Quantitative Real-Time Polymerase Chain Reaction Assay for Detection of *Pectobacterium wasabiae* Using YD Repeat Protein Gene-Based Primers

Myeong Ho Kim, Min Seok Cho, Byoung Kyu Kim, Hyeon Jin Choi, Jang Ho Hahn, ChangKug Kim, and Man Jung Kang, National Academy of Agricultural Science, Rural Development Administration, 441-707 Suwon, Republic of Korea; Seong Hwan Kim, Department of Microbiology, Dankook University, 330-714 Cheonan, Republic of Korea; and Dong Suk Park, National Academy of Agricultural Science, Rural Development Administration, Republic of Korea

Abstract

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The aim of this study was to develop a quantitative polymerase chain reaction (qPCR) assay for specific detection of *Pectobacterium wasabiae* using a primer pair based on the YD repeat protein gene for amplification of a 140-bp DNA fragment from infected wasabi (*Wasabia japonica*), a member of the crucifer family. The soft rot caused by *P. wasabiae* is an emerging disease that is present in many wasabi-producing areas. However, specific and reliable methods for identifying the pathogen are not available. Therefore, a qPCR primer set for accurate diagnosis of *P. wasabiae* was developed from publically available genome sequences. A SYBR Green qPCR primer set was designed

based on a YD repeat protein gene of *P. wasabiae* WPP163 because it is known that this gene is structurally diverse among species, pathovars, or subspecies. The specificity of the primer set was evaluated using genomic DNA from 5 isolates of *P. wasabiae*, 5 different species of *Pectobacterium*, and 16 other pathogenic reference bacteria. The primer set used in the PCR assay successfully amplified a 140-bp amplicon for all five *P. wasabiae* strains. No amplification was obtained from 29 other pathogenic bacteria. The assay was also able to detect at least two genomic DNA, or 3 CFU per reaction, when using calibrated cell suspension.

Wasabi (*Wasabia japonica* (Miq.) Matsum.), a member of the crucifer family, is one of the popular spices in many Asian countries, especially in Korea and Japan (10). It is currently grown commercially in many countries in the world, including Korea, Japan, New Zealand, Israel, Brazil, Thailand, Taiwan, Canada, and the United States (3). It is a perennial aromatic herb that is cultivated on gravel beds laid in mountain streams at altitudes of about 200 to 800 m above sea level, where water temperatures range from 10 to 18°C during the year (7,18).

Pectobacterium wasabiae (5,7) has a narrow host range, having previously only been associated with Japanese horseradish. However, recent characterization of *Pectobacterium* spp. causing soft rotting in New Zealand has identified *P. wasabiae* isolates pathogenic to potato (16). The soft rot often occurs when the temperature of irrigation water exceeds 20°C, because of a decline in water flow during prolonged dry weather conditions, or when gravel beds are muddied by flooding from heavy rains (7,19). The pathogen inhabits rhizome tissues and fibrous roots independently or in a complex without causing bacterial soft rot symptoms as long as the temperature of irrigation water has been maintained below 18°C (7). However, there is no report on a specific and reliable method for identifying *P. wasabiae* from the crucifer family in the world thus far. Therefore, it is urgent to establish an accurate, rapid, sensitive, and practical method for detection of the pathogen from wasabi.

Currently, molecular assays based on the 16S-23S rDNA internal transcribed spacer region, *gyrB*, and *rpoD* are used for the detection of pathogenic bacteria. However, the sequences of these

genes are highly conserved because they differ at only two or three bases with respect to analogous regions in closely related species (5,12,15). However, it has been reported that YD repeat proteins (*Rhs* repertoires) are ubiquitous and comprise six structurally distinct lineages within the family Enterobacteriaceae, and there is considerable intergenomic variation in *Rhs* repertoires (8). YD repeats proteins are the repetitive dipeptide sequence tyrosine-aspartate. They contain two tandem copies of a 21-residue extracellular repeat that is found in gram-negative, gram-positive, and animal proteins. The repeat is named for a YD dipeptide, the most strongly conserved motif of the repeat. These repeats appear in general to be involved in binding carbohydrate. YD repeats may be free on the bacterial surfaces for host interactions as well (2,13).

Therefore, we have investigated the YD repeat protein gene to develop a specific primer set for identifying *P. wasabiae*. The primers will be used to develop a quantitative polymerase chain reaction (qPCR) assay for the quantitative and specific detection of *P. wasabiae* in wasabi, which will allow for the rapid and specific detection of *P. wasabiae*, thereby simplifying diagnosis and monitoring of the pathogen.

Materials and Methods

Bacterial strains, culture condition, and DNA extraction.

Bacterial strains were obtained from the Korean Agricultural Culture Collection in the Republic of Korea and the Belgian Coordinated Collections of Micro-organisms. Bacterial strains used in this study are listed in Table 1. *Pectobacterium*, *Erwinia* and *Rhizobium* strains were incubated 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; *Xanthomonas* strains were grown on YGC medium (D-[+]-glucose, 1.0%; CaCO₃, 3.0%; yeast extract, 0.5%; and agar, 1.5%) at 28°C for 3 days; and other microbes were grown on Luria-Bertani agar (tryptone, 1%; yeast extract, 0.5%; sodium chloride, 1%; and agar, 1.5%) at 28 to 37°C for 1 to 3 days (1). Genomic DNA from bacterial strains was extracted using the bacterial genomic DNA extraction kit supplied by Qiagen (Qiagen, Hilden, Germany). All genomic DNA were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Corresponding author : D. S. Park, E-mail: dspark@rda.go.kr

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Primer design and conventional PCR assays. A primer set, PW7011F (5'-CTATGACGCTCGCGGGTTCGTGTT-3') and PW7011R (5'-CGGCGGCGTCGTAGTGGAAAGTC-3'), was designed using PrimerSelect in Lasergene (version 7.2.1; DNASTAR Inc., Madison, WI) for the YD repeat protein gene of *P. wasabiae* WPP163 (GenBank accession CP001790.1, region 385930..390069, protein ID ACX86200.1), with a predicted PCR product of 140 bp. The nucleotide sequences of the genes were assessed for specificity and variety among *Pectobacterium* spp. through BLASTn searches (14). Conventional PCR reactions were carried out with a PTC-225 thermocycler (MJ Research, Watertown, MA). PCR was performed in 50 µl of reaction mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.2 mM each dNTP, 20 pM of each primer, 2 units of *Taq* polymerase (Promega Corp., Madison, WI), and 25 ng of genomic DNA from a given bacterial strains. Reactions were run for 35 cycles, each consisting of 60 s at 94°C, 30 s at 67°C, and 60 s at 72°C, with an initial denaturation of 5 min at 94°C and a final extension of 10 min at 72°C. Amplified products were separated on 1.5% agarose gel by electrophoresis at 100 V for 60 min, 1× Tris-acetate-EDTA buffer, then stained with LoadingStar (DYNEBIO, Republic of Korea).

Sensitivity and specificity of the SYBR Green qPCR assay. The SYBR Green qPCR assay was carried out in a 20-µl reaction mixture containing KAPA SYBR FAST qPCR Master Mix (2×) Universal (KAPA Biosystem, Inc.). The amplification mixture consisted of 10 µl of KAPA SYBR FAST qPCR Master Mix (2×) Universal and 5 pM each of PW7011F/R and 5 ng of purified DNA from each sample. As for *P. wasabiae*, 5 fg is roughly equivalent to one bacterial genome (11,17,20). Therefore, the bacterial genomic DNA was diluted from 5 ng to 5 fg using 10-fold dilutions.

SYBR Green qPCR assay was performed in the CFX96 real-time PCR system (Bio-Rad Laboratories, Inc.) with the following

conditions: 95°C for 3 min and 45 cycles of 95°C for 10 s, 67°C for 20 s, and a melting curve of 65 to 95°C with an increment of 0.5°C. The concentration of the cloned DNA was measured using a fluorometer and the corresponding copy number was calculated using the equation $(4) \text{ copies}/\mu\text{l} = [6.022 \times 10^{23} (\text{copy}/\text{mol}) \times \text{DNA amount (g)}]/[\text{DNA length (bp)} \times 660 (\text{g}/\text{mol}/\text{bp})]$.

Detection of pathogen by bio-PCR. For the bio-PCR assay, the pathogen was grown overnight on nutrient agar medium and resuspended in sterile distilled water to an optical density of 600 nm ($\text{OD}_{600} = 0.1$). Bacterial suspensions were inoculated by the prick method (7) on independent leaves (*W. japonica* 'Dalma') of 3-year-old healthy plants grown in the greenhouse. Leaf tissue was collected from an area 1 cm from the inoculation point at 3 days post inoculation, as depicted in Supplementary Figure S1. Each sample was dipped in 500 µl of sterile distilled water in a 1.5-ml tube for 30 min. A 2-µl sample from the rinse water was directly used in the SYBR Green PCR assays as described above.

Results

In silico specificity test of designed primer set. Specificity of the primer set (PW7011F/R) based on a YD repeat protein (GenBank accession CP001790.1, protein ID ACX86200.1) was tested by similarity search against the sequence database at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). When comparing the sequence of the 140-bp fragment amplified by the primer set, the closest match in a BLASTn (version 2.2.25) search revealed only 67% sequence identity with *Pantoea ananatis* (GenBank accessions AP012032.1 and CP001875.2). BLAST searches with the predicted protein sequence (BLASTx) revealed that the closest similarity was to the *Rhs* family protein from *Enterobacter cloacae* subsp. *cloacae* (American Type Culture Collection 13047, GenBank accession YP_003613625.1) but it only had a maximum identity of 60%. Thus, the sequence region to

Table 1. Bacterial strains used in this study

Number	Scientific name	Source ^a	Geographic origin	Host	Assay ^b
1	<i>Pectobacterium wasabiae</i>	LMG 8444 ^T	Japan	Japanese horseradish	+
2	<i>P. wasabiae</i>	KACC 10406	...	...	+
3	<i>P. wasabiae</i>	KACC 14078	Republic of Korea	...	+
4	<i>P. wasabiae</i>	KACC 10868	Japan	Japanese horseradish	+
5	<i>P. wasabiae</i>	KACC 10869	Japan	Japanese horseradish	+
6	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	LMG 2404 ^T	Denmark	<i>Schizanthus</i> sp.	-
7	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	LMG 2399	United Kingdom	<i>Solanum tuberosum</i>	-
8	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	LMG 2400	Japan	<i>Brassica rapa</i>	-
9	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	LMG 2401	United States	<i>Daucus carota</i> var. <i>sativus</i> , soft rot	-
10	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	LMG 2405	Israel	<i>Persea americana</i>	-
11	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	LMG 2406	United Republic of Tanzania	<i>Schizanthus</i> sp.	-
12	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	KACC 10343	Republic of Korea	Japanese horseradish	-
13	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	KACC 10347	Republic of Korea	Japanese horseradish	-
14	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	KACC 13968	Republic of Korea	Potato	-
15	<i>P. carotovorum</i> subsp. <i>odoriferum</i>	KACC 13954	Republic of Korea	Chinese cabbage	-
16	<i>P. atrosepticum</i>	KACC 10477 ^T	United Kingdom	<i>Solanum tuberosum</i> , stem rot	-
17	<i>P. betavascularum</i>	LMG 2466 ^T	United States	<i>Beta vulgaris</i> , vascular strand	-
18	<i>P. cacticida</i>	LMG 2720	Arizona United States	<i>Carnegiea gigantea</i>	-
19	<i>Erwinia rhapontici</i>	LMG 2688 ^T	United Kingdom	<i>Rheum rhabarbarum</i>	-
20	<i>E. persicina</i>	KACC 10053 ^T	Japan	Tomato	-
21	<i>E. tracheiphila</i>	KACC 10084 ^T	United States	<i>Cucumis melo</i>	-
22	<i>E. chrysanthemi</i>	KACC 10062 ^T	United States	<i>Chrysanthemum morifolium</i>	-
23	<i>E. herbicola</i>	LMG 2565	Canada	Cereals	-
24	<i>E. nigrifluence</i>	LMG 2694 ^T	Zimbabwe	Knee laceration	-
25	<i>Enterobacter agglomerans</i>	LMG 1286 ^T	United States	<i>Juglans regia</i> , bark canker	-
26	<i>Pantoea ananatis</i>	LMG 2676	United States	<i>Puccinia graminis</i> f. sp. <i>tritici</i> , uredia	-
27	<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	KACC 10331	Republic of Korea	...	-
28	<i>Pseudomonas syringae</i> pv. <i>syringae</i>	LMG 5082	United Kingdom	<i>Zea mays</i>	-
29	<i>P. fuscovaginae</i>	LMG 2158 ^T	Japan	<i>Oryza sativa</i>	-
30	<i>P. graminis</i>	KACC 10803 ^T	Germany	Grasses	-
31	<i>Rhizobium radiobacter</i>	KACC 10736	...	<i>Malus</i> sp.	-
32	<i>Ralstonia solanaceum</i>	KACC 10814 ^T	United States	<i>Lycopersicon esculentum</i>	-
33	<i>Serratia marcescens</i>	KACC 11961 ^T	Czech Republic	Pond water	-
34	<i>Escherichia coli</i>	LMG 2092 ^T	Denmark	Urine	-

^a Superscript "T" indicates type strain.

^b Conventional or real-time assay; + and - indicate species detected or not detected, respectively.

be amplified with the designed primer set revealed no significant matches in both BLASTn and BLASTx searches.

Specificity test of primers. The PW7011F/R primers were tested against five strains of *Pectobacterium wasabiae*. As expected, the 140-bp gene fragment was amplified. All other strains, including other *Pectobacterium* spp., did not amplify with the primer set (Fig. 1).

Sensitivity test by SYBR Green qPCR. We used SYBR Green qPCR assay of *P. wasabiae* with the PW7011F/R primers to generate a standard curve by plotting the mean threshold cycle (Ct) ($n = 3$) versus the logarithmic concentration of genomic DNA, cloned DNA, and density of the bacterial cells (range of 5×10^0 to 5×10^{-6} ng/ μ l, 1.45×10^9 to 1.45×10^3 copies/ μ l, and 3.0×10^5 to 3.0×10^0 CFU/ μ l, respectively; Fig. 2; Table 2). Standard regression analysis

Fig. 1. Specific polymerase chain reaction amplification of a YD repeat protein from *Pectobacterium wasabiae* with the primer set PW7011F/R. Lane M is the size marker (1-kb DNA plus ladder; Gibco BRL) while lanes 1–5 were *P. wasabiae* strains; lanes 6–34 included strains from other *Pectobacterium* spp. along with strains from species of *Erwinia*, *Pantoea*, *Xanthomonas*, *Pseudomonas*, *Rhizobium*, *Ralstonia*, *Serratia*, and *Escherichia*; and lane 35 was a negative control (distilled water).

Fig. 2. Real-time polymerase chain reaction (PCR) with SYBR Green and primers PW7011F/R for quantitative amplification of *Pectobacterium wasabiae* LMG8444. **A.** Fluorescence signal in relation to the amount of template. For each assay, cloned amplified target DNA using purified DNA (5×10^0 ng/ μ l) was diluted 10-fold (sample numbers 1–7) and used as a template in the PCR. **B.** Standard curve derived from amplification plot shown in A. **C.** Melting-curve analysis is shown as the negative first derivative of the relative fluorescence units ($-d(RFU)/dT$) plotted versus temperature; (primer dimer product: 79.0°C, amplified product: 87.5°C).

of the linear part of the slope gave a coefficient of -3.540 , which yielded a PCR efficiency of 91.6% (Fig. 2B). Analysis of the melting temperature and melting peaks of *P. wasabiae* by SYBR Green qPCR revealed a reproducible melting temperature of 87°C and specific peaks (Fig. 2C).

A sensitivity test was performed using 10-fold dilution series of genomic DNA and bacterial suspension of *P. wasabiae*. The minimum detection level of genomic DNA and bacterial suspension by SYBR Green qPCR assay was approximately 5×10^0 fg/ μl and 3 CFU/reaction of *P. wasabiae*, respectively. Standard curves showed

Table 2. Mean threshold cycle (Ct), end-point fluorescence, and 10-fold serially diluted *Pectobacterium wasabiae* LMG8444 genomic DNA and bacterial suspensions by SYBR-Green quantitative polymerase chain reaction^a

Genomic DNA		Bacterial suspensions	
ng/ μl	Ct values	CFU/ μl	Ct values
5 ng	16.45 ± 0.25	ND	ND
500 pg	19.29 ± 0.16	3.0×10^5	24.45 ± 0.26
50 pg	22.49 ± 0.10	3.0×10^4	23.94 ± 0.16
5 pg	26.16 ± 0.17	3.0×10^3	26.62 ± 0.29
500 fg	29.50 ± 0.29	3.0×10^2	30.13 ± 0.11
50 fg	33.14 ± 0.11	3.0×10^1	33.69 ± 0.24
5 fg	36.11 ± 0.19	3.0×10^0	36.59 ± 0.52

^a ND = not determined.

Fig. 3. Specific detection of *Pectobacterium wasabiae* by SYBR Green bio-polymerase chain reaction in infected leaves using the primer set PW7011F/R. **A**, Fluorescence intensity corresponding to a YD repeat protein from *Pectobacterium wasabiae* amplified using PW7011F/R. Sample 1, *P. wasabiae* LMG8444 genomic DNA; sample 2, *P. wasabiae* LMG8444 cell suspension (optical density at 600 nm = 0.1); samples 3–6, infected wasabi leaves; samples 7–11, healthy wasabi leaves and no template control). **B**, Melting curve analysis generated during polymerase chain reaction. Amplified products derivative of relative fluorescence units ($-d(RFU)/dT$) is plotted as a function of temperature (primer dimer product: 79.0°C , amplified product: 87.5°C).

that there was a linear correlation between the Ct values and the concentrations of input DNA ($R^2 = 0.998$, slope = -3.346) or cell suspension ($R^2 = 0.995$, slope = -3.235) of the pathogen. A standard curve for density of the cell suspension was generated, except for 3.0×10^5 CFU/ μl , due to an overlap between 3.0×10^5 and 3.0×10^4 CFU/ μl Ct values (Table 2).

Detection of pathogen by bio-PCR. The primer set PW7011F/R could also detect the *P. wasabiae* by SYBR Green PCR in four symptomatic wasabi leaves. Amplified products derived from all symptomatic samples showed fluorescence in the SYBR Green PCR assay, and the expected DNA fragment of 140 bp was amplified by SYBR Green PCR, whereas no amplicons were obtained from healthy leaves. The Ct values of cell suspensions ($\text{OD}_{600} = 0.1$) ranged from 24.10 to 24.38, whereas the Ct values of inoculated samples ranged from 28.26 to 30.55 (Fig. 3).

Discussion

The primary hurdle in developing specific, easily used diagnostic markers for any pathogenic bacteria has been the difficulty in finding unique features whether they are bacterial surface antigens or gene sequences. Rapid detection and accurate identification of pathogens is important to guide responses for the prevention or elimination of plant pathogens. In particular, early disease detection is necessary for assessing the health status of plants before the transplantation of seedlings to fields.

The molecular assay for identification of *Pectobacterium* (formerly *Erwinia*) spp. is often difficult and controversial. The analyses of 16S rRNA gene, *gyrB*, and *rpoD* are very widely used for the molecular identification of bacteria. However, these regions do not sufficiently distinguish between the closely related species of *Pectobacterium* (6,9). In this study, we developed a primer set for the specific detection of *P. wasabiae* using available genome sequence information in GenBank. The species-specific primers derived from the whole genome sequences of *P. wasabiae* WPPI63 (GenBank accession CP001790.1). The YD repeat protein gene sequence (GenBank accession ACX86200.1) was analyzed by blast searches and identified to be highly variable among *Pectobacterium* spp. The YD repeat proteins (*Rhs* repertoires) are very dynamic among enterobacterial genomes, because of gains and losses in the repeated gene. In contrast, the main structures of *Rhs* genes are evolutionarily conserved, indicating that *Rhs* sequence diversity is driven not by rapid mutation but by the relatively slow evolution of novel core–tip combinations. Nevertheless, the function of these proteins is still unknown and the conditions under which they are expressed have also been difficult to define (8).

Therefore, a SYBR Green qPCR assay for the detection of *P. wasabiae* based on the YD repeat protein was developed in this study. In general, when cell suspensions or tissue extracts are directly used for a PCR, the appearance of nonspecific amplification bands is a common problem, which hampers the interpretation of the results (14). However, no spurious bands were observed in any of the samples analyzed using the PW7011F/R primer set, indicating that the primer set is very specific to *P. wasabiae* and reliable for the detection and identification of this pathogen. This method is faster and more economical and practical than the traditional methods.

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