Light Modulates Virulence Factors in Strain Aac5 of Acidovorax citrulli

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Abstract

Background: Acidovorax citrulli causes bacterial fruit blotch (BFB), a disease that poses a global threat to watermelon and melon production. Despite the economic importance of BFB, relatively little is known about the regulation of A. citrulli pathogenesis. Light has a role in regulating the virulence factors of many plant pathogenic bacteria; however, the role of light in A. citrulli is unknown. This study aimed to investigate the roles of light in A. citrulli pathogenicity. Results: We found that light suppressed swimming motility and biofilm formation in A. citrulli Aac5 strain. Furthermore, we found that A. citrulli Aac5 strain exhibited significant differences in colony morphology under light and dark conditions. In fact, the twitching motility of A. citrulli Aac5 strain also was suppressed cultured by light conditions compared to dark conditions. In addition, the expression of hrpG, hrpX, and hrcC genes, which are important for type 3 secretion system (T3SS), was inhibited under light compared to dark conditions. However, in A. citrulli host interactions, light suppressed ROS production and enhanced callose deposition by watermelon when induced by A. citrulli; in addition, light enhanced the ability of A. citrulli to infect watermelon. These results suggest that light affected the pathogenicity of A. citrulli Aac5 strain by regulating its virulence factors and watermelon immune response. Conclusions: Light as an important environmental factor plays an important role in regulating the occurrence of plant diseases. BFB as an important watermelon disease seriously threatens the development of the industry. However, there are few reports on the impact of environmental factors on the disease. In our studying, we report for the first time that light can inhibit the expression of major virulence factors, such as swimming motility, biofilm formation, twitching motility, and even key gene of T3SS in A. citrulli Aac5 strain. But surprisingly, the light enhances the pathogenicity of A. citrulli Aac5 strain for natural host watermelons. The important phenotypes of host immune response are also different
between ROS and callose deposition. These phenomena reflect the close relationship between BFB occurrence and light factor, which is of great significance for the study of BFB.

Background

As a major environmental stimulus, light regulates plant physiology, including photosynthesis [1], stomatal opening and closing [2], and defense responses [3, 4]. While adapting to light signals, some of these physiological functions in plants also affect many plant pathogenic microorganisms[5-7]. Especially in some plant pathogenic bacteria, such as *Xanthomonas* spp., light plays an important regulatory role on pathogenicity [8-10]. For example, Río-Álvarez *et al.* [11] revealed that light suppressed motility and biofilm formation in *Pseudomonas syringae* pv. *tomato* DC3000 strain. Similarly, Moriconi showed that sigma factor genes and their downstream targets linked to bacterial growth, virulence, and quorum sensing were inhibited in *Pseudomonas syringae* pv. *tomato* in a strictly light-dependent manner [12]. In *Xanthomonas axonopodis* pv. *citri*, Kraiselburd reported that motility and biofilm formation of a wild-type strain are significantly different under light and dark conditions [13]. Kraiselburd also reported that light conditions had an effect on the counteraction of plant immune responses during infection [14]. However, because of differences in the light environment, pathogens, and host, there are also differences in the regulation of light on the virulence factors of plant pathogenic bacteria. For example, McGrane *et al.* (2015) showed that there was a difference in the motility between *Pseudomonas syringae* pv. *tomato* and *Pseudomonas syringae* pv. *syringae* in light and dark conditions, although they are genetically closely related. Further, during the long-term history of evolution, pathogens and hosts have co-evolved [15]. In response to differences in the expression of virulence factors of pathogens under light
and dark conditions, host plants have also evolved adaptive mechanisms [4], as follows: (i) by providing signals for the deployment of defensive barriers; (ii) by influencing the general energy supply and, thus, the “fuel” available to launch and sustain responses against invaders; and (iii) by inducing the production of reactive oxygen species (ROS) in chloroplasts and peroxisomes. The plant immune system mainly includes pathogen- or microbe-associated molecular patterns (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI)[16-19]; pathogens first need to overcome PTI[20, 21]. The difference in plant-immune response dependence on light environment can be reflected in the PTI signaling pathway, such as ROS and callose deposition[22, 23], which are important markers for PTI.

*Acidovorax citrulli* is the causal agent of bacterial fruit blotch (BFB), a disease that poses a global threat to watermelon and melon production[24]. Previous studies have shown motility and biofilm formation as the main virulence factors in *A. citrulli* [25, 26]. Moreover, type 3 secretion system (T3SS) is the key pathogenicity factor of *A. citrulli* [24]. Zhang et al. (2018) reported that *hrpG* and *hrpX* were key regulators of the expression of T3SS in *A. citrulli*, and thus, can be used as an indicator of T3SS expression to detect the influences of environmental factors on *A. citrulli*. Despite the economic importance of BFB, relatively little is known about the regulation of *A. citrulli* pathogenesis, especially the role of the light in *A. citrulli* is unknown. The effects of light on swimming motility, biofilm formation, and T3SS gene expression of *A. citrulli* have also not been elucidated.

Here, we tested our hypothesis that light affects virulence factors of *A. citrulli* strain Aac5. In addition, we investigated the plant immune response induced by *A. citrulli* under light and dark conditions. Our results suggest that light affects virulence factors, and thus, the pathogenicity of *A. citrulli*.

**Results**
Light suppresses the motility of A. *citrulli*

Motility plays a positive regulatory role in the pathogenesis of A. *citrulli* [26]. To investigate whether motility is affected by light, we tested the swimming motility of strain Aac5 of A. *citrulli*. The results showed a significant reduction ($p<0.05$) in swimming motility on 0.3% agar plates in the wild-type strain Aac5 (WT) under light compared with darkness (Fig. 1A). Furthermore, we tested the expression of the *fliC* gene in strain Aac5, which is a key gene for motility in A. *citrulli* [26], by using quantitative reverse-transcription polymerase chain reaction. The results showed that light suppressed the expression of the *fliC* gene (Fig. 1B).

Light suppresses biofilm formation by A. *citrulli*

Previous studies reported that biofilm formation was involved in A. *citrulli* virulence and pathogenicity [25, 27, 28]. We quantified biofilm formation by crystal violet staining with qualitative and quantitative tests. The qualitative test showed that strain Aac5 in the dark condition exhibited more biofilm formation in darkness than in the light (Fig. 2A). Further, the quantitative test showed that strain Aac5 condition exhibited significantly enhanced biofilm formation in the dark compared with the light condition (Fig. 2B). Thus, our results indicated that light suppressed biofilm formation by strain Aac5.

Differences in colony morphology of A. *citrulli* under light and dark conditions

To examine the role of light on colony morphology of A. *citrulli*, wild-type Aac5 was
cultured and incubated on nutrient agar plates in the light or dark for 72 h. The results showed that strain Aac5 showed black dots when cultured in the dark compared to that under light conditions (Fig. 3), indicating that light changed the colony morphology of A. *citrulli*.

**Light supresses twitching motility of A. *citrulli***

Past reports indicate that colony morphology of A. *citrulli* was in contact with twitching motility[29, 30]. To examine the role of light on twitching motility of A. *citrulli*, wild-type Aac5 was cultured and incubated on nutrient agar plates in the light or dark for 48 h. The results showed that strain Aac5 showed stronger twitching motility when cultured in the dark compared to that under light conditions (Fig. 4), indicating that light supresses the twitching motility of A. *citrulli*.

**Light suppresses T3SS-related gene expression in A. *citrulli***

T3SS is the key pathogenicity factor of A. *citrulli* [24]. To test whether light affects T3SS-related gene expression, we selected *hrpG*, *hrpX*, and *hrcC* as marker genes. *hrpG* and *hrpX* are key T3SS regulators in Aac5 (Zhang *et al.*, 2018), while *hrcC* is an important T3SS structural gene in A. *citrulli* [31]. Wild-type strain Aac5 was incubated in the dark for 24 h in T3SS-inducing media, followed by incubation in the light for 2 h. The qPCR results showed that mRNA levels of *hrpG*, *hrpX*, and *hrcC* in the light significantly declined compared with the corresponding levels after incubation in the dark (Fig. 5). These results indicate that light suppressed T3SS-related gene expression in A. *citrulli*.

**Light enhances callose deposition**
of host watermelon inoculated with A. *citrulli*

As a response of the plant immunity, system callose deposition can be induced by mechanical wounding, physiological stress, and phytopathogen infection [32, 33]. To test whether light affects A. *citrulli*-induced callose deposition in watermelon, callose deposition was monitored in leaves inoculated with strain Aac5 under light and dark conditions; flg22 was used as a positive control. 24 h after infection, inoculated leaves were stained for callose with aniline blue and cytological observations were performed at the sites of infection by UV-fluorescence microscopy. Callose deposition was identified as bright-green dots on leaves or veins (Fig. 6A) and quantified using Image J software. The qualitative assay showed that strain Aac5 induced greater callose deposition in the light than in the dark condition. Additionally, the quantitative test showed that Aac5-inoculated watermelon leaves exhibited significantly increased callose deposition in the light, compared with the dark condition; further, they exhibited significantly less callose deposition in the dark condition compared with flg22 treatment (positive control) (Fig. 6B). These results indicate that light enhanced callose deposition in watermelon leaves inoculated with A. *citrulli*.

**Light suppresses A. *citrulli*-induced ROS production by watermelon**

Based on the callose deposition test, we inferred that light might affect ROS production by the host when infected with A. *citrulli*. Host watermelon leaves were inoculated with strain Aac5 under light and dark conditions, and ROS production was detected by qualitative and
quantitative tests after 24 h. The results showed that strain Aac5 induced greater ROS production in the dark than under light conditions or by flg22 used as positive control (Fig. 7), indicating that darkness enhanced ROS production by watermelon leaves infected with *A. citrulli*.

**Light enhances *A. citrulli* pathogenicity with host watermelons**

Based on the above results with comprehensive analysis, we hypothesized that light may affect the pathogenicity of *A. citrulli*. To test our hypothesis, watermelon leaves were syringe-infiltrated with wild-type Aac5. The inoculum was prepared by growing wild-type Aac5 to logarithmic phase and adjusting $1 \times 10^4$ cfu/ml. After infiltrating the watermelon leaves, plants were incubated under light or dark conditions for 96 h (10 mM MgCl$_2$ was used as negative control). The results showed that the infection ability of wild-type Aac5 in the light was significantly stronger than in the dark from the onset of symptoms (Fig. 8A). An *in vivo* growth test with host watermelon showed that after four days, wildtype strain Aac5 was larger in the light than in the dark (Fig. 8B), indicating that light enhanced the pathogenicity of *A. citrulli*.

**Discussion**

In this study, we evaluated the role of light on virulence factors of *A. citrulli* wild-type strain Aac5 and watermelon responses to *A. citrulli* infection, namely ROS production and callose deposition. Light suppressed the swimming motility (Fig. 1) and biofilm formation (Fig. 2). Previous studies suggested that swimming motility and biofilm formation were important virulence factors in *A. citrulli* [24]. For example, Bahar *et al.* (2011) reported
that the fliC mutant of A. citrulli, which is involved in swimming motility, showed a significant reduction in virulence in seed transmission assays and stem and foliage inoculations, compared to the wild-type. Consistently, Luo reported that a biofilm-defective A. citrulli mutant showed reduced virulence [34]. Additionally, Wang et al. [28] also reported that swimming motility and biofilm formation were important for the virulence of A. citrulli. In fact, swimming motility and biofilm formation also play key roles in pathogenicity for other phytopathogenic bacteria, such as Xanthomonas spp.[35-37], and Pseudomonas syringae [38, 39]. In addition, colony morphology of A. citrulli was different in light or dark conditions (Fig. 3). Hansen et al.[40] reported that the absence of a flagellum leads to altered colony morphology, biofilm development, and virulence in Vibrio cholerae O139. Our own results showed differences in biofilm and colony morphology concomitant with a difference in swimming motility under light or dark conditions. Studies in the past have shown that the morphological changes of A. citrulli are associated with twitching motility[30, 41]. Our own results showed that the twitching motility of A. citrulli was suppressed under light conditions. This confirms the importance of light as an environmental factor for A. citrulli. Differences in A. citrulli colony-morphology under light or dark conditions may also determine the virulence factor differences reported herein. In a previous study, we confirmed hrpG and hrpX as key T3SS regulators in A. citrulli that play an important role in the pathogenicity of the microbe [42]. We hypothesized that T3SS-related gene expression may be affected by light; hrpG and hrpX were used as indicator genes of T3SS-related gene expression: our results showed that light suppressed the expression of both genes at the transcriptional level. In addition, hrcC played a key role in T3SS function in A. citrulli [31], and light suppressed hrcC expression at the transcriptional level in strain Aac5. These results clearly indicate that T3SS expression was suppressed by light.
It is well known that ROS play an important role in pathogen–host interactions[43]. We showed that A. *citrulli*-induced ROS production by host watermelon plants cultivated under light conditions were different from that in plants cultivated under dark conditions. Interestingly, ROS production was more in the dark than in the light conditions. Callose deposition induced by pathogens plays a key role in their interaction with hosts, which is often used as an important indicator of PTI signaling[44]. We confirmed that A. *citrulli*-induced callose deposition was enhanced by light condition in comparison with dark conditions. This finding, together with the results described for ROS production, strongly suggest that ROS production and callose deposition induced by A. *citrulli* under light and dark condition is different. In fact, subsequent pathogenic experiments confirmed that light enhanced the pathogenicity of A. *citrulli* for watermelon. Although the expression of virulence factors, swimming motility, biofilm formation, twitching motility, is inhibited under light conditions, it is indeed stronger in the dark from the perspective of A. *citrulli*-infected watermelon virulence assay compared to light conditions. Recently, studies have reported that watermelon is more resistant to nematodes under far red light which is similar to darkness compared to light condition[6]. This is basically consistent with our results. We think this may be related to the resistance of watermelon. As we all know, watermelon is originated to the tropics[45] and like lighting, and in history outbreak of watermelon fruit blotch was happened at the Mariana islands that a place full of sunshine[46]. This may have made a choice during the evolution process in the interaction between watermelons and A. *citrulli* under light and dark conditions. More importantly, strain Aac5 was isolated from Taiwan province of China that a place full of sunshine. The classic theory of the disease triangle tells us that the disease is inseparable from environmental factors[47]. These results also tell us that the occurrence of diseases is not only the interaction between pathogenic virulence factors and hosts. It is actually a
complicated process, and the occurrence of diseases requires the participation of environmental factors, for examples, light or dark conditions.

Conclusions

This is the first report demonstrating the roles of light in A. *citrulli* strain Aac5 pathogenicity. Overall, our study results showed that light inhibited virulence factors including swimming motility, biofilm formation, twitching motility and T3SS, and further affected the pathogen-host interaction. However, light enhanced the pathogenicity of A. *citrulli*.

Methods

**Strains and culture conditions**

The A. *citrulli* Aac5 strain was grown on King’s B (KB) or T3SS-inducing medium [42](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. Liquid cultures of the strains were grown in sterilized test tubes containing KB, or T3SS-inducing broth continuously agitated at 200 rpm on a rotary shaker (DDHZ-300; Taicang Experimental Instrument Factory, Jiangsu, China). When required, the growth media were supplemented with the following antibiotics: ampicillin (Amp), 100 μg/mL. For assays, bacterial concentrations were estimated by optical density (OD₆₀₀) using a spectrophotometer (Evolution 300 UV/VIS; Thermo Scientific, Waltham, MA, USA).

**Swimming motility assay**

To determine the roles of light and dark conditions in swimming motility, assays were performed on A. *citrulli* wild-type Aac5 strain, as previously described [48], with slight modifications. Briefly, 3 μL of overnight cultures of Aac5 strain was inoculated onto the surface of a semi-solid agar plate containing 0.03% Bacto Peptone, 0.03% yeast extract, and 0.3% agar. Cells were cultured at 25°C under the light and dark conditions, and
colony diameters of the strains were measured 84 h after inoculation. The experiment was conducted three times with twelve replicates each time.

**Biofilm formation assay**

To determine the roles of light and dark conditions in biofilm formation, assays were performed using *A. citrulli* wild-type Aac5 strains, as previously described [42]. Briefly, 24-well plates (Corning, NY, USA) were pre-loaded with T3SS-inducing liquid medium and inoculated with a 1:1000 dilution of overnight cultures of *A. citrulli* Aac5 strain. The plates were incubated in a tilted position at 28°C under light and dark conditions 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 OD$_{590}$ of the stained-cell suspensions with a spectrophotometer. The experiment was conducted three times with twelve replicates each time.

**Colony morphology observation assay**

The colony morphology observation assay was performed as described previously [29]. Colony morphology observation was performed with wild-type Aac5 strain. Briefly, Aac5 strain was cultured on Nutrient agar medium under the light and dark conditions for 72 h. Pictures were taken with the Olympus BX83 to observe and record the colony morphology. The experiment was conducted three times with three replicates each time.

**Twitching motility assay**

The twitching motility assay was performed as described previously[28]. The twitching motility assay was performed with wild-type Aac5 strain. Briefly, Aac5 strain was cultured
on Nutrient agar medium under the light and dark conditions for 48 h. Pictures were taken with the Olympus BX83 to observe and record the colony morphology. The experiment was conducted three times with three replicates each time.

**Callose deposition assay**

The callose deposition assay was performed as described previously [32]. Four-week-old watermelon leaves (cv. Ruihong, China) were syringe-infiltrated with wild-type Aac5 strain (~10^8 CFU/mL) and flg22 (100 μM). Whole leaves were collected 24 h after infiltration and stained with aniline blue mounted in 50% glycerol. Briefly, whole leaves were immersed in 5 ml of alcoholic lactophenol (1 volume of phenol: glycerol:lactic acid:water [1:1:1:1] and 2 volume of ethanol). The leaves were evacuated for 15 min, then placed at 65°C and incubated until they were completely cleared of chlorophyll (15-30 min). The leaves were transferred into fresh alcoholic lactophenol for additional 24 h. To detect callose, cleared leaves were rinsed with 50% ethanol, rinsed with water, and then stained for 30 min in 150 mM K_2HPO_4 containing 0.01% aniline blue. Samples were mounted in 50% glycerol and pictures were taken using BX63 (Olympus, Beijing, China). Image J software was used for analyzing the image. The experiment was conducted three times with twelve replicates each time.

**ROS Assay**

The qualitative test of ROS was performed as described previously [49]. The three-week-old watermelon leaves (cv. Ruihong, Chian) inoculated with cell suspensions of A. citrulli Aac5 (~10^8 CFU/mL). The infiltrated leaves were stained by placing in 1 mg/ml DAB (3, 3-diaminobenzidine tetrahydrochloride) solution (pH 3.8) for 12 h. The stained leaves were then bleached with trichloreacetic acid (1.5 g/L) in ethanol: chloroform (4:1 v/v) for 48 h; the solution was changed once during the next 48 h of incubation. The leaf segments were
afterward transferred into microscopy solution (50% glycerol) for storage. The accumulation of hydrogen peroxide was visualized by the production of reddish-brown color due to polymerization of DAB. The stained leaves were photographed and the experiment was conducted three times with 3 replicates every time.

Quantitative reverse-transcription (qRT-)PCR

Total RNA was extracted from wild-type *A. citrulli* Aac5 cultured in T3SS-inducing liquid medium up to OD$_{600}$=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 Remover (Toyobo, Shanghai, China). The mRNA levels were quantified by qPCR using KOD SYBR qPCR Mix (Toyobo, Shanghai, China) on an Applied Biosystems 7500 instrument (ABI, Waltham, MA, USA). The primers used for qRT-PCR are listed in Table S1. Each sample was assayed in triplicate. *rpoB* was used as a reference gene. Relative expression of genes of interest was calculated by the 2$^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The experiment was conducted three times with three replicates each time.

The pathogenicity assay

The pathogenicity assay was performed as described previously[50]. The watermelon leaves (cv. Ruihong, China) were inoculated with cell suspensions of *A. citrulli* Aac5 ($\sim 10^4$ CFU/mL). The infiltrated leaves were treated under the light and dark conditions for 96 h, and followed by taking pictures. The experiment was conducted three times with three replicates each time.

Statistical analysis
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* values less than 0.05 were considered significant.

**Abbreviations**

BFB: Bacterial Fruit Blotch; T3SS: Type 3 secretion system; ROS: reactive oxygen species.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interest.

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**Authors’ contributions**

XXZ and TCZ designed the experiments; XXZ performed the experiments; XXZ, MZ, YWY, LLY, JPY, ZHF, WG and XB analyzed the data; XXZ and MZ wrote the manuscript; all authors read and approved the final manuscript.
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**Figures**

**Figure 1**

Effect of light on the motility of *Acidovorax citrulli* strain Aac5. (A) Bacterial swimming motility was examined after 84 h on semi-solid agar plate under light and dark conditions in wild-type Aac5. (B) Relative mRNA levels of fliC were determined using quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The rpoB gene was used as a reference gene. Values are a representative of three independent experiments, each including three replicates. Error bars indicate standard error of the means. Asterisks indicate significant differences between means as per Student’s t-test (p < 0.05). (TIF 113kb)
Figure 2

Effect of light on biofilm formation by Acidovorax citrulli strain Aac5. (A) Qualitative detection of bacterial biofilm formation in wild-type Aac5 under light and dark conditions. The arrow shows the biofilm. (B) Quantitative detection of bacterial biofilm formation of wild-type Aac5 under light and dark conditions. Values are representative of three independent experiments, each including 12 replicates. Error bars indicate standard error of the means. Asterisks indicate significant differences between means as per Student’s t-test (p < 0.05). (TIF 476kb)
Figure 3

Effect of light on colony morphology of Acidovorax citrulli strain Aac5. The experiment was conducted three times, each including three replicates. Pictures were taken after a 72 h incubation period on nutrient agar plates. (TIF 333kb)
Effect of light on twitching motility of Acidovorax citrulli strain Aac5. The experiment was conducted three times, each including three replicates. Pictures were taken after a 48 h incubation period on nutrient agar plates. (TIF 241kb)
Figure 5

Effect of light on T3SS-related gene expression in strain Aac5 at the transcriptional level. Values are representative of three independent experiments, each including three replicates. Error bars indicate standard error of the means. Asterisks indicate significant differences between means as per Student’s t-test (p < 0.05). (TIF 175kb)
Figure 6

Effect of light on callose deposition of host watermelon leaves inoculated with strain Aac5. (A) Qualitative detection of callose deposition. (B) Quantitative detection of callose deposition. Values are representative of three independent experiments, each including 12 replicates. Error bars indicate standard error of the means. Asterisks indicate significant differences between means as per Student’s t-test (p < 0.05). (TIF 724kb)
Effect of light on ROS production by host watermelon leaves inoculated with Aac5.

The DAB staining experiment was conducted three times, each including three replicates. (TIF 479kb)
Figure 8

Effect of light on the pathogenicity of Acidovorax citrulli strain Aac5. (A) Qualitative detection of virulence assay. (B) Quantitative detection of virulence assay. The experiment was conducted three times, each including three replicates. (TIF 781kb)

Supplementary Files

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