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DEVELOPMENT OF SPECIFIC REAL-TIME PCR PRIMERS FOR DETECTING *PSEUDOMONAS SYRINGAE* PV. *ACTINIDIAE* IN CHINA

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Abstract

Pseudomonas syringae pv. *Actinidae* (Psa) is the most devastating disease of kiwifruit cultivation. In this study, to detect Psa in China high specifically and sensitively, a method of real-time PCR was developed. The specific primers YF32/YR32 was set for the 590 bp *hrp*W gene fragment, with amplicon of 248bp in length. The standard curves of real-time PCR clearly showed a suitable condition of real-time PCR and an excellent linear of the data. The specificity and sensitivity assay showed this method could specifically discriminate between Psa and Psa-related pseudomonads and sensitivity threshold was 100fg/μL. In the actual effect validation experiments, the results of all of 5 twig samples with symptoms were positive, and 3 of 5 symptomless samples were positive with minimum DNA concentration 8.45×10^{-5}ng/μL. The methods of this study could make an important contribution for the prevention and diagnosis of the bacterial canker disease of kiwifruit in China.

Key words: Real-time PCR primer; *Pseudomonas syringae* pv. *Actinidae*; China.

Introduction

Kiwifruit (*Actinidia chinensis*) originated in China, but due to improved cultivation techniques from New Zealand it gradually spread to other countries. Kiwifruit has become the primary focus of global fruit production with high economic value. China is the largest producer of kiwifruit in the world with a cultivated area of over 81,450 hectares and an annual output of 670,000 tons (Statistics of the Chinese Horticultural Society Kiwi Branch in 2014). But *Pseudomonas syringae* pv. *actinidiae* (Psa) has been first reported causing kiwifruit bacterial canker in several countries, including Japan (Serizawa *et al*., 1989), Korea (Koh & Lee, 1992), Italy (Scortichini, 1994), China(Liang *et al*., 2000), New Zealand (Everett *et al*., 2011),Turkey(Bastas & Karakaya, 2012), Greece (Holeva *et al*., 2015) and Georgia (Meparishvili *et al*., 2016) . The widespread outbreak of bacterial canker of kiwifruit has become an important limiting factor for the cultivation of kiwifruit. Therefore, it is very essential to explore a more reliable Psa detection method and take control measures before the occurrence of obvious symptoms.

Traditionally, the detection of Psa is based on 16S–23S rDNA sequencing (Balestra *et al*., 2009; Reesgeorge *et al*., 2010), or new type PCR technology, including ERIC-PCR (Ferrante & Scortichini, 2009), KN-PCR (Koh & Nou, 2002) and RG-PCR (Rees-George *et al*., 2010). Nevertheless, these methods are nonspecific, and cannot discriminate Psa from similar bacteria *P. syringae* pv. *theae*. To improve the accuracy and specificity of the assays, more and more sensitive and reliable method are gradually established. For example, a duplex-PCR with high specificity is applied to infected kiwifruit matrices (Gallelli *et al*., 2011a); two PCR-assays (direct-PCRs) are applied to symptomless kiwifruit material (Gallelli *et al*., 2011b); a PCR and nested-PCR is applied to bleeding sap samples (Biondi *et al*., 2013); a multiplex PCR is applied to populations from different geographical origin (Balestra *et al*., 2013).

Two types of PSA strains have recently been found, one is highly virulent strains of Psa-V (Young, 2012) and the other is less virulent strains of PsD (Butler *et al*., 2013). Psa-D strains cause only minor or no damage and phylogenetically relate to Psa-V (Butler *et al*., 2013; Hill *et al*., 2015). A real-time PCR assay has been carried out to detect specifically Psa-V strains (Gallelli *et al*., 2013). However, the bacteria strains used in the experiment do not come from China and do not take Chinese kiwifruit samples for validation.

It has been reported that the design of primers in the *hrp*W gene can detect specifically Psa and there is no amplification for PsD strain (Gallelli *et al*., 2011a). In this study, in order to detect Chinese Psa high specifically and sensitively, a real-time PCR method with new primers designed for *hrp*W gene fragment was developed, and applied to the infected samples collected from orchards in China.

Materials and Methods

Bacterial strains: All of strains used in primer design in this experiment were listed in Table 1. The strain of *P. syringae* pv. *actinidiae* were originated from different provinces in China and have been identified by molecular and pathogenicity experiments. Other similar bacteria were provided by the **ATCC®** Standards Development Organization**.** All the strains were stored in the laboratory after purified cultivation.

DNA preparation: A single colony of strains were picked to a liquid LB medium, cultivated at 25°C on a 250r / min shaker for 16-24h. Bacterial genomic DNA were extracted using the Bacteria DNA Kit (TIANGEN Biotech, China). Fresh infected and healthy kiwifruit twig were cut into pieces in sterile mortar, fully grounded by adding liquid nitrogen for two-three times. Plant tissue genomic DNA were extracted using the Plant Genomic DNA kit (TIANGEN Biotech, China). The quality of all genomic DNA samples were determined using a NanoDrop 200 spectrophotometer (Bio-Rad Laboratories Co., Ltd.). All tested samples were stored at -20°C.

Table 1. Strains of *Pseudomonas syringae* pathovars or similar strains of *Pseudomonas* used in this study.

Strain	Specie or pathovars	Source
P2101	*Pseudomonas syringae* pv. *actinidiae*	China, Sichuan
P2102	*Pseudomonas syringae* pv. *actinidiae*	China, Jiangxi
P2103	*Pseudomonas syringae* pv. *actinidiae*	China, Shanxi
P2104	*Pseudomonas syringae* pv. *actinidiae*	China, Hubei
P2105	*Pseudomonas syringae* pv. *actinidiae*	China, Zhejiang
DC3000	*Pseudomonas. syringae* pv. *tomato*	UK
NCPPB 48	*Pseudomonas syringae* pv. *morsprunorum*	Italy
NCPPB 3969	*Pseudomonas syringae* pv. *syringae*	Italy
NCPPB 537	*Pseudomonas syringae* pv.*lachryma*	USA
Bs#91	*Pseudomonas syringae* pv. *alisalensis*	USA
NCPPB 1883	*Pseudomonas*. syringae pv. *glycinea*	USA
NCPPB 2598	*Pseudomonas*. syringae pv. *theae*	Japan
NCPPB 3873	*Pseudomonas*. *avellanae*	Italy
NCPPB 2445	*Pseudomonas*. *corrugata*	UK
NCPPB 635	*Pseudomonas*. *viridiflava*	Switzerland

Sequence analysis and primer design: To obtain a highly specific Psa primer, highly conserved 590 bp *hrp*W gene fragment among different Psa-V strains and phylogenetic related bacteria (*P. syringae* pv. *syringae*, *P. viridiflava*, *P. syringae* pv. *tomato*, *P. syringae* pv. *theae*, and *P. avellanae*) were compared. The pair of primers YF32/YR32 (5'- CTGCAACAGGCGACGGCGAGGC -3', 5'- CATAGGCTTCTGGTTTTCTTCCTGATCC -3') were screened out, with expected amplicon of 248bp in length, were used in the real-time PCR.

Construction of the recombinant plasmid: The specific fragment was amplified using YF32/YR32 as the primers and Psa genomic DNA as a template. Assays were performed in a total volume of a 20μL reaction mixture containing 10μL of 2 × Taq PCR Master Mix, with 0.5μM of each forward and reverse primer, and 10ng DNA. Cycling conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 10s, 66°C for 30 s, 72°C for 30s, and a final extension at 72°C for 5 min. The amplification fragment were purified, then connected with pUC57 vector. The ligation products were transformed into DH5α competent cells, and recombinant were screened out and sequenced.

Establishment of the standard curve of real-time PCR: The recombinant plasmid of 10-fold serial dilutions as a positive control were used to build a standard curve of real-time PCR. Total reaction were 20μL, containing 10μL of the SYBR Premix Ex TaqTM, and 0.5μM of each forward and reverse primer. Cycling conditions were 95°C for 3 min, followed by 45 cycles of 94°C for 10s, 68°C for 15s, 72°C for 30s, and a final extension of at 72°C for 10min. The linear regression curves were drawn with the threshold cycles (Cq) of each reaction as a vertical coordinate, the logarithmic starting quantity of recombinant plasmid concentrations as a horizontal coordinate.

Specificity and sensitivity detection of real-time PCR: The sensitivity of primer YF32 / YR32 was tested using serially diluted Psa genomic DNA as a template for amplification. The diluted concentration was10ng/μL, 1ng/μL, 100pg/μL, 10pg/μL, 1pg/μL, 100fg/μL, 10fg/μL.

The specificity of the primers YF32/YR32 was tested on the following set of genomic DNA: infected twigs, healthy twigs, Psa pathogenic bacteria, and 10 Psa-related pseudomonads.

Specificity and sensitivity assay of primers were carried out by both real-time PCR and conventional PCR. Each sample was assayed for three times, and ddH_2O was included as negative control. The real-time PCR conditions were the same as the standard curve of real-time PCR building. The conventional PCR protocols were the same as recombinant plasmid construction.

Validation of real-time PCR method: To validate this method of real-time PCR, 5 twig samples with symptoms and 5 twig samples without symptoms collected from orchards from Mary to May/2016 in different China regions were analyzed, and healthy twig were sampled as negative controls. 30 twig per sample were randomly selected, and cut in half. Then samples were treated as follows: each sample was soaked in 400mL sterilized PBS buffer containing 0.2% Tween-20, shaken for 2 hours at 25°C and prepared as an eluent; 1ml eluent was pipetted into 100mL LB medium for 24h, incubated at 120 rpm with 25°C for 72 hours. DNA extraction was performed with 2mL sample solution. The real-time PCR test was repeated 3 times for each sample.

Results

Construction the recombinant plasmid: The purified recombinant plasmid was sent to Beijing Genomics Institute (BGI) for sequencing. The results confirmed that amplification fragment connected with the vector was 248bp in length (Fig. 1).

Establishment of the standard curve of real-time PCR: The standard curves using recombinant plasmid of 10-fold serial dilutions as templates clearly showed that amplification efficiency reached 115.6% ,and the correlation coefficient reached 0.997 (Fig. 2). Indicating a suitable condition of real-time PCR and an excellent linear relationship of the data.

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1 CAGCCTGATA GCCAGGCTCC TTTCCAAAAC AACGGCGGGC TCGGTACACC GTCGGCCGAT
61 AGCGGGGGCG GCGGTACACC GGATGCGACA GGTGGTGGCG GCGGTGGCGG TGATACTCCG
121 AGCGCAGCAG GTGGCGGCGG CGGTGGTGGC GATACTCCGA CCGCAACAGG TGGTGGCGGC
181 GGCGGTGGCG GTACACCCAC TGCAACAAGT GGCGGCGGTA GCAGCACACC CACTGCAACA
241 GGCGACGGCG AGGCTGGCGT AACACCGCAA ATCACTCCCC AGCTGGCCAA CCCTGGCCGT
301 ACCTCAGGCA CCGGCTCGGT TTCGGACACC GCAGGTTCTA CCGAGCAAGC CGGCAAGATC
361 AATGTGGTGA GAGACACCAT CAAGGTCGGC GCTGGCGAAG TCTTCGACGG CCACGGCGCA
401 ACCTTTACCG CCGACAAGTC GATGGGTAAC GGGGATCAGG AAGAAAACCA GAAGCCTATG
461 TTCGAGCTGG CTGAAGGCGC TACGTTGAAG AATGTGAACC TGGGTGAGAA CGAAGTGGAT
501 GGCATCCACG TGAAAGCCAA AAACGCTCAG GAAGTCACCA TTGACAACGT
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Fig. 1. Nucleotide sequences of PSA *hrpW* gene fragment. Underlined sequences are forward and reverse primers YF32/YR32; Shadow sequences are amplicon of 248bp in length generated by primer YF32/YR32.

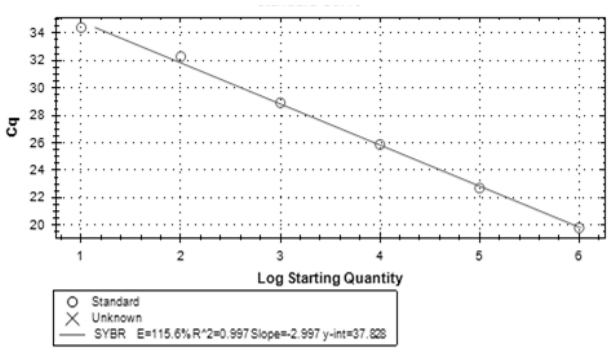

Fig. 2. The standard curve of positive recombinant plasmid by real-time PCR.

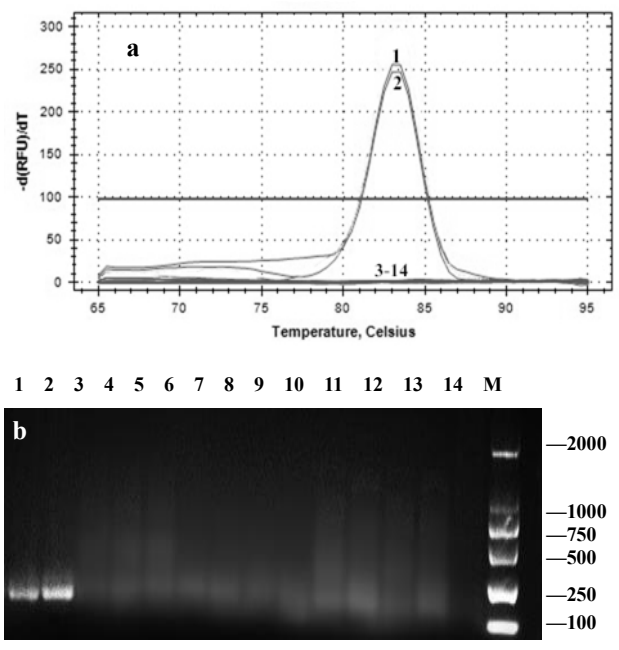

Fig. 3. The specificity detection of primers YF32/YR32 by real-time PCR amplification(a) and conventional PCR (b). 1, infected twig; 2, PSA pathogenic bacteria; 3, healthy twig; 4, *Pseudomonas. syringae* pv. *tomato*; 5, *Pseudomonas syringae* pv. *morsprunorum*; 6, *Pseudomonas syringae* pv. *syringae*; 7, *Pseudomonas syringae* pv.*lachryma*; 8, *Pseudomonas syringae* pv. *alisalensis*; 9, *Pseudomonas. syringae* pv. *glycinea*; 10, *Pseudomonas. syringae* pv. *theae*; 11, *Pseudomonas. avellanae*; 12, *Pseudomonas. Corrugate*; 13, *Pseudomonas. viridiflava*; 14, ddH_2O

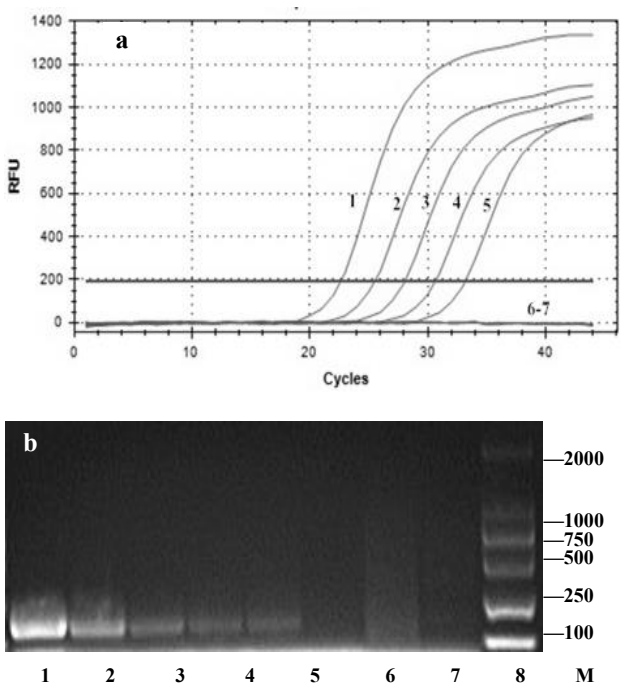

Fig. 4. The sensitivity detection of primers YF32/YR32 by real-time PCR amplification (a) and conventional PCR (b). (a) 1-6, 1ng/μL, 100pg/μL, 10pg/μL, 1pg/μL, 100fg/μL, 10fg/μL Psa genomic DNA; 7, ddH_2O. (b) 1-7, 10ng/μL, 1ng/μL, 100pg/μL, 10pg/μL, 1pg/μL, 100fg/μL, 10fg/μL Psa genomic DNA; 8, ddH_2O; M, DL2000.

Specificity and sensitivity detection of real-time PCR: The results of specificity of conventional PCR and real-time PCR assays showed the genomic DNA of the infected twigs and Psa pathogenic bacteria could produce a 248 bp specific band or single-peak of solubility curves (Fig. 3). Nevertheless, the 10 Psa-related pseudomonads, healthy twigs did not yield any fluorescent signals above the default background threshold and any bands by electrophoretic analysis after conventional PCR, which illustrated that the primers had excellent specificity and reaction condition was optimized. The sensitivity threshold of the real-time PCR was 100fg/μL (Fig. 4a). As a comparison, the sensitivity of conventional PCR, which was parallel tested, showed a threshold limit was 1pg/μL (Fig. 4b). This confirmed that the primers had excellent sensitivity.

Validation of real-time PCR method: The results of all of 5 twig samples with symptoms were positive in the actual effect validation experiments of the real-time PCR method, with maximum DNA concentration 2.18ng/μL. Meanwhile, the more obvious the symptoms appear, the higher the DNA concentration was. In the validation experiments of 5 symptomless samples, the results of 3 twig samples were positive, with minimum DNA concentration 8.45×10^{-5}ng/μL (Table 2).

Discussion

Plant pathogen *hrp* (hypersensitive reaction and pathogenicity gene) genes involved in and mediated an anaphylactic reaction, could determine the pathogenicity of

pathogen (Lindgren, 1997). The *hrp*W gene encoded an effector protein that was involved in the regulation of plant-pathogen interactions, and could be used in specific primers design of conventional or real-time PCR to detect plant pathogens (Zaccardelli *et al*., 2005; Ivey & Miller, 2010; Cai *et al*., 2011). There was a close phylogenetic relationship between Psa-V strains and other related pseudomonads, so it was difficult to identify at molecular level from each other (Butler *et al*., 2013; Petriccione *et al*., 2015). In this paper, the primers YF32/YR32 targeting a fragment of the *hrpW* gene could distinguish Psa-V from other similar pseudomonads. It was believed that *hrpW* gene used to design PCR primers was very effective to detect plant pathogenic bacteria, and might be possible to adjust the host range or the adaptability to environmental factors.

To specifically detect highly virulent strains of Psa-V, obviously it was quite necessary to have a rapid and reliable method. Recently, a sensitive nested PCR method had been developed, but it could not directly identify strains of Psa-V and PsD (Biondi *et al*., 2013), and additional experimental steps made it more limited. Nevertheless, real-time PCR was relatively flexible, cheap and simple with fewer intermediate steps than conventional PCR, and it could avoid irrelevant interference. Furthermore, whether each cycle produced non-specific amplification could be estimated by analyzing the melting curve. This meant that the residual diagnostic values could be read in the melting curve even if any cross-reactions occurred in the experimental results.

Compared to RG-PCR and duplex-PCR for Psa detection, this real-time PCR method with specific primers (YF32/YR32) had higher sensitivity, especially tested on symptomless twigs. In order to detect Psa in symptomless twigs that may had a low bacterial charge, DNA extraction step was necessary. Even if a low number of symptomless samples were tested, some results were positive after DNA extraction. However, the false negative results still could not be avoided because of special amplification problems. Further research was under way to assess the reliability of this method application to symptomless samples.

Despite phytosanitary departments of many countries which made the great efforts to contain the spread of this pathogen, the disease still continued to extend its boundaries. There are evidences to confirm that Psa could migrate to non-kiwifruit plants. There would may be a new way of spreading this disease both locally and internationally (Liu *et al*., 2016), so more measures should be taken to prevent and control effectively bacterial canker disease of kiwifruit.

Table 2. The DNA detection of Psa in kiwifuit samples by real-time PCR.

Detection NO.	Sample name	Origin of China	DNA content (ng/μL)
1-1	Twig without symptomatic appearance	Zhejiang Jiang-shan	$1.12(\pm2.45)\times10^{-4}$
1-2	Twig without symptomatic appearance	Hubei Yi-chang	N/A
1-3	Twig without symptomatic appearance	Sichuan Pu-jiang	N/A
1-4	Twig without symptomatic appearance	Jiangxi Yi-chun	$8.45(\pm3.09)\times10^{-5}$
1-5	Twig without symptomatic appearance	Shanxi Yang-lin	$5.72(\pm1.51)\times10^{-4}$
2-1	Twig with symptomatic appearance	Zhejiang Jiang-shan	0.92±0.02
2-2	Twig with symptomatic appearance	Hubei Yi-chang	1.96±0.24
2-3	Twig with symptomatic appearance	Sichuan Pu-jiang	2.18±0.12
2-4	Twig with symptomatic appearance	Jiangxi Yi-chun	0.25±0.01
2-5	Twig with symptomatic appearance	Shanxi Yang-lin	1.53±0.75

Conclusion

The specific primers YF32/YR32 targeting a fragment of the *hrpW* gene was screened out, with amplicon of 248bp in length. The standard curves using recombinant plasmid of 10-fold serial dilutions as templates indicated a suitable condition of real-time PCR and an excellent linear relationship of the data. The specificity and sensitivity assay showed this method could specifically discriminate between Psa and Psa-related pseudomonads and sensitivity threshold was 100fg/μL. In the actual effect validation experiments, the results of all of 5 twig samples with symptoms were positive, and 3 of 5 symptomless samples were positive with minimum DNA concentration 8.45×10^{-5}ng/μL.

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