Natural Variation in Virulence of *Acidovorax citrulli* Isolates That Cause Bacterial Fruit Blotch in Watermelon, Depending on Infection Routes

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Acidovorax citrulli causes bacterial fruit blotch in *Cucurbitaceae*, including watermelon. Although *A. citrulli* is a seed-borne pathogen, it can cause diverse symptoms in other plant organs like leaves, stems and fruits. To determine the infection routes of *A. citrulli*, we examined the virulence of six isolates (Ac0, Ac1, Ac2, Ac4, Ac8, and Ac11) on watermelon using several inoculation methods. Among six isolates, DNA polymorphism reveals that three isolates Ac0, Ac1, and Ac4 belong to Clonal Complex (CC) group II and the others do CC group I. Ac0, Ac4, and Ac8 isolates efficiently infected seeds during germination in soil, and Ac0 and Ac4 also infected the roots of watermelon seedlings wounded prior to inoculation. Infection through leaves was successful only by three isolates belonging to CC group II, and two of these also infected the mature watermelon fruits. Ac2 did not cause the disease in all assays. Interestingly, three putative type III effectors (Aave_2166, Aave_2708, and Aave_3062) with intact forms were only found in CC group II. Overall, our results indicate that *A. citrulli* can infect watermelons through diverse routes, and the CC grouping of *A. citrulli* was only correlated with virulence in leaf infection assays.

Keywords : bacterial fruit blotch, clonal complex groups, effector genes, infection, virulence

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Bacterial fruit blotch (BFB) is caused by the Gram-negative bacterium *Acidovorax citrulli* (previously known as *Acidovorax avenae* subsp. *citrulli*) in *Cucurbitaceae*, including watermelon (*Citrullus lanatus*) and melon (*Cucumis melo*) (Schaad et al., 1978, 2008; Willems et al., 1992). The disease was first reported in Georgia, USA in 1965 (Webb and Goth, 1965), and has been reported worldwide (Burdman and Walcott, 2012). The typical symptoms caused by *A. citrulli* are water-soaking regions in cotyledons and leaves followed by necrotic symptoms, wilting and necrosis on stems, and irregularly-shaped water-soaked lesions on fruits (Schaad et al., 2003).

A. citrulli isolates have been divided into two groups. Group I is moderately aggressive on all cucurbit host plants except watermelon, while group II causes more severe symptoms on watermelon than on other host plants (Walcott et al., 2004). These groups coincide with the Clonal Complex (CC) groups I and II, based on multi-locus sequence typing analysis (Feng et al., 2009). In addition, CC groups can be easily distinguished by DNA sequence polymorphisms in the housekeeping gene *gltA*: the isolates in CC group I have C, G, and A, while the isolates in CC group II have G, A, and C at the positions 439, 442, and 451 from the start codon, respectively (Song et al., 2015; Yan et al., 2013).

A. citrulli is known as a seed-borne pathogen, and infected seeds have been considered as the primary inoculum for BFB epidemics (Hopkins and Thompson, 2002; Latin and Hopkins, 1995; Rane and Latin, 1992). One of the known infection routes of *A. citrulli* into seeds is through a pollinated female flower (Walcott et al., 2003). Particularly, pathogen infection through the pistil causes deep infection into the cotyledon part of the mature seed, resulting in longer bacterial survival in seeds (Block and Shepherd, 2008;

Dutta et al., 2012). The cotyledons grown from infected seeds may be the main source for a secondary inoculum for spreading *A. citrulli* to neighboring healthy seedlings (Chalupowicz et al., 2015). Thus, seed decontamination is an important strategy for BFB management (Kubota et al., 2012).

Because BFB symptoms occur on a range of plant organs such as leaves and fruits of watermelon, *A. citrulli* must have the ability to infect host plants through other organs besides flowers. Previous reports showed that *A. citrulli* could infect melon leaves after spray inoculation and moved to stems (Alves et al., 2010; Neto et al., 2006). *A. citrulli* was also able to colonize and move through xylem vessels to probably reach fruits and seeds (Bahar et al., 2009). Moreover, this pathogen passively moved to soil after irrigation from infected cotyledons of melon seedlings to roots of neighboring seedlings, and eventually migrated to stems in the neighboring hosts (Chalupowicz et al., 2015). However, a detailed dissection of infection routes and their relationship with virulence of *A. citrulli* in host plants still remains to be determined.

Thus, in this study, we hypothesized that *A. citrulli* could infect host plants through diverse routes and so focused on investigating possible routes of *A. citrulli* infection other than flowers in watermelon. In addition, we examined if CC grouping is related with infection routes and virulence of *A. citrulli* isolates.

Materials and Methods

Bacterial isolates and inoculum preparation. Six *A. citrulli* isolates isolated from watermelon showing disease symptom (Ac0, Ac2, Ac4, Ac8, and Ac11 isolates) or its root stock (Ac1 isolate) were used in this study (Rahimi-Midani et al., 2018) (Table 1). One was obtained from Koran Agricultural Culture Collection (KACC17005;

Ac0) and five from Nongwoo Bio Co. (Suwon, Korea; Ac1, Ac2, Ac4, Ac8, and Ac11). Each isolate was confirmed by PCR with the species-specific primers SEQID4 (5'-TCGTCATTACTGAATTTCAACA-3') and SEQID5 (5'-CCTCCACCAACCAATACGCT-3') amplifying the 16/25S rRNA gene region (Makizumi et al., 2011; Schaad et al., 2000). For monitoring *A. citrulli* populations in watermelon after inoculation, a rifampicin-resistant strain of each isolate was generated as previously described (Choi et al., 2016) and used for plant assays. *A. citrulli* isolates and strains were stored at -80°C with 25% glycerol and cultured at 26°C on King's B medium (containing proteose peptone 20 g, K_2HPO_4 1.5 g, MgSO_4 1.5 g, glycerol 10 ml, agar 15 g in 1,000 ml distilled water) with 50 mg/l rifampicin.

To prepare inoculum for plant assays, rifampicin-resistant *A. citrulli* strains were pre-cultured in 5 ml of King's B broth in a 14 ml round bottom culture tube (SPL Life Sciences Co., Ltd., Pocheon, Korea) at 26°C overnight in a shaking incubator at 180 rpm. One hundred microliters of overnight cultures were used to re-culture them in 20 ml of King's B broth in a 50 ml snap tube (SPL Life Sciences Co., Ltd.) overnight. After centrifugation of bacterial culture at 8,000 rpm for 3 min, the pellet was washed with 20 ml of 10 mM MgCl_2 . After washing once, the pellet was re-suspended with the same buffer and diluted to 10^6 cfu/ml as an inoculum.

Detection of *A. citrulli* by PCR. The PCR method with SEQID4F and SEQID5R primer set was used to detect *A. citrulli* isolates from watermelon seeds, leaves, seedlings, or plants. PCR amplification was conducted in a 10 μl reaction with $2\times$ pre-mixed Taq (Enzynomics, Daejeon, Korea) and 1 μl of plant extracts, according to the manufacturer's instructions. The PCR cycle was as follows: 95°C for 5 min followed by 30 cycles of PCR consisting of denaturation

Table 1. *Acidovorax citrulli* isolates used in this study

Isolates	Strain names ^a	Isolation sites	Isolated hosts	DNA polymorphism ^b (439, 442, 451)	Clonal complex ^c	Sources
Ac0	KACC17005	Suwon, South Korea	Watermelon	G, A, C	II	KACC
Ac1	NWBSC074	Gimje, South Korea	Watermelon rootstock	G, A, C	II	Nongwoo Bio Co.
Ac2	NWBSC107	Haman, South Korea	Watermelon	C, G, A	I	Nongwoo Bio Co.
Ac4	NWBSC109	Buyeo, South Korea	Watermelon	G, A, C	II	Nongwoo Bio Co.
Ac8	NWBSC196	Miryang, South Korea	Watermelon	C, G, A	I	Nongwoo Bio Co.
Ac11	NWBSC206	Nonsan, South Korea	Watermelon	C, G, A	I	Nongwoo Bio Co.

^aNames, originated from Park et al. (2017) and Rahimi-Midani et al. (2018).

^bDNA polymorphism at the indicated base pair position from the start codon of *gltA* gene.

^cBased on DNA polymorphism in the housekeeping gene *gltA* (Song et al., 2015; Yan et al., 2013).

at 95°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Five microliters of PCR products was separated by electrophoresis at 135 V for 15 min on a 1.5% agarose gel in 0.5× TBE buffer with a 1 kb DNA ladder (Sigma-Aldrich, St. Louis, MO, USA).

Inoculation of *A. citrulli* by soil mixing. Soil contaminated with this bacterium was produced by pouring 50 ml of each bacterial isolate into 165 g of sterile soil (3×10^5 cfu/g). Right after, five seeds of watermelon cultivar ‘Speed Plus’ (Nongwoo Bio Co.), which was commercially available, were sown in this soil in each hole of a 32-hole tray (Seoul Bio, Eumseong, Korea), and 20 seeds were used for each isolate. The tray was kept in an incubator at 26°C, and symptom development in the seedlings was monitored for 2 weeks. The severity of disease symptom in the seedlings was scored based on the following disease indices: 0, no symptom; 1, weak water-soaking spots on cotyledon; 2, strong water-soaking or necrosis spots on cotyledon; 3, stem bending; 4, stem falling; 5, whole plant death (Supplementary Fig. 1A). To measure the bacterial titer in the seedling, the hypocotyls from 4 seedlings was ground after surface sterilization with 1% sodium hypochlorite for 30 s and washing twice with distilled water. Then, the extracts were re-suspended with 500 µl of 10 mM MgCl₂ and serially diluted. The extracts were placed on King’s B (KB) agar plate with 50 µg/ml rifampicin for growth of bacterial colonies. These experiments were repeated twice with the similar results.

Inoculation of *A. citrulli* by soil drenching. Five-day-old seedlings were treated with 10^6 cfu/ml of bacterial suspension by pouring it near seedlings in a pot, and six seedlings were used per each isolate. To check if wounding in roots can increase infection by *A. citrulli*, the 5-day-old watermelon seedlings were pulled out, and their roots were cut by scissors. After they were re-planted, each bacterial suspension (10^6 cfu/ml) or 10 mM MgCl₂ as a mock treatment was poured into soil near the seedlings. Six seedlings were used per each isolate. Then, symptom development was observed for one week, and the disease severity in the seedlings was scored, as described above (Supplementary Fig. 1A). These experiments were repeated twice with the similar results.

Spray inoculation of *A. citrulli*. Three-week-old watermelon plants were sprayed with 10 mM MgCl₂ or bacterial suspension with 0.02% silwet to make them completely wet, and 8 to 12 seedlings per each isolate were used. The treated plants were covered with clear vinyl for 24 h to

retain humidity. On the seventh day after spray inoculation (dai), a bacterial titer in leaves was measured after surface sterilization. The disease severity in the leaves was scored based on the following disease indices: 0, no symptom; 1, yellowing and marginal necrosis; 2, complete necrosis (Supplementary Fig. 1B). These experiments were repeated twice with the similar results.

Syringe or vacuum infiltration of *A. citrulli*. For syringe infiltration, five fully developed leaves of 3-week-old watermelon seedlings per treatment were infiltrated with 10 mM MgCl₂ or bacterial suspension (10^4 cfu/ml) using a needleless syringe. For vacuum infiltration, the aerial parts of 3-week-old seedlings were dipped into each *A. citrulli* culture suspension (10^6 cfu/ml) in the vacuum infiltration system (Rocker410, SPARMAX, Taipei, Taiwan) with the vacuum pump on to let bacterial cells enter into the leaves. In both cases, development of disease symptoms in leaves was monitored for 2 weeks, and, to measure a bacterial titer in the infiltrated leaves, four leaf discs per treatment were collected with a 6-mm diameter cork-borer every 2 dai. The disease severity in the leaves was scored based on disease index scale in Supplementary Fig. 1B.

Inoculation of *A. citrulli* by fruit injection. For fruit inoculation, the surface of mature harvested watermelon fruits was punctured with a 200 µl pipette tip at a depth of 2.5 cm, and 50 µl of bacterial suspension (10^6 cfu/ml) per each isolate was injected inside the puncture. Development of disease symptom was monitored for 2 to 3 weeks. Then, the fruits were cut and symptoms inside the fruits were observed. To determine if *A. citrulli* could enter into seeds, all seeds from watermelon fruit injected with Ac1 isolate, which caused the most severe symptom in fruit, were harvested 10 and 40 dai. After surface sterilization of seeds, each seed was ground separately with 500 µl of 10 mM MgCl₂ buffer. After 30 min shaking, the extracts were diluted 10 times and then placed on KB medium for colony development. These experiments were repeated twice with the similar results.

Effector gene prediction and profiling. Prediction of open reading frames (ORFs) of putative effector genes was performed by Artemis v.17.0.1 (<http://www.sanger.ac.uk/science/tools/artemis>). To check the intact putative effector genes in *A. citrulli* isolates, the genome sequence was used for Ac0 isolate (Park et al., 2017), and gene-specific primers (Supplementary Table 1) were designed, and PCR with those primers was performed for other five isolates. The PCR products were purified and sequenced.

Statistical analysis. For bacterial titers, Duncan's multiple range test ($P < 0.05$) was performed with SAS (version 9.4 for Windows; SAS Institute Inc., Cary, NC, USA). For disease severity, non-parametric statistics were performed by Kruskal-Wallis test using statistiXL (v. 1.8, 2008, statistiXL–Nedlands, WA, Australia, <http://www.statistiXL.com>).

Results

Grouping and preparation of rifampicin-resistant *A. citrulli* isolates. Six *A. citrulli* natural isolates isolated from watermelon showing disease symptoms or watermelon

root stock from different locations in South Korea were obtained (Table 1). By checking DNA sequences of the *gltA* housekeeping gene in these six isolates, they were divided into two groups: Ac2, Ac8, and Ac11 in CC group I and Ac0, Ac1, and Ac4 in CC group II (Table 1). To distinguish *A. citrulli* isolates used in this study from other bacteria, their spontaneous mutants resistant to rifampicin antibiotics were generated and used for further experiments.

Infection efficiency and virulence of six *A. citrulli* isolates from soil to watermelon seeds. To examine if *A. citrulli* can infect watermelon seeds during germination in soil, soil was infested with 3×10^5 cfu/g of each iso-

Fig. 1. Infection efficiency and virulence of six *Acidovorax citrulli* isolates through soil. (A) The seeds were sowed in soil infested with 10^6 cfu/ml of each indicated isolate. 10 mM $MgCl_2$ buffer was used as a mock control. Watermelon seedlings showing typical symptoms, water-soaked spots, blotch or wilting, at 7 days after inoculation (dai). The red and yellow arrows in left pictures indicate severe blotch and weak symptom, respectively. Right pictures are representatives of seedlings showing the most severe symptoms. (B) The disease severity by bacterial isolates at 10 dai and 13 dai that was scored according to the following disease index scale: 0, no symptoms; 1, slight water-soaking spots on cotyledon; 2, massive water-soaking or necrosis spots on cotyledon; 3, bending of stem; 4, falling stem; 5, necrosis of whole plant (Supplementary Fig. 1A). Bars represent standard error ($n = 20$). (C) Confirmation of the presence of inoculated bacteria by PCR. The colonies isolated from infiltrated leaves were confirmed by PCR using the 16/25S rRNA gene primer set. M, 1 kb ladder marker; P, Ac0 genomic DNA (positive control); N, H_2O (negative control). (D) Bacterial cell numbers in watermelon seedlings at 7 dai and 10 dai. Y-axis represents mean $\log_{10}$ cfu/g of watermelon, and bars indicate standard error ($n = 4$). The letters on top of error bars show results from Duncan's multiple range test ($P < 0.05$).

late, and then watermelon seeds were sowed. First, the germination rate was almost over 90% for all treatments. Second, symptom development and disease severity were monitored for 2 weeks. Common symptoms in the young seedlings were water-soaking at the cotyledons, later turning to leaf necrosis and eventually wilting and necrosis of the whole seedlings (Fig. 1A). These symptoms were observed in seedlings grown in soil infested with all isolates except Ac2 (Fig. 1A). Disease severity caused by six

isolates showed similar patterns in 10 and 13 dai (Fig. 1B). Disease severity was the highest and similar in Ac0, Ac4, and Ac8, followed by Ac1 and Ac11. The Ac2 isolate consistently failed to cause obvious disease symptom even two weeks after inoculation. Third, bacterial titers per gram of seedlings were measured at 7 and 10 dai. Before measuring the titers, the bacteria isolated and grown on medium with rifampicin were confirmed by PCR (Fig. 1C). Consistent with disease severity, Ac0, Ac4, and Ac8 isolates showed

Fig. 2. Infection efficiency and virulence of six *Acidovorax citrulli* isolates through wounding in roots. The watermelon seedlings were pulled out to injure the roots on the fifth day after sowing and their roots were cut by scissors. After they were re-planted, each bacterial suspension (10^6 cfu/ml) or 10 mM $MgCl_2$ as a mock treatment was poured into soil near seedlings. (A) Watermelon seedlings showing symptoms at 2 days after inoculation (dai). In photos, left and right pots represent non-cut and cut groups, respectively. The arrows indicate symptomatic watermelon. (B) The disease severity by bacterial isolates at 2 dai that was scored according to disease index scale in Supplementary Fig. 1A. Bars represent standard error ($n = 6$). (C) The bacterial concentration in non-cut- or cut- roots of watermelon seedlings at 2 dai. Y-axis indicate the average of bacterial cell numbers per seedling ($\log_{10}$ cfu/root). Bars represent standard error ($n = 3$) and the letters on top of error bars show results from Duncan's multiple range test ($P < 0.05$).

high titers compared with other isolates at 7 dai (Fig. 1D). Interestingly, the Ac11 isolate titer was relatively high although disease severity caused by the isolate was low. Surprisingly, about 10^4 cfu/g of Ac2 isolate was detected at both 7 and 10 dai (Fig. 1D), indicating that Ac2 can be

transmitted through soil and propagate inside the seedlings without symptom development. Overall Ac0, Ac4, and Ac8 were highly virulent in watermelon regardless of CC group, and Ac2 was almost non-pathogenic. These results indicate that *A. citrulli* can infect watermelon seeds during

Fig. 3. Infection efficiency and virulence of *Acidovorax citrulli* isolates infected by spraying on watermelon seedlings. Three-week-old seedlings were sprayed with bacterial suspension (10^6 cfu/ml + 0.02% silwet). Two hours after spraying, seedlings were covered with transparent plastic bags to keep moist inside for 24 h. (A) Symptoms on leaves 7 days after inoculation (dai). (B) The disease severity at 7 dai that was scored according to disease index scale in Supplementary Fig. 1B ($n = 4$). (C) Measurement of bacterial titers in aerial part of watermelon seedlings at 7 dai. Y-axis indicates the average of bacterial cell numbers per aerial part of each seedling ($\text{Log}_{10}\text{cfu/ea}$). Bars represent standard error ($n = 4$) and the letters on top of error bars show results from Duncan's multiple range test ($P < 0.05$).

germination in soil and CC grouping is not correlated with the level of virulence in the given condition.

Infection efficiency and virulence of *A. citrulli* isolates through roots. To determine if *A. citrulli* isolates can infect watermelon seedlings through seedling roots, 5-day-old watermelon seedlings with or without wounded roots were inoculated with *A. citrulli* isolates by soil drenching with a 10^6 cfu/ml bacterial suspension. Then, disease severity and bacterial titers were measured. Disease symptoms appeared in the wounded seedlings at only 2 dai, but symptoms were

not observed in the non-cut roots (Fig. 2A). Interestingly, only Ac0 and Ac4 isolates, which belong to CC group II, caused severe disease symptoms (Fig. 2B). *A. citrulli* infection in seedlings without wounding in roots was poor, with bacterial titers of all *A. citrulli* isolates lower than 10^3 cfu per root (Fig. 2C). However, infection efficiency dramatically increased through root cutting before inoculation, with bacterial titers detecting up to 10^8 cfu per root for *A. citrulli* isolates Ac0 and Ac4 (Fig. 2C). Although Ac4 and Ac8 isolates did not caused disease symptoms, bacterial population of the two isolates were relatively high (Fig.

Fig. 4. Virulence of *Acidovorax citrulli* isolates infected by syringe infiltration into leaves of 3-week-old watermelon and melon plants. (A) Symptoms in watermelon leaves after infiltration with 10^4 cfu/ml of each indicated isolate at 6 days after inoculation (dai). (B) The disease severity at 6 dai that was scored according to disease index scale in Supplementary Fig. 1B ($n = 4$). (C) Bacterial growth in the watermelon leaves. Y-axis indicates the average of $\text{Log}_{10}\text{cfu}/\text{cm}^2$ in the infected watermelon leaves. Bars represent the standard error ($n = 9$; 9 leaf discs/treatment) and the letters on top of error bars show results from Duncan's multiple range test ($P < 0.05$).

2C). These results indicate that it is difficult for *A. citrulli* to infect watermelon seedlings through intact roots after germination, but wounding can help *A. citrulli* infect roots.

Infection efficiency and virulence of *A. citrulli* isolates through the leaf surface. One symptom caused by *A. citrulli* is water-soaking in leaves, followed by necrosis. Therefore, the infection efficiency of *A. citrulli* isolates through the leaf surface was determined by the spray

inoculation method. Three-week-old watermelon plants were sprayed with 10^6 cfu/ml of each *A. citrulli* isolate. In this case, 0.02% silwet was added to the bacterial suspension, and plants were covered by transparent plastic bags to hold humidity for 24 h after spraying. Necrotic disease symptoms in leaves inoculated with Ac0, Ac1, or Ac4 isolates, which belong to CC group II, were obvious at 7 dai (Fig. 3A). Among these three, Ac1 caused the most severe symptoms (Fig. 3B). However, Ac2, Ac8, and Ac11 iso-

Fig. 5. Virulence of *Acidovorax citrulli* isolates after inoculation into fully ripe watermelon fruits. Watermelon fruits were inoculated with 10^6 cfu/ml of *A. citrulli* isolates using a pipette at a depth of 0.5 cm from the surface and incubated at room temperature for 40 days. (A) Symptoms in the surface of or inside the watermelon fruits at 24 days after inoculation (dai) with 10^6 cfu/ml of each indicated isolate. The mark at the bottom right represents the extent of disease. (B) Confirmation of the presence of inoculated bacteria by PCR using a 16/25S rRNA gene primer set. The fruit extract was cultured on medium for 24 h at 26°C, and colonies on medium were used for PCR. M, 1 kb ladder marker; P, Ac0 genomic DNA (positive control); N, H₂O (negative control). (C) Percentage of infected seeds collected from watermelons inoculated with Ac1 isolate (10^6 cfu/ml). All seeds were collected at 10 dai and 40 dai, and bacterial titers inside each seed were counted. The percentage of seeds with each range of bacterial titer was calculated by the following equation: (number of seeds with each range of bacterial titer/total seed number) $\times$ 100.

lates, which belong to CC group I, did not show symptoms at the same time point (Fig. 3B). Bacterial titers of Ac0, Ac1, and Ac4 isolates were 10^9 cfu per aerial part of each seedling (cfu/ea) (Fig. 3C). Although bacterial titers of Ac8 and Ac11 reached around 10^7 cfu/ea, Ac2 was not detected (Fig. 3C). These results indicate that *A. citrulli* isolates belonging to CC group II can efficiently infect leaves and cause necrotic symptoms in leaves, but the infection efficiency of isolates belonging to CC group I is poor.

Virulence of *A. citrulli* isolates infiltrated into watermelon leaves. To bypass the entry step through leaves, 10^4 cfu/ml of each *A. citrulli* isolate was infiltrated directly into leaves of 3-week-old watermelon plants using a needleless syringe. Similar to the results from spray inoculation, Ac0, Ac1, and Ac4 isolates caused severe necrosis (Fig. 4A). Bacterial titers of these isolates reached more than 10^{10} cfu per cm^2 of inoculated leaves (cfu/ cm^2) at 4 dai (Fig. 4C). However, Ac8 and Ac11, which did not cause any disease symptom with spray inoculation, caused mostly yellowing in the inoculated regions, while Ac2 did not consistently cause disease symptom (Fig. 4B). Bacterial titers of Ac8 and Ac11 reached 10^7 cfu/ cm^2 , which was similar to the level by spray inoculation at four dai (Fig. 4C). Unlike with the spray inoculation method, Ac2 multiplied almost up to 10^6 cfu/ cm^2 at 4 dai, indicating that, although the entry from the leaf surface was poor, Ac2 had the ability to grow inside the leaf tissue. In addition to syringe infiltration, vacuum infiltration of the whole plants with 10^4 cfu/ml of the same Ac isolates with 0.02% silwet was performed. Overall results were very similar to those of syringe infiltration, although disease symptom development was weaker and bacterial titers were lower (Supplementary Fig. 2).

Virulence of *A. citrulli* isolates in mature watermelon fruits and their infectivity to seeds. The most typical symptom caused by *A. citrulli* in watermelon is fruit blotch. To determine virulence of *A. citrulli* isolates in mature watermelon fruits, 10^6 cfu/ml of each isolate was inoculated using a pipette at a depth of 0.5 cm from the surface, and symptom development in fruits and infectivity to seeds inside were monitored for 40 days after inoculation. Blotch symptoms were developed inside the inoculation sites, although symptoms were not obvious outside the fruits. Among the six isolates, Ac1 and Ac4 caused severe blotch symptoms inside, Ac0, Ac8, and Ac11 caused little, and Ac2 did not cause (Fig. 5A). Inoculation made at a lower depth showed the same results (data not shown). The presence of inoculated Ac isolates at sites showing symptoms was confirmed by PCR with an *A. citrulli*-specific primer

set after isolating bacteria from a fruit extract (Fig. 5B). Finally, infectivity of *A. citrulli* isolates to seeds was determined only with the Ac1 isolate, which caused severe symptom in fruits. A total of 144 and 108 seeds were collected from the inoculated watermelons at 10 dai and 40 dai respectively, and bacterial titers were measured. At 10 dai, only approximately 9% of seeds (13 out of 144 seeds) were pathogen-free and seeds carrying 10^2 to 10^4 cfu/seed were dominant, while at 40 dai, all seeds were infected with more than 10^2 cfu/seed and seeds carrying 10^5 to 10^7 cfu/seed were dominant (Fig. 5C), indicating that Ac1 infects seeds very efficiently and more bacteria colonize inside seeds as time goes on.

Profiles of putative effector genes of *A. citrulli* isolates.

Sixteen putative effector genes were found in the genome of *A. citrulli* strain AAC00-1 from previous reports (Bahar and Burdman, 2010; Eckshtain-Levi et al., 2014) and genome searching with Artemis v.17.0.1. Profiles of those effector genes were determined in Ac0 isolate, using the genome sequence (Park et al., 2017) and PCR with gene-specific primers and DNA sequencing for other five isolates (Table 2). Ac0, Ac1, and Ac4 isolates carried all genes, while Ac2, Ac8 and Ac11 isolates carried only thirteen intact genes. The latter three isolates carried two non-functional effector genes, Aave_2166 and Aave_3062, which encode homologs of AvrBsT and AvrRxo1 of *X. euvesicatoria*, respectively, due to deletion of certain bases or transposon insertion, resulting in early translational stop. Moreover, these isolates lacked the Aave_2708 gene, which encodes the XopJ homolog of *X. euvesicatoria*. Because only Ac0, Ac1 and Ac4 among the six isolates showed high virulence through leaves, these three putative effector genes (Aave_2166, Aave_2708, and Aave_3062) may be important for leaf infection or CC grouping.

Discussion

In this study, we showed that *A. citrulli* could infect watermelon through diverse routes, including seeds during germination, roots, leaves, and fruits, which are equivalent to sites where disease symptoms develop under the natural conditions (Latin and Hopkins, 1995). However, this statement appears correct only when many *A. citrulli* isolates are considered as a single population because not all isolates can infect through all possible routes. Our study showed that the infection routes of certain *A. citrulli* isolates might be limited because three out of six *A. citrulli* isolates were virulent only when they contacted certain organs (Table 3). This indicates that virulence of *A. citrulli*

Table 2. Profiles of putative effector genes in six *Ac* isolates confirmed by PCR with gene-specific primers and DNA sequencing

Putative effector gene ^a	Protein homolog ^b	Identity (%) ^b	Positives (%) ^b	<i>Acidovorax citrulli</i> isolates					
				Ac0	Ac1	Ac2	Ac4	Ac8	Ac11
Aave_0277	HopG1 of <i>Ralstonia solanacearum</i>	65	78	+	+	+	+	+	+
Aave_1373	AvrXv3 of <i>Xanthomonas euvesicatoria</i>	50	62	+	+	+	+	+	+
Aave_1548	HopW1-1 (HopPmaA) of <i>Pseudomonas syringae</i> pv. <i>maculicola</i>	53	70	+	+	+	+	+	+
Aave_2166	AvrBsT of <i>X. euvesicatoria</i>	66	81	+	+	Del ^c	+	Del ^c	Del ^c
Aave_2173	AvrBs1 of <i>Xanthomonas gardneri</i>	45	63	+	+	+	+	+	+
Aave_2708 (Aave_2938)	XopJ of <i>X. euvesicatoria</i>	100	100	+	+	–	+	–	–
Aave_2801	Rsc0782 of <i>R. solanacearum</i>	46	57	+	+	+	+	+	+
Aave_2802	HopD1 of <i>P. syringae</i> pv. <i>tomato</i>	26	41	+	+	+	+	+	+
Aave_2876	HopH1 of <i>P. syringae</i>	42	62	+	+	+	+	+	+
Aave_3051	HopF2 of <i>P. syringae</i> pv. <i>antirrhini</i>	37	65	+	+	+	+	+	+
Aave_3062	AvrRxo1 of <i>X. translucens</i>	71	81	+	+	In ^d	+	Del ^e	Del ^e
Aave_3237	Lytic murein transglycosylase of <i>A. citrulli</i>	100	100	+	+	+	+	+	+
Aave_3452	AvrPphE of <i>R. solanacearum</i>	44	57	+	+	+	+	+	+
Aave_3462	PopP3 (YopJ family) of <i>R. solanacearum</i>	35	50	+	+	+	+	+	+
Aave_3502	HopAO1 (HopD2) of <i>P. syringae</i>	48	63	+	+	+	+	+	+
Aave_4606	RipAY of <i>R. solanacearum</i>	42	58	+	+	+	+	+	+
Aave_4728	RipBI protein of <i>R. solanacearum</i>	33	47	+	+	+	+	+	+

+, Presence of indicated genes; –, absence of indicated genes.

^aObtained from Eckshtain-Levi et al. (2014), Fujiwara et al. (2016), Lo et al. (2017), Potnis et al. (2012), and Washington et al. (2016).

^bProtein homologs and amino acid identity and positives by BLASTP at GenBank database.

^cDeletion of 120 bp including a start codon causing no intact proteins.

^dInsertion of a 876-bp transposase within an ORF causing early termination.

^eDeletion of one base within an ORF causing frame shift and early termination.

isolates might be different depending on where they first contact a host plant. Only two among six *A. citrulli* isolates could efficiently infect watermelon through all infection routes tested, although its virulence level varied (Table 3). The virulence in an organ-specific manner might be one of characteristics of *A. citrulli* causing BFB in watermelon.

Plant-pathogenic bacteria, which mostly have semi-biotrophic lifestyle, seem to have preferred organs for infection such as roots and leaves. Depending on the infection routes, disease symptoms are normally developed at the initial infection organ, at distal organs, or in the entire plants (Bové and Garnier, 2002). If pathogens infect leaves of host plants like *Pseudomonas syringae* pv. *tomato* and *X. euvesicatoria* in tomato (Boureau et al., 2002; Potnis et al., 2015), then they normally cause spot-type symptoms at the infection sites. However, if pathogens infect vascular systems or roots, like *Ralstonia solanacearum* in tomato, *X. oryzae* pv. *oryzae* in rice, and *Erwinia amylovora* in apple and pear (Mew et al., 1993; Tans-Kersten et al., 2001; Van-neste, 2000), they cause wilting or blight-type symptoms in the entire plants or twigs. Some of plant-pathogenic bac-

teria can cause disease symptoms in multiple organs of the host plants. *P. syringae* pv. *tomato* and *X. euvesicatoria* in tomato cause disease symptoms not only in leaves, but also in fruits. More obviously, *P. syringae* pv. *actinidiae*, the pathogen that causes bacterial canker in kiwifruit, can cause disease symptoms in leaves, flowers, and stems (Scortichini et al., 2012). However, the knowledge about organ-specific virulence of each plant-pathogenic bacterium is limited, except in pathogens mainly infecting leaves. Therefore, it would be worthy to determine the genes controlling virulence in an organ-specific manner and eventually discover the underlying molecular mechanisms.

A. citrulli isolates having different infectivity in diverse routes in watermelon may be good materials to study this subject, because profiles of effector genes are positively correlated with infectivity in an organ-specific manner, as summarized in Tables 2 and 3. Generally, effector genes encoding proteins delivered into host plant cells are virulence determinants in plant-pathogenic bacteria (Alfano and Collmer, 2004; Bogdanove et al., 1996). Combinations of effector genes in a single pathogenic bacterium control

Table 3. Summary of disease severity by *Acidovorax citrulli* isolates in watermelon depending on inoculation methods and sites

Inoculation method	Soil mixing	Soil drenching		Spray inoculation	Syringe infiltration	Vacuum infiltration	Fruit injection
Plant stages	Mature seeds	5-Day-old seedlings	5-Day-old seedlings	3-Week-old seedlings	3-Week-old seedlings	3-Week-old seedlings	Mature fruits
Infection sites	Seeds and developing roots	Developed healthy roots	Wounded roots	Whole seedlings	Leaves	Whole seedlings	Fruit surface
Inoculum concentration (cfu/ml)	3×10^5 (cfu/g)	10^6	10^6	10^6	10^4	10^4	10^6
Natural isolates							
Ac0	++*	–	+++	++	+++	+	+
Ac1	+	–	–	+++	+++	+	+++
Ac2	–	–	–	–	–	–	–
Ac4	+++	–	+++	+++	++	+	+++
Ac8	+++	–	–	–	+	–	++
Ac11	+	–	–	–	+	–	+

*Level of disease severity. +++, very severe; ++, severe; +, weak; –, very mild or no disease symptom developed.

its virulence level including infection strategy. The function of three effector genes, Aave_2166, Aave_2708, and Aave_3062, may be critical for infection in leaves because Ac2, Ac8, and Ac11 isolates lacking these functional genes could not cause disease through leaves with the spray inoculation (Tables 2 and 3).

Aave_2166 encodes a homolog to AvrBsT, a gene with acetyltransferases enzyme activity reported from *X. euvesicatoria* (Cheong et al., 2014; Kim et al., 2010). The 39-kDa AvrBsT protein is a member of the YopJ family of effectors in plant and animal pathogens (Ciesiolka et al., 1999; Escobar et al., 2001). AvrBsT is also a well-known host determinant because *X. euvesicatoria* carrying AvrBsT is impossible to cause the disease in pepper, but it can infect tomato (Minsavage et al., 1990). In pepper, AvrBsT suppresses the hypersensitive response induced by another effector protein AvrBs1 of *X. euvesicatoria* (Kim et al., 2010; Szczesny et al., 2010), and this suppression is dependent on SNF1-related kinase, SnRK1, which is located in the plant cell cytoplasm (Szczesny et al., 2010). Interestingly, Aave_2173 in *A. citrulli* is a homolog of AvrBs1 in *X. gardneri* (Table 2). Thus, it will be worthwhile to determine the relationship between Aave_2166 and Aave_2173 in watermelon. Aave_2708 is a homolog of XopJ, which also belongs to the YopJ family. XopJ inhibits vesicle trafficking and protein secretion to suppress cell wall associated defense responses and degrades the proteasome subunit RPT6 of the host cell to suppress salicylic-acid mediated plant defense (Bartetzko et al., 2009; Üstün and Börnke, 2015; Üstün et al., 2013). Aave_3062 is homologous to AvrRxo1 of *X. euvesicatoria*. The AvrRxo1 locus

contains two ORFs: AvrRxo1-ORF1 with a polynucleotide kinase domain and its interactor AvrRxo1-ORF2 (Han et al., 2015). *A. citrulli* carries the Aave_3063 gene next to Aave_3062, which is homologous to AvrRxo1-ORF2. This suggests that Aave_3062 might function similarly to AvrRxo1. The protein structure of AvrRxo1 is similar to a zeta (ζ) toxin, which is a part of the toxin-antitoxin system in *Streptococcus* (Triplett et al., 2016). Moreover, it has recently been revealed that AvrRxo1 phosphorylates NAD *in planta* and its kinase catalytic sites are necessary for its toxic and resistance-triggering phenotypes (Shidore et al., 2017). Leaves in plants are composed of metabolically active cells including photosynthetic activity compared with cells in roots or fruits (Sonnewald and Fernie, 2018). It can be postulated that the three effectors have functions to interfere with host mechanisms in leaves and to promote diseases. Therefore, they may possess roles to determine infectivity in different organs. Nevertheless, further research should be done to elucidate how these three effector proteins control virulence of *A. citrulli* in an organ-specific manner.

A. citrulli causes disease symptoms in fruits, cotyledons, leaves, and whole plants. This bacterium has been considered a seed-borne pathogen (Burdman and Walcott, 2012), and several works have been done to study other infection routes of this pathogen with diverse natural isolates. When we consider how *A. citrulli* enters seeds initially, it seems that it travels into xylem vessels from an initial infection site (Bahar et al., 2009) and moves to the seeds during development. This means that there are other initial infection sites, and to determine these would be very critical to our

understanding of the complete disease cycle of *A. citrulli* and eventually for disease management. In this study, we used several inoculation methods and showed that this pathogen can infect host plants through seeds during germination, wounded roots, leaves, and fruits. Our inoculation strategies mimic the conditions or steps that *A. citrulli* may face in watermelon plants from seeds to mature fruits. Moreover, our results show several possible occasions where *A. citrulli* can enter host plants such as the seed germination step in the nursery, the transplanting step in the greenhouse or field, flowering or fruit developing steps, or overhead irrigation.

A. citrulli is normally divided into two CC groups, based on genetic differences (Eckshtain-Levi et al., 2014). However, the correlation between these groups and level of virulence or infection routes in host plants is not clear. In this study, we showed that, among several inoculation methods, the spray inoculation (infection through leaves) could match CC grouping with virulence in watermelon leaves and the three genes, Aave_2166, Aave_2708, and Aave_3062, with the intact form were found in only CC group II. Other than this, there were no obvious correlations between the two aspects. It remains to be determined that CC grouping might correlate with virulence in host plants that we have not tested, such as melon and cucumber.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (<http://www.ppjonline.org/>).

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