for us to clone and construct the pBBRNolacGUS. This study was supported by the Beijing Natural Science Foundation (NO. 6162023) and the Fund for Modern Agro-industry Technology Research System (CARS-25).

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00507/full#supplementary-material

**Supplementary Table 1 | Primers used in the study.**

**Supplementary Image 1 |** The seedling syringe-infiltration assays of Acidovorax citrulli. (A) Response of watermelon cotyledons to syringe infiltration with Acidovorax citrulli wildtype strain Aac5, ΔhrpG and the complemented hrpG mutant (ΔhrpG-comp). The experiment was conducted three times.

**Supplementary Image 2 |** Programmed cell death assay for Aac5_2166 gene. The Aac5_2166 full-length gene sequence with primers was cloned and inserted into 1132 vector carrying the 35S promoter and eGFP tag, and transformed into the GV3101 strain to generate the strain 1132-2166. The suspension was inoculated into N. benthamiana leaves and the empty vector 1132 and buffer were used as negative controls. The photo was taken after 3 days. The experiment was conducted five times.

**Supplementary Image 3 |** Western blotting showed that effector Aac5_2166 lost the ability to express the protein in ΔhrpG mutant. WT-2166, wild-type strain Aac5 transformed with pBBR2166 carrying 4×FLAG tag. ΔhrpG-2166, ΔhrpG transformed with pBBR2166 carrying 4×FLAG tag. These strains were cultured with liquid T3SS induced medium up to OD_{600} = 0.6. To extract intracellular protein, 4 ml of cell suspension was treated with Protease Inhibitor Cocktail (Bimake, Shanghai, China). The lysate was centrifuged at 10,000 × g and 4 °C for 3 min, and the cells were resuspended in 200 μL of 4×Laemmli Sample Buffer (Bio-Rad, Beijing, China) and heated for 10 min. To extract secreted proteins, the

**FIGURE 8 |** Effects of hrpG and hrpX genes on the regulation of type 3 effectors in Acidovorax citrulli. (A) Real-time quantitative RT-PCR was used to determine Aac5_2166 mRNA levels. Putative type 3 secreted effector (T3E) Aac5_2166 gene expression was assayed through RT-qPCR in wild-type strain Aac5 and the hrpG and hrpX deletion mutant strains, using specific primers. The data shown are means ±standard deviation of duplicate samples from one representative experiment and are reported as fold induction relative to expression of WT. The rpoB gene was used as a reference gene. The experiment was performed three times, with similar results obtained each time. Asterisks above bars indicate significant differences as determined by t-test, p < 0.05; (B) The detection of promoter activity of the putative T3E Aac5_2166 was determined by assaying GUS activity. The pBBR2166GUS was constructed with Aac5_2166 native promoter and GUS reporter gene. The wild-type strain Aac5 transformed with vector pBBR2166GUS carrying the GUS reporter gene, WT-2166GUS; the hrpG mutant transformed with vector pBBR2166GUS carrying the GUS reporter gene, ΔhrpG-2166GUS; the hrpG mutant transformed with vector pBBR2166GUS carry GUS reporter gene, ΔhrpX-2166GUS; the wild-type strain Aac5 transformed with pBBRNolacGUS without promoter sequence, WT-GUS (negative control). Each column shows the mean and standard deviation. All experiments were performed three times, with similar results obtained each time. Different letters above bars indicate significant differences as determined by one-way analysis of variance (ANOVA) and Tukey's honest significant differences test, p < 0.05.
REFERENCES

Ancona, V., Lee, J. H., Chatnparapat, T., Oh, J., Hong, J. I., and Zhao, Y. (2015). The bacterial alarmones (p)pGpp activates the type III secretion system in Erwinia amylovora. J. Bacteriol. 197, 1433–1443. doi: 10.1128/JB.02551-14

Bahar, O., Goffer, T., and Burdman, S. (2009a). Type IV pili are required for virulence, twitching motility, and biofilm formation of Acidovorax avenae subsp. citrulli. Mol. Plant-Microbe Interact. 22,909. doi: 10.1094/MPMI-22-8-9099

Bahar, O., Kritzman, G., and Burdman, S. (2009b). Bacterial fruit blotch of melon: screens for disease tolerance and role of seed transmission in pathogenicity. Eur. J. Plant Pathol. 123, 71–83. doi: 10.1007/s10658-008-9345-7

Bahar, O., Levi, N., and Burdman, S. (2010). The cucurbit pathogenic bacterium Acidovorax citrulli requires a polar flagellum for full virulence before and after host-tissue penetration. Mol. Plant-Microbe Interact. 24, 1040–1050. doi: 10.1094/MPMI-2011-0041

Block, A., Li, G., Fu, Z. Q., and Alfano, J. R. (2008). Phytopathogen type III effector weaponry and their plant targets. Curr. Opin. Plant Biol. 11, 396–403. doi: 10.1016/j.pbi.2008.06.007

Bogdanove, A. J., Beer, S. V., Bonas, U., Boucher, C. A., Collmer, A., and Coplin, D. L. (1996). Unified nomenclature for broadly conserved hrp genes of phytopathogenic bacteria. Mol. Microbiol. 20, 681–683. doi: 10.1046/j.1365-2958.1996.5731077.x

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu. Rev. Plant Biol. 60, 379–406. doi: 10.1146/annurev.arplant.57.032905.105346

Burdman, S., and Walcott, R. (2012). Acidovorax citrulli: generating basic and applied knowledge to tackle a global threat to the cucurbit industry. Mol. Plant Microbe Interact. 13, 805–815. doi: 10.1094/MPMI-2012-00810x

Büttner, D., and Bonas, U. (2010). Regulation and secretion of Cui, Y., Zou, L., Zou, H., Li, Y., Zakria, M., and Chen, G. (2013). HrpE3 is a type III effector activates jasmonate signaling by directly targeting JAZ transcriptional repressors. PLoS Pathog. 9:e1003715. doi: 10.1371/journal.ppat.1003715

Johnson, K. L. (2010). The effector activator of Seed-to-Seedling Transmission of Acidovorax citrulli. Ph. Dissertation, The University of Georgia, Athens, GA.

Johnson, K. L., Minsvang, G. V., Le, T., Jones, J. B., and Walcott, R. R. (2011). Efficacy of a nonpathogenic Acidovorax citrulli strain as a biocontrol treatment for bacterial fruit blotch of cucurbits. Plant Dis. 95, 697–704. doi: 10.1094/PDIS-09-10-0660

Kim, J. G., Park, B. K., Yoo, C. H., Jeon, E., Oh, J., and Hwang, I. (2003). Characterization of the Xanthomonas axonopodis pv. glycines Hrp pili pathogenicity island. J. Bacteriol. 185, 3155–3166. doi: 10.1128/JB.185.8.3155-3166.2003

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., and Roop, R. M. (1995). Four new derivatives of the broad-host-range cloning vector pBR3MCS, carrying different antibiotic-resistance cassettes. Gene 167, 176. doi: 10.1016/0378-1101/000854-1

Kvitko, B. H., and Collmer, A. (2011). Construction of Pseudomonas syringae pv. tomato DC3000 mutant and polymutant strains. Methods Mol. Biol. 712, 109–128. doi: 10.1007/978-1-61737-998-7_10

Laub, M. T., and Goulain, M. (2007). Specificity in two-component signal transduction pathways. Annu. Rev. Genet. 41, 121–145. doi: 10.1146/annurev.genet.41.010407.150223

Li, R. F., Lu, G. T., Li, L., Su, H. Z., Feng, G. F., and Chen, V. (2014). Identification of a putative cognate sensor kinase for the two-component response regulator HrpG, a key regulator controlling the expression of the hrp genes in Xanthomonas campestris pv. campestris. Environ. Microbiol. 16, 2053–2071. doi: 10.1111/1462-2920.12207

Liu, L., Wang, Y., Cui, F., Fang, A., Wang, S., and Wang, J. (2017). The type III effector AvrXccB in Xanthomonas campestris pv. campestris targets putative methyltransferases and suppresses innate immunity in Arabidopsis. Mol. Plant Pathol. 18, 768–782. doi: 10.1111/mpp.12435

Liu, P., Zhang, W., Zhang, L. Q., Liu, X., and Wei, H. L. (2015). Supramolecular structure and functional analysis of the type III secretion system in Pseudomonas fluorescens 2P24. Front. Plant Sci. 6:1190. doi: 10.3389/fpls.2015.01190

Livel, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25, 402–408. doi: 10.1006/meth.2001.1262

Murata, Y., Tamura, N., Nakaho, K., and Mukaihara, T. (2006). Mutations in hrpB pathogenicity operon of Xanthomonas campestris pv. oryzicola which affects Hrp pili production and virulence. Mol. Plant-Microbe Interact. 19, 884–895. doi: 10.1094/MPMI-19-0884

Spa, and Fli secretion systems. Mol. Plant-Microbe Interact. 8, 845–854. doi: 10.1094/MPMI-8-0845

Guo, Y., Figureiredo, F., Jones, J., and Wang, N. (2011). HrpG and HrpX play global roles in coordinating different virulence traits of Xanthomonas axonopodis pv. citri. Mol. Plant-Microbe Interact. 24, 649–661. doi: 10.1094/MPMI-09-10-0209

Huang, D. L., Tang, D. J., Liao, Q., Li, X. Q., He, Y. Q., and Feng, J. X. (2009). The Zur of Xanthomonas campestris is involved in hypersensitive response and positively regulates the expression of the hrp cluster via hrpX but not hrpG. Mol. Plant-Microbe Interact. 22,321. doi: 10.1094/MPMI-22-3-0321

Jehle, A. K., Lipschis, M., Albert, M., Fallahzadeh-Mamaghani, V., Fürst, U., and Mueller, K. (2013). The receptor-like protein RemAX of Arabidopsis detects the microbe-associated molecular pattern eMax from Xanthomonas. Plant Cell 25, 2330–2340. doi: 10.1105/tpc.113.118033

Jiang, C. H., Wu, F., Yu, Z. Y., Xie, P., Ke, H. J., and Li, H. W. (2015). Study on screening and antagonistic mechanisms of Bacillus amyloliquefaciens 54 against bacterial fruit blotch (BFB) caused by Acidovorax avenae subsp. citrulli. Microbiol. Res. 170, 95–104. doi: 10.1016/j.micres.2014.08.009

Jiang, S., Yao, J., Ma, K. W., Zhou, H., Song, J., and He, S. Y. (2013). Bacterial effector activates jasmonate signaling by directly targeting JAZ transcriptional repressors. PLoS Pathog. 9:e1003715. doi: 10.1371/journal.ppat.1003715
Noël, L., Thiere, F., Nennstiel, D., and Bonas, U. (2001). cDNA-AFLP analysis unravels a genome-wide hrpG-regulon in the plant pathogen Xanthomonas campestris pv. vesicatoria. Mol. Microbiol. 41, 1271–1281. doi: 10.1046/j.1365-2958.2001.02567.x

Nomura, K., Mecsey, C., Lee, Y. N., Imboden, L. A., Chang, J. H., and He, S. Y. (2011). Effector-triggered immunity blocks pathogen degradation of an immunity-associated vesicle traffic regulator in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 108, 10774–10779. doi: 10.1073/pnas.1103388108

Ren, Z. G., Hou, L., Song, Z. G., and Zhang, L. Q. (2009). Screening of the pathogenicity mutants of Acidovorax avenae subsp. citrulli and cloning of the hrcR gene. Acta Physiopathol. Sin. 39, 501–506.

Ren, Z. G., Jiang, W. J., Ni, X. Y., Lin, M., Zhang, W., and Tian, G. Z. (2014). Multiplication of Acidovorax citrulli in plants during infection of melon seedlings requires the ability to synthesize leucine. Plant Pathol. 63, 784–791. doi: 10.1111/ppa.12156

Rossier, O., Wengelnik, K., Hahn, K., and Bonas, U. (1999). The Xanthomonas Hrp type III system secretes proteins from plant and mammalian bacterial pathogens. Proc. Natl. Acad. Sci. U.S.A. 96, 9368–9373. doi: 10.1073/pnas.96.19.9368

Sambrook, J., and Russell, D. (2001). Molecular Cloning: A Laboratory Manual (Third Edition). New York, NY: Cold Spring Harbor Laboratory Press.

Schaad, N. W., Postnikova, E., Sechler, A., Claffin, L. E., Vidaver, A. K., Jones, J. B., Schulte, R., and Bonas, U. (1992). A Two-component signaling system VgrRS directly senses extracytoplasmic and intracellular iron to control bacterial adaptation under iron-depleted stress. PLoS Pathog. 12:e1006133. doi: 10.1371/journal.ppat.1006133

Wang, T., Guan, W., Huang, Q., Yang, Y., Yan, W., Sun, B., et al. (2016). Quorum-sensing contributes to virulence, twitching motility, seed attachment and biofilm formation in the wild type strain Aae-5 of Acidovorax citrulli. Microb. Pathog. 100, 133–140. doi: 10.1016/j.micpath.2016.09.039

Wang, T., Sun, B., Yang, Y., and Zhao, T. (2015). Genome sequence of Acidovorax citrulli group 1 strain PBS65 causing bacterial fruit blotch of melons. Genome Announc. 3, e00327–e00315. doi: 10.1128/genomeA.00327-15

Wengelnik, K., Marie, C., Russel, M., and Bonas, U. (1996a). Expression and localization of HrpA1, a protein of Xanthomonas campestris pv. vesicatoria essential for pathogenicity and induction of the hypersensitive reaction. J. Bacteriol. 178, 1061–1069. doi: 10.1128/jb.178.4.1061-1069.1996

Wengelnik, K., Van den Ackerveken, G., and Bonas, U. (1996b). HrpG, a key hmr regulatory protein of Xanthomonas campestris pv. vesicatoria is homologous to two-component response regulators. Mol. Plant-Microbe Interact. 9, 704–712. doi: 10.1094/MPMI-9-0704

Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K., and De Ley, J. (1992). Transfer of several phytopathogenic Pseudomonas species to Acidovorax as Acidovorax avenae subsp. avenae subsp. nov. Int. J. Syst. Bacteriol. 42, 107–119. doi: 10.1099/00207713-42-1-107

Xin, X. F., Nomura, K., Aung, K., Velásquez, A. C., Yao, J., and Boutrout, F. (2016). Bacteria establish an aequous living space in plants crucial for virulence. Nature 539, 524–529. doi: 10.1038/nature20166

Xue, X. B., Zou, L. F., Ma, W. X., Liu, Z. Y., and Chen, G. Y. (2014). Identification of 17 HrpX-regulated proteins including two novel type III effectors, XOC_3956 and XOC_1550, in Xanthomonas oryzae pv. oryzicola. PLoS ONE 9:e93205. doi: 10.1371/journal.pone.0093205

Yan, L., Hu, B., Chen, G., Zhao, M., and Walcott, R. R. (2017). Further evidence of cucurbit host specificity among Acidovorax citrulli groups based on a detached melon fruit pathogenicity assay. Phytopathology 107, 1305–1311. doi: 10.1094/PHYTO-11-16-0416-R

Yan, S., Yang, Y., Wang, T., Zhao, T., and Schaad, N. W. (2013). Genetic diversity analysis of Acidovorax citrulli in China. Eur. J. Plant Pathol. 136, 171–181. doi: 10.1007/s10658-012-0152-9

Yan, W. R., Wang, T. L., Yang, Y. W., Dai, L. Y., and Zhao, T. C. (2015). Biological function analysis of hrcN gene of Acidovorax citrulli. Acta Phytopathol. Sin. 45, 33–40. doi: 10.1392/j.cnki.aps.2015.01.005

Yuan, J., and He, S. Y. (1996). The Pseudomonas syringae Hrp regulon and secretion system controls the production and secretion of multiple extracellular proteins. J. Bacteriol. 178, 6399–6402. doi: 10.1128/jb.178.21.6399-6402.1996

Zivanovic, M., and Walcott, R. R. (2017). Further characterization of genetically distinct groups of Acidovorax citrulli strains. Phytopathology 107, 29–35. doi: 10.1094/PHYTO-06-16-0245-R

Conflict of Interest Statement: 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. Copyright © 2018 Zhang, Zhao, Yan, Yang, Guan, Walcott and Zhao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.