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Rapid and Specific Detection of *Acidovorax avenae* subsp. *citrulli* Using SYBR Green-Based Real-Time PCR Amplification of the YD-Repeat Protein Gene

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The aim of this study was to develop a SYBR Green-based real-time PCR assay for the rapid, specific, and sensitive detection of *Acidovorax avenae* subsp. *citrulli*, which causes bacterial fruit blotch (BFB), a serious disease of cucurbit plants. The molecular and serological methods currently available for the detection of this pathogen are insufficiently sensitive and specific. Thus, a novel SYBR Green-based real-time PCR assay targeting the YD-repeat protein gene of *A. avenae* subsp. *citrulli* was developed. The specificity of the primer set was evaluated using DNA purified from 6 isolates of *A. avenae* subsp. *citrulli*, 7 other *Acidovorax* species, and 22 of non-targeted strains, including pathogens and non-pathogens. The AC158F/R primer set amplified a single band of the expected size from genomic DNA obtained from the *A. avenae* subsp. *citrulli* strains but not from the genomic DNA of other *Acidovorax* species, including that of other bacterial genera. Using this assay, it was possible to detect at least one genome-equivalents of the cloned amplified target DNA using 5×10^0 fg/μl of purified genomic DNA per reaction or using a calibrated cell suspension, with 6.5 colony-forming units per reaction being employed. In addition, this assay is a highly sensitive and reliable method for identifying and quantifying the target pathogen in infected samples that does not require DNA extraction. Therefore, we suggest that this approach is suitable for the rapid and efficient diagnosis of *A. avenae* subsp. *citrulli* contaminations of seed lots and plants.

Keywords: *Acidovorax avenae* subsp. *citrulli*, bacterial fruit blotch, YD-repeat protein, detection, real-time PCR

Introduction

Acidovorax avenae subsp. *citrulli* causes bacterial fruit blotch (BFB), a serious disease of cucurbit plants [3]. Infested seeds served as the primary inoculum for BFB outbreaks, particularly in transplant seedling production systems [12]. This pathogen is an aerobic mesophilic gram-negative bacterium belonging to the beta subdivision of the Proteobacteria. It was first recognized as *Pseudomonas pseudoalcaligenes* subsp. *citrulli* in the United States in 1965 [22, 25]. Currently, BFB is one of the most devastating diseases encountered by watermelon-seed producers throughout the world. The significant economic losses caused by this pathogen also affect the cucumber and pumpkin industries of many countries [3]. As in the case for many bacterial diseases, chemical control of BFB has been difficult to achieve [3]; although pesticide treatments can reduce the *A. avenae* subsp. *citrulli* populations in infected seeds, they sometimes fail to eliminate this pathogen from seedlings [3, 21]. Thus, disease management through the development of resistant cultivars remains an important control strategy. However, attempts to screen watermelon cultivars for resistance to *A. avenae* subsp. *citrulli* have yielded inconsistent results [28]. Therefore, screening must be conducted with a

precise disease-score rating and deep expertise in *in planta* assays. Several screening methods for detecting BFB have been developed [3, 21, 28]. The most widely employed assays for *A. avenae* subsp. *citrulli* infections of seeds are seedling grow-out and blotter assays, but these methods require planting seed samples and subsequently observing the seedlings for symptoms or signs of BFB [9]. In addition, pathogenicity assays, DNA-fingerprint profiling, and whole-cell fatty-acid analyses of strains of *A. avenae* subsp. *citrulli* have revealed that they are phenotypically and genotypically complex. Based on the results of these analyses, *A. avenae* subsp. *citrulli* has been divided into at least two very different groups [2, 3, 27].

PCR-based techniques provide useful alternatives for the specific detection and identification of *A. avenae* subsp. *citrulli* that might overcome the diagnostic limitations associated with the problems of screening methods and pathogen diversity. However, there are few reports of sensitive and specific PCR-based assays for the identification and diagnosis of *A. avenae* subsp. *citrulli* in plants or seed lots that do not require DNA extraction [2, 9, 19, 10, 24, 28, 29, 31, 32]. Moreover, in general, TaqMan probe-based assays are more expensive than SYBR Green-based assays. Therefore, it is important to develop a simple and efficient method for the sensitive and specific detection of *A. avenae* subsp. *citrulli*.

The YD-repeat proteins are ubiquitous proteins that comprise six structurally distinct lineages within the Enterobacteriaceae, which exhibit significant intergenomic variation in their YD repertoire [13], suggesting that the YD-repeat protein gene would be useful for diagnostic PCR assays of these bacteria. In this study, a species-specific primer set based on the YD-repeat protein gene of *A. avenae* subsp. *citrulli* AAC00-1 was designed and employed in a highly specific assay for the detection of this pathogen in watermelon seeds and plants.

Materials and Methods

Bacterial Strains and DNA Extraction

Bacterial strains were obtained from the Korean Agricultural Culture Collection (KACC), the Belgian Coordinated Collections of Micro-Organisms (BCCM), and the National Collection of Plant Pathogenic Bacteria (NCPPB). The bacterial strains used in this study are listed in Table 1. All of the reference strains used in this study were selected according to the phylogenetic tree in the NCBI taxonomic database and the strains used in other studies [28, 29]. The *Acidovorax, Burkholderia, Erwinia, Pectobacterium, Rhizobium,* and *Ralstonia* strains were grown on nutrient agar (peptone: 0.5%; NaCl: 0.5%; yeast extract: 0.2%; Lab-Lemco beef extract: 0.1%; and agar: 1.5%) at 28°C for 1 to 2 days; the *Xanthomonas* strains were grown on YGC medium (D- (+)-glucose: 1.0%; $CaCO_3$: 3.0%; yeast extract: 0.5%; and agar: 1.5%) at 28°C for 3 days; and the other microbes were cultured on Luria-Bertani agar (tryptone: 1%; yeast extract: 0.5%; sodium chloride: 1%; and agar: 1.5%) at 28°C to 37°C for 1 to 3 days [1]. The genomic DNA was isolated from the *Acidovorax* strains and the other bacterial strains using a Genomic DNA Prep Kit (SolGent, Korea) according to the manufacturer's protocol. The quantity and purity of the bacterial genomic DNA was evaluated by measuring its absorbance using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Primer Design and Specificity

In this study, primers specific for a 158 bp fragment of the YD-repeat protein gene of *Acidovorax avenae* subsp. *citrulli* AAC00-1 (GenBank Accession No. NC_008752; region: 2237201..2242513; protein ID YP_970406) were designed using the PrimerSelect program in the Lasergene (ver. 7.2.1; DNASTAR Inc. Madison, WI, USA) software (Table 2). Conventional PCR amplification was conducted using a PTC-225 thermocycler (MJ Research, Watertown, MA, USA). The PCR mixture contained 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM of each dNTP, 20 pM of each primer, 2 units of *Taq* polymerase (Promega Corp., Madison, WI, USA), and 25 ng of genomic DNA from the given bacterial strains. The PCR procedure entailed 35 cycles, each of which consisted of 60 sec at 94°C, 30 sec at 56°C, and 1 min at 72°C, with an initial denaturing step of 5 min at 94°C and a final extension step of 10 min at 72°C. The PCR products, stained using LoadingStar (DYNEBIO, Republic of Korea), were separated on 1.5% agarose gels by electrophoresis at 100 V for 60 min in 0.5× TAE buffer. The SYBR Green-based real-time PCR assay was conducted using a total volume of 20 µl of a reaction mixture containing 10 µl of SYBR Premix Ex Taq (Takara Bio, Inc., Japan), 5 pM of each AC158F/R primers, and 5 ng of purified DNA. The SYBR Green-based real-time PCR assay was performed using a CFX96 real-time PCR system (Bio-Rad Laboratories, Inc., USA) under the following conditions: 95°C for 30 sec, 45 cycles of 95°C for 5 sec, and 56°C for 30 sec, using a melting curve of 65°C to 95°C, with increments of 0.5°C.

Limit of Detection (LOD) of the SYBR Green-Based Real-Time PCR Assay

The LOD of the SYBR Green-based real-time PCR assay was determined using 10-fold diluents of a plasmid into which the amplified product of the target gene had been cloned and genomic DNA, and suspensions of *A. avenae* subsp. *citrulli* cells (range, 1.48×10^9 to 1.48×10^3 copies/µl, 5×10^0 to 5×10^{-6} ng/µl, and 6.5×10^4 to 6.5×10^0 CFU/µl, respectively) in a 20 µl reaction mixture containing 10 µl of SYBR Premix Ex Taq and 5 pM each of the AC158F/R primers. Approximately 5 fg of *A. avenae* subsp. *citrulli* genomic DNA corresponds to one bacterial genome [14, 15, 20, 26]. Thus, this genomic DNA was diluted from 5 ng to 5 fg

Table 1. Bacterial strains used in this study.

No.	Scientific name	Source[a]	Biological origin	Geographic origin	Result[b]
1	*Acidovorax avenae* subsp. *citrulli*	NCPPB 3679^T	*Citrullus lanatus*	USA	+
2	*A. avenae* subsp. *citrulli*	NCPPB 3244	*Citrullus lanatus*	USA	+
3	*A. avenae* subsp. *citrulli*	LMG 2254	*Citrullus lanatus*	USA	+
4	*A. avenae* subsp. *citrulli*	LMG 5483	*Citrullus lanatus*		+
5	*A. avenae* subsp. *citrulli*	NCPPB 4204	*Citrullus lanatus*	Brazil	+
6	*A. avenae* subsp. *citrulli*	NCPPB 4203	*Cucumis melo*	Brazil	+
7	*A. avenae* subsp. *avenae*	NCPPB 1011^T	*Zea mays*	USA	-
8	*A. avenae* subsp. *cattleyae*	NCPPB 961^T			-
9	*A. konjaci*	NCPPB 3698^T	*Amorphophalus rivieri*	Japan	-
10	*A. valerianellae*	NCPPB 4283^T	*Valerianella radiata*		-
11	*A. delafieldii*	LMG 5943^T	Soil	USA	-
12	*A. facilis*	LMG 6598	Lawn soil		-
13	*A. temperans*	LMG 7169^T	Human	Sweden	-
14	*Burkholderia glumae*	LMG 2196^T	*Oryza sativa*	Japan	-
15	*B. gladoli*	LMG 2216^T	*Gladiolus* sp.	USA	-
16	*B. platarii*	LMG 9035^T	*Oryza sativa*	Japan	-
17	*B. cepacia*	LMG 1222^T	*Allium cepa*	USA	-
18	*B. cartophyophvlli*	LMG 2155^T	*Dianthus caryophyllus*	USA	-
19	*B. andropogonis*	LMG 2129^T	*Sorghum bicolor*	USA	-
20	*B. cenocepacia*	KACC 12021^T	Sputum	UK	-
21	*Pseudomonas libanensis*	KACC 10809	Spring water		-
22	*P. fuscovaginae*	KACC 10676^T	*Oryzae sativa*	Japan	-
23	*P. graminis*	LMG 21661^T	Grasses	Germany	-
24	*P. ludensis*	LMG 13517^T	Prepacked beef		-
25	*P. aeruginosa*	KACC 2386^T			-
26	*Pectobacterium atrosepticum*	KACC 10477^T	*Solanum tuberosum*	UK	-
27	*Erwinia tracheiphila*	KACC 2707^T	*Cucumis melo*	USA	-
28	*Pantoea ananatis*	LMG 2676	*Puccinia graminis*	USA	-
29	*P. agglomerans*	LMG 2565	Cereals	Canada	-
30	*Rhizobium radiobacter*	KACC 10736	*Malus* sp.		-
31	*Xanthomonas axonopodis* pv. *vesicatoria*	LMG 905			-
32	*X. orizae* pv. *oryzae*	KACC 10331		Republic of Korea	-
33	*X. campestris* pv. *campestris*	KACC 10913^T	*Brassica oleracea*	UK	-
34	*Ralstonia solanasearum*	KACC 10814^T	*Lycopersicon esculentum*	USA	-
35	*R. pickettii*	LMG 5942	Patient	USA	-

[a]A superscripted “T” indicates a type strain.

[b]+ and – indicate that the species was detected or not detected, respectively, using both conventional and real-time PCRs.

using 10-fold dilutions. The SYBR Green-based real-time PCR assay was conducted as described above. The copy number of the plasmid DNA was calculated using the following equation [7]: Copies/µl = [6.022 × 10^{23} (copy/mol) × amount of DNA (g)]/[DNA length (bp) × 660 (g/mol/bp)]. To determine the LOD, primers specific for the 16S-23S ITS region were used under the following reaction conditions [29]: 95°C for 30 sec, 45 cycles of 95°C for 5 sec and 53°C for 30 sec, using a melting curve of 65°C to 95°C with increments of 0.5°C.

Detection of *A. avenae* subsp. *citrulli* in Infected Samples Using Direct PCR

For the pathogenicity tests, the pathogen was grown overnight on nutrient agar at 28°C and then the cells were suspended in

Table 2. Primers used in this study.

Primer	Sequences (5′-3′)	Annealing temp.	Amplicon size	Gene	Reference
AC158F	CTTGGTGCTCCATGCTCGA	56°C	158 bp	YD-repeat protein[a]	This study
AC158R	GGCTTGGTTGCGAATTCACT				
SEQID4	GTCATTACTGAATTTCAACA	53°C	246 bp	16S-23S ITS region of rDNA	Walcott *et al*. [29]
SEQID5	CCTCCACCAACCAATACGCT				

[a]The listed sequences are found in GenBank Accession No. NC_008752; region: 2237201..2242513; protein ID YP_970406.

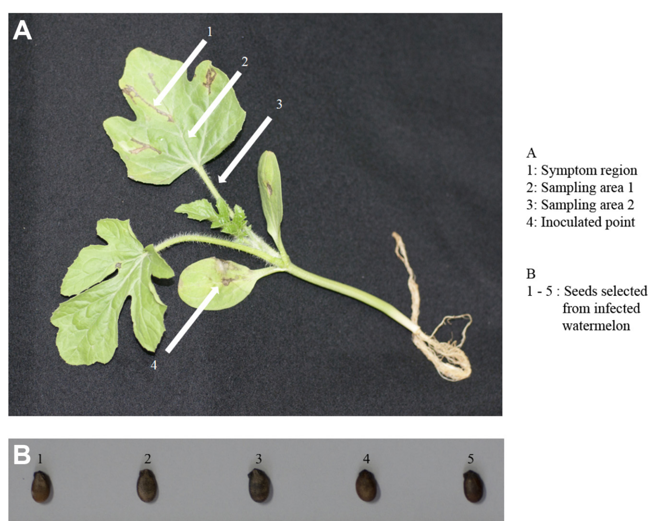

Fig. 1. Sampled area of watermelon seedlings.
Image of a watermelon seedling with the sampled areas and inoculation point indicated. Inoculation with *Acidovorax avenae* subsp. *citrulli* NCPPB 3679 was performed using the prick method; dpi = days post inoculation. (**A**) Infected watermelon plant. 1: infected area at 7 dpi; 2: sampled region 1; 3: sampled region 2; 4: inoculation point. (**B**) Infected watermelon seeds.

sterile distilled water and brought to an optical density (OD_{600}) of 0.1. The bacterial cells were inoculated on individual cotyledons of 1-week-old seedlings (*Citrulus lanatus* cv. 'Speed') grown in a greenhouse, using the prick method [8]. Naturally infected watermelon seeds were provided by Nongwoo Bio, Republic of Korea. Six watermelon seeds were randomly selected, cut using a sterile scalpel, and soaked in 500 µl of sterile distilled water for 30 min. Artificially inoculated leaf and stem tissues were removed from the watermelon seedlings at 7 dpi (days post inoculation) as depicted in Fig. 1. Each sample was dipped in 500 µl of sterile distilled water in a 1.5 ml tube for 30 min. Two microliters of the rinse water was directly used in the SYBR Green-based direct PCR assays as described above. The *in planta* testing was performed in triplicate for each sample. After PCR amplification, a melting-curve analysis was performed to ensure that only one amplicon was produced.

Results

In Silico Specificity Test of the Designed Primer Set

To determine their annealing specificity, potential primers based on the sequence of the gene encoding a YD-repeat protein (GenBank Accession No. NC_008752; protein ID YP_970406) were tested by conducting similarity searches of the NCBI database (http://www.ncbi.nlm.nih.gov/). When the sequences of the 158 bp products amplified using the primers were compared, no significant matches were found using both BLASTn and BLASTx searches.

Specificity of the Conventional PCR and SYBR Green-Based Real-Time PCR Assays

The specificity of the conventional PCR and SYBR Green-based real-time PCR assays performed using the selected pair of primers was tested using six strains of *A. avenae* subsp. *citrulli* as well as other strains. As expected, the 158-bp amplified product was produced using conventional PCR, and SYBR Green-based real-time PCR yielded an amplified product with a fluorescence intensity indicating that a single amplicon was present in *A. avenae* subsp. *citrulli*. Genomic DNA isolated from the other *Acidovorax* strains and the reference bacterial strains was not amplified using this primer set (Fig. 2 and Table 1).

Limit of Detection of the SYBR Green-Based Real-Time PCR Assay

We used the SYBR Green real-time PCR assay of *A. avenae* subsp. *citrulli* using the AC158F/R primer set to determine the LOD, using a standard curve created by plotting the mean threshold cycle (*Ct*) (n = 3) for logarithmically decreased concentrations of plasmid DNA (Fig. 3A) and genomic DNA and densities of cell suspensions (Table 3).

It was possible to quantify at least one genome-equivalents of the target DNA within purified DNA using the assay that was developed. The assay showed a good linear response (R^2 = 0.997). Standard regression analysis of the linear part of the slope yielded a coefficient of -3.486,

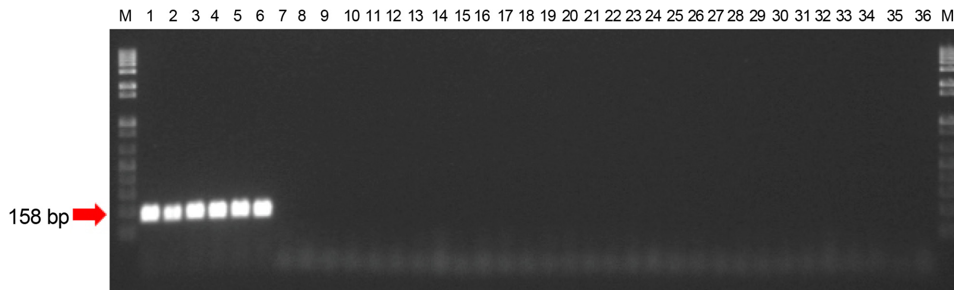

Fig. 2. Specific polymerase chain reaction amplification of a region of the YD-repeat protein gene of *Acidovorax avenae* subsp. *citrulli* using the AC158F/R primer set.

Lane M contains the size standards (1 kb Plus DNA ladder; Gibco BRL), lanes 1 to 6 contain the products of *A. avenae* subsp. *citrulli* strains, lanes 7 to 13 contain the products of other *Acidovorax* species, and lanes 14 to 35 contain the products of the *Burkholderia* strains and the *Pseudomonas*, *Pectobacterium*, *Erwinia*, *Pantoea*, *Rhizobium*, *Xanthomonas*, and *Ralstonia* strains that are listed in Table 1. Lane 36 represents the negative control (distilled water).

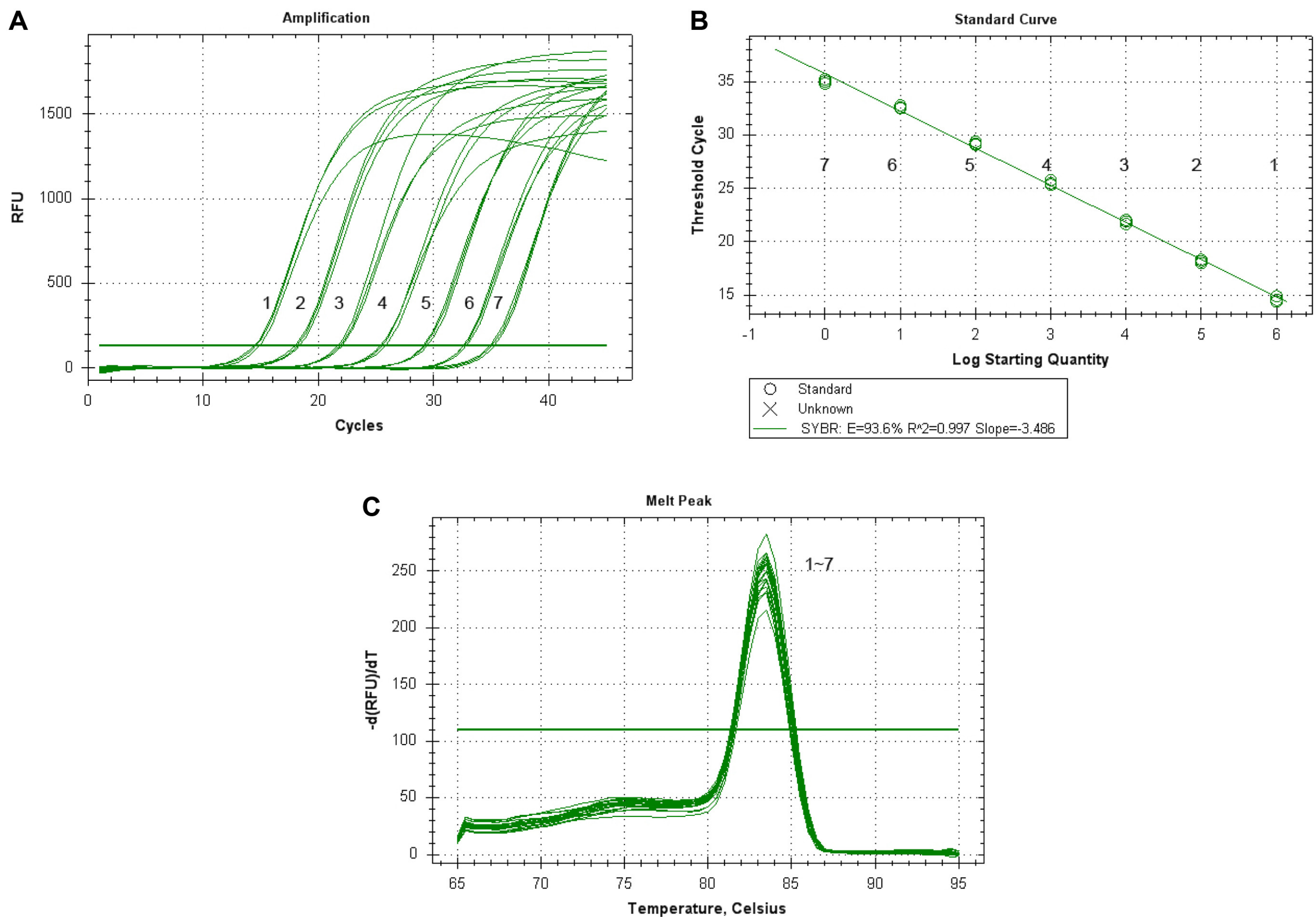

Fig. 3. SYBR Green-based real-time PCR using the AC158F/R primers for the quantitative amplification of an *Acidovorax avenae* subsp. *citrulli* product.

(**A**) The intensities of the fluorescence signal in relation to the amount of template used. Purified cloned target DNA (5 ng/µl) that was diluted 10-fold (sample numbers 1–7) was used as the template in each assay. (**B**) The standard curve derived from the amplification plot shown in panel A. (**C**) Results of the melting-curve analysis. The relative fluorescence units (–d(RFU)/dT) of the amplified products was plotted as a function of temperature. The melting temperature of the amplified product was 83°C. A high peak indicates the amplified product.

which corresponded to a PCR efficiency level of 93.6% (Fig. 3B). Analysis of the melting temperature and melting peaks of *A. avenae* subsp. *citrulli* DNA obtained using SYBR Green-based real-time PCR revealed a reproducible melting temperature of 83°C and specific melting peaks (Fig. 3C).

The LODs of the SYBR Green-based real-time PCR assay were determined using a 10-fold dilution series of genomic DNA and of suspensions of *A. avenae* subsp. *citrulli* cells and were found to be 5 fg/µl and 6.5 CFU/µl, respectively (Table 3). The standard curves indicated that there was a linear correlation between the *Ct* values and the concentration of the input DNA or the cell suspension, as follows: genomic DNA (R^2 = 0.996, slope = -3.636, and PCR efficiency = 88.4%) and bacterial suspension (R^2 = 0.996, slope = -3.425, and PCR efficiency = 95.9%) of the pathogen (data not shown). The SEQID 4^m/5 primers [29] provided false-negative PCR results when the genomic DNA concentration was low or the pathogen population was small (Table 3).

Detection of *A. avenae* subsp. *citrulli* in Infected Samples Using Direct PCR

Using the AC158F/R primer set for SYBR Green-based direct PCR, it was possible to detect *A. avenae* subsp. *citrulli* in five artificially infected watermelon leaves or stems or five naturally infected seeds. The predicted 158 bp product, which exhibited fluorescence, was amplified from all of these infected materials using this method, whereas no amplicons were obtained from healthy watermelon leaves, stem tissues, or seed samples. The *Ct* values obtained using the cell suspensions (OD_{600} = 0.1) ranged from 24.10 to 24.38, whereas the *Ct* values obtained using infected leaves, stem tissues, and seed samples ranged from 20.61 to 24.54, 24.11 to 30.27, and 26.13 to 29.32, respectively (Fig. 4).

Discussion

BFB is considered a serious threat mainly for the watermelon industry. Early detection of this disease is very important when assessing the health status of a watermelon nursery before the seedlings are transplanted in the fields. The recent increase in BFB outbreaks on cucurbits worldwide is due to changes in the climate, such as increasing temperatures that favor the survival and dispersion of the pathogen [27]. In addition, currently available pathogen detection assays are insufficiently sensitive, and *A. avenae* subsp. *citrulli*–infested plants can be asymptomatic under suboptimal conditions while the disease is developing [3].

The 16S ribosomal RNA gene has long been used for sequence-based microbial classification. However, only two or three bases of the analogous regions of closely related species differ, and this region has been amplified only from isolates of *A. avenae* subsp. *citrulli.* Additionally, maintaining the specificity of the relevant primers requires very strict control of the annealing temperature [5].

The database of entire genomic sequences of microbes is rapidly growing and will be useful for disease diagnosis. Pathogen detection has become a very dynamic field characterized by convergence technology. Automation and electronic data management are vital to increasing the efficiency of detecting pathogenic bacteria. Nevertheless, despite recent progress in pathogen identification techniques, most of the currently available diagnostic methods have

Table 3. Mean *Ct* values, and end-point fluorescence intensities for 10-fold serially diluted *Acidovorax avenae* subsp. *citrulli* NCPPB 3679 genomic DNA and cell suspensions, obtained using SYBR-Green-based real-time PCR.

Genomic DNA			Cell suspension		
ng/µl	*Ct* value[a]	*Ct* value[b]	CFU/µl	*Ct* value[a]	*Ct* value[b]
5 ng	15.35 ± 0.23	29.30 ± 0.30	6.5×10^6	ND[d]	ND
500 pg	18.82 ± 0.23	33.89 ± 0.59	6.5×10^5	ND	ND
50 pg	22.55 ± 0.21	37.29 ± 0.97	6.5×10^4	22.39 ± 0.09	23.03 ± 0.64
5 pg	26.20 ± 0.37	-[c]	6.5×10^3	25.91 ± 0.28	29.21 ± 0.11
500 fg	29.94 ± 0.32	-	6.5×10^2	29.21 ± 0.16	33.84 ± 0.47
50 fg	33.30 ± 0.91	-	6.5×10^1	32.83 ± 0.48	37.96 ± 1.29
5 fg	37.11 ± 0.88	-	6.5×10^0	36.06 ± 0.67	-

[a]AC158 F/R primer set.

[b]SEQID4m/SEQID5 primer set (Walcott *et al*. [29]).

[c]-, not detected.

[d]ND, not determined.

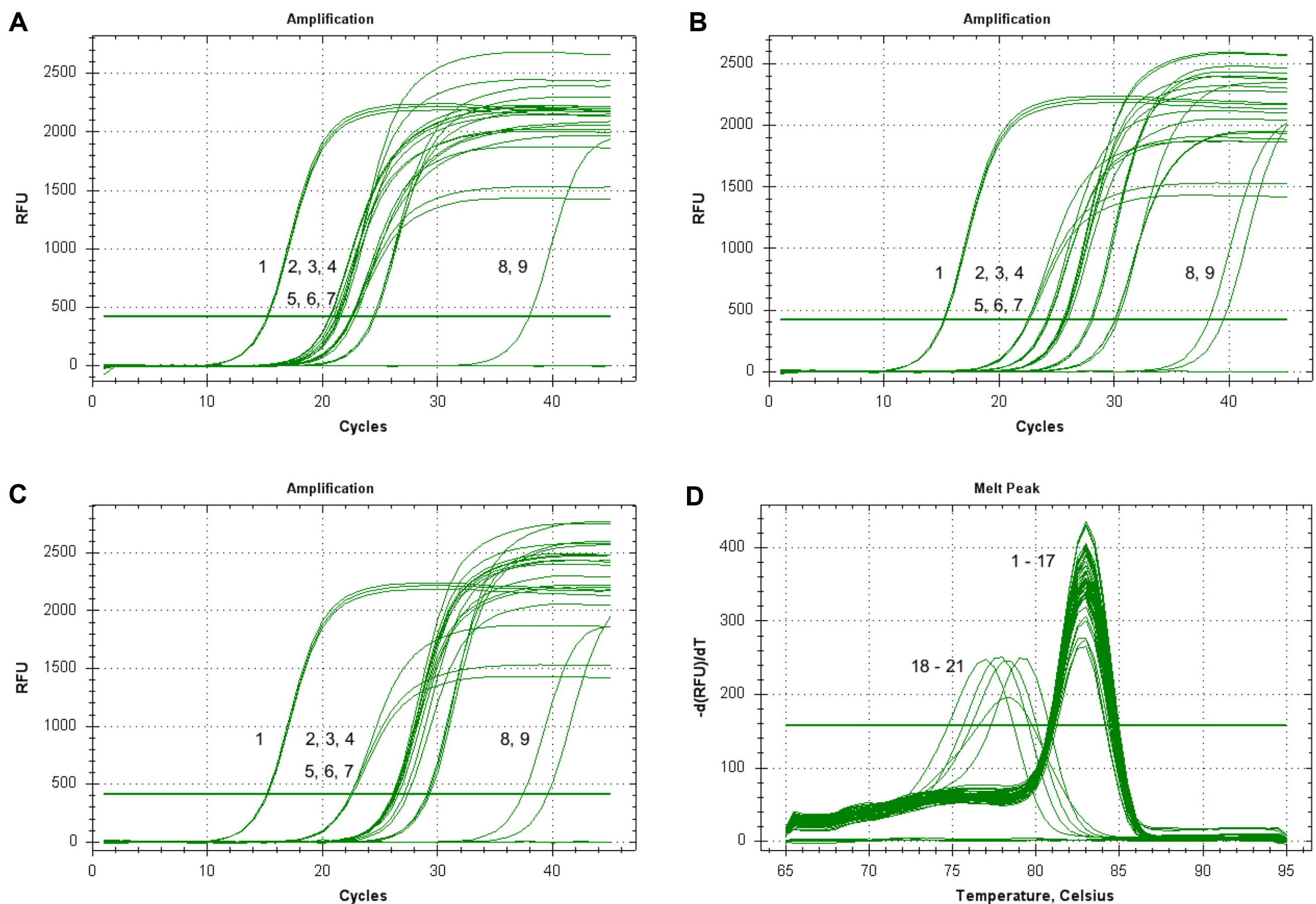

Fig. 4. Specific detection of *Acidovorax avenae* subsp. *citrulli* in infected watermelon samples using the AC158F/R primer set for SYBR Green-based direct PCR.

The fluorescence intensity corresponding to the fragment of the YD-repeat protein gene of *A. avenae* subsp. *citrulli* that was amplified using the AC158F/R primer set. (**A**) Sample 1, *A. avenae* subsp. *citrulli* NCPPB 3679 genomic DNA; sample 2, *A. avenae* subsp. *citrulli* NCPPB 3679 cell suspension; samples 3–7, infected watermelon leaves; sample 8, healthy watermelon leaf; sample 9, no-template control. (**B**) Sample 1, *A. avenae* subsp. *citrulli* NCPPB 3679 genomic DNA; sample 2, *A. avenae* subsp. *citrulli* NCPPB 3679 cell suspension; samples 3–7, infected watermelon stems; sample 8, healthy watermelon stem; sample 9, no-template control. (**C**) Sample 1, *A. avenae* subsp. *citrulli* NCPPB 3679 genomic DNA; sample 2, *A. avenae* subsp. *citrulli* NCPPB 3679 cell suspension; samples 3–7, naturally infected watermelon seeds; sample 8, healthy watermelon seed; sample 9, no-template control. (**D**) Results of the melting-curve analysis. The relative fluorescence units (-d(RFU)/dT) corresponding to the amplified products were plotted as a function of temperature (primer-dimer products: 77.0–79.0°C; amplified product: 83°C). sample 1, *A. avenae* subsp. *citrulli* NCPPB 3679 genomic DNA; sample 2, *A. avenae* subsp. *citrulli* NCPPB 3679 cell suspension; Samples 3–7, infected watermelon leaves; samples 8–12, infected watermelon stems; samples 13–17, naturally infected watermelon seeds; samples 18–20, healthy watermelon leaf, stem, and seed; and sample 21, no-template control.

limitations, including the need for laborious sample preparation, the lack of specificity, the requirement for bulky instrumentation, and slow data-readout rates [23].

In this study, we exploited the genomic information that has been deposited in a public database (http://www.ncbi.nlm.nih.gov) to develop a SYBR Green-based direct PCR assay for the detection and identification of *A. avenae* subsp. *citrulli* in infected plant samples. The nucleotide sequences of the YD-repeat protein gene of *Acidovorax* species were assessed for specificity and variety through BLAST searches of the public genomic database. A species-specific primer set based on the YD-repeat protein gene of *A. avenae* subsp. *citrulli* (GenBank Accession No. NC_008752; protein ID YP_970406) showed high sensitivity and specificity for detecting the pathogen in seed lots and plants. The YD-repeat proteins (Rhs repertoires) of the Enterobacteriaceae are reported to be highly dynamic, which has been attributed to repeated gains and losses of genes. In contrast,

the key structures of the *rhs* genes have been evolutionarily conserved, indicating that the sequence diversity of these genes is driven not by rapid mutation, but by the slow evolution of novel core/tip combinations. Comparison of the C-terminal tips and dissociated fragments of YD-repeat protein genes indicated that although C-termini diverged greatly within a locus, each distinct sequence was conserved in the related strains and, indeed, in related species and genera. However, the function of these proteins is unknown and the conditions under which they are expressed have also been difficult to define [4, 13, 18, 19]. The YD-repeat proteins contain repetitive tyrosine-aspartate dipeptides. These proteins have two tandem copies of a 21-residue extracellular repeat that is found in gram-negative and gram-positive bacterial strains and in animals. The YD repeat is named for the YD dipeptide, the most strongly conserved motif of the repeat. These repeats appear to be involved in binding carbohydrates. The YD repeats may be found free on the surface of bacteria for host interactions as well [6, 11, 14, 16].

Here, we present that our novel molecular marker is capable of detecting *A. avenae* subsp. *citrulli* in watermelon and it can reliably distinguish the pathogen from other closely related species and genera. The developed SYBR Green PCR assay for the quantitative detection of *A. avenae* subsp. *citrulli* in infested samples is a fast, accurate, and sensitive surveillance tool, and more sensitive and reliable than previous results (Table 3).

In conclusion, as a result of the high sensitivity and specificity of the SYBR Green real-time PCR assay with its relatively rapid and simple procedure, this method is rapid and less cumbersome than other diagnostic methods for the identification of *A. avenae* subsp. *citrulli* strains. The described method can be used to detect a low level of *A. avenae* subsp. *citrulli* from seed and plant samples without the use of selective media, additional biochemical tests, or DNA extraction.

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