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Field detection of canker-causing bacteria on kiwifruit trees: *Pseudomonas syringae* pv. *actinidiae* is the major causal agent

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ABSTRACT

Bacterial canker of kiwifruit, caused by *Pseudomonas syringae* pv. *actinidiae* (*Psa*), is thought to be responsible for severe damage to kiwifruit cultivation and production worldwide. However, several etiology issues impede disease management. As similar symptoms on kiwifruit could be affected by *P. syringae* pv. *syringae* (*Pss*), it is not known whether *Psa* is the only pathogen causing the current canker disease. In this study, we identified a *P. syringae* strain M209 isolated from canker tissue of kiwifruit based on biochemical characteristics, pathogenicity test and multi-locus sequence analysis. M209 caused mild canker lesions and leaf spots on kiwifruit, and it belongs to *P. syringae* genomospecies 2, showing distinct differences compared to the previously reported *Psa*-LV (less virulent) strains in pathogenicity and classification. We developed a multiplex PCR to evaluate the distribution of *Psa*, *Pss* and M209-representing populations. The PCR procedure was optimized and further validated for detecting pure isolates, as well as in artificially and naturally infected plant tissues. We used the PCR assay to detect 130 representative samples from 50 orchards in Shaanxi Province of China during 2013–2014, which covers the largest area of kiwifruit cultivation. The results indicated that *Psa* was the dominating pathogenic microbe responsible for canker symptoms on kiwifruit, while *Pss* and M209 were infrequent and less important pathogens in kiwifruit orchards in Shaanxi. In summary, a new canker-inducing strain on kiwifruit was identified, a reliable multiplex PCR for detecting three canker-inducing *P. syringae* populations was developed and applied, and the pathogen causing severe kiwifruit canker disease was determined in Shaanxi such that *Psa* should be the focus of disease management.

1. Introduction

Kiwifruit (*Actinidia spp.*), an economically important crop, is cultivated worldwide. *Pseudomonas syringae* is a phytopathogenic bacterium affecting a wide variety of plants worldwide, and it can be differentiated into 57 pathovars (Bull et al., 2010). Thus far, two *P. syringae* pathovars have been reported to affect the woody canes of kiwifruit, *P. syringae* pv. *actinidiae* (*Psa*) and *P. syringae* pv. *syringae* (*Pss*).

Psa is the causal agent of bacterial canker of kiwifruit, which caused relevant economic losses in Japan and Korea in the 1990s (Takikawa et al., 1989; Serizawa et al., 1994; Koh et al., 1996, 2002), and it is devastating kiwifruit orchards in all major areas of kiwifruit production worldwide since the "2008 outbreak" in Italy (Balestra et al., 2009; Ferrante and Scortichini, 2010; Everett et al., 2011; Scortichini et al., 2012; Zhao et al., 2013; McCann et al., 2013). Symptoms of the disease include woody-tissues canker (bleeding cankers on the trunk and leader, wilting and death of canes, leaders or affected vines) and leaf spots (brown angular lesions surrounded by a distinct yellowish halos).

Noticeably, similar symptoms caused by *Pss* have also been reported. A disease called "pseudomonas canker" from California caused by *Pss* resulted in canker symptoms on kiwifruit, including leaf wilt, cane blight, dried bark, brown tissue, and rusty red exudate (Opgenorth et al., 1983; Rees-George et al., 2010). A subsequent "bacterial canker" from Iran also caused by *Pss* showed symptoms of darkening and shriveling along the bark (Mazarei and Mostofipour, 1994). There are also reports that *Pss* could infect phylloplane organs (leaves, twigs and floral buds) of kiwifruit

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(Balestra and Varvaro, 1997; Rossetti and Balestra, 2008).

We also isolated a bacterial strain, M209, from kiwifruit that caused canker symptoms. The strain was identified as *P. syringae*, belonging to genomospecies 2, based on morphological, physiological, biochemical characteristics and molecular analyses. The strain's pathogenicity is weak, similar to *Psa*-LV, which was first reported in New Zealand and subsequently considered a new, unnamed pathovar (Chapman et al., 2012; McCann et al., 2013; Ferrante and Scortichini, 2010), but it could cause slight canker symptoms on kiwifruit canes. The molecular features of M209 distinguish it from any other *Pseudomonas* species and *P. syringae* pathovars that potentially affect kiwifruit.

These undefined complex pathogenic *P. syringae* pathovars that affect kiwifruit lead to confusion and difficulties in disease management. They had significant differentiation on susceptibility and spectrum against bactericides (e.g., antibiotics and copper compounds) *in vitro*. As canker disease severely damages kiwifruit, it is urgent to investigate their contributions to kiwifruit canker. PCR protocols for detecting plant pathogenic bacteria have been widely used (Palacio-Bielsa et al., 2009). To detect *Psa*, several PCR protocols have been developed (Koh and Nou, 2002; Rees-George et al., 2010; Gallelli et al., 2011; Balestra et al., 2013; Gallelli et al., 2014). A PCR method developed by Sorensen et al. (1998) is specific to *Pss*, which contains the *syrB* gene. And the *syrB* gene has been successfully detected in *Pss* strains isolated from kiwifruit by southern hybridization (Little et al., 1998). We designed a pair of specific primers (M209F/R) for detecting M209 based on *dnaA* sequence, which has been used to classify closely related strains of bacteria (Schneider et al., 2011). The primer pair was proven to be specific by a blast algorithm on NCBI. A unique fragment of 411 bp was produced with the primer pair M209F/R from M209, but not from all other strains, including *Pss* and *Psa*.

Therefore, in this study, we identified a new low-virulent *P. syringae* strain M209 inducing both canker lesions and leaf spots on kiwifruit as well as developed a multiplex PCR for detection of *Psa*, *Pss* and strain M209 to investigate the distribution of the three populations in the field and to evaluate the independent or dependent contributions of them to bacterial canker of kiwifruit. Our data indicated that *Pss* and M209 were infrequent and unimportant pathogens in kiwifruit orchards in Shaanxi province of China, and they required less attention when controlling the *Psa* epidemic.

2. Materials and methods

2.1. Bacterial strains and culture

Twelve characterized *Psa* strains were isolated from Shaanxi province of China (Zhao et al., 2013), and 78 uncharacterized bacterial isolates were from symptomatic tissues of both *Actinidia deliciosa* and *Actinidia chinensis* during 2010–2014 in Shaanxi. The 12 characterized *Psa* strains included strains M7 (GenBank Assembly ID: GCA_000344495.1) and M228 (GCA_000344475.2), whose genomes were sequenced. These isolates were from kiwifruit cultivating areas including Meixian County, Zhouzhi County, Chang'an District, Yangling District and Weinan City, which are all geographically along the north flank of Qinling Mountains in Shaanxi province. In addition, *Psa* strains 7285 (Italy, 2009), 7286 (Italy, 2009), ICMP 18800 (New Zealand, 2010), ICMP 18708 (New Zealand, 2010) and ICMP 9853 (Japan, 1984), and *Pss* strain B64, *P. syringae* pv. *tomato* strain DC3000, *P. syringae* pv. *lachrymans* strain M301315, *Pseudomonas savastanoi* pv. *savastanoi* strain NCPPB 639 and *P. savastanoi* pv. *glycinea* strain B076 were also included. All bacterial strains were grown on Luria Bertani (LB) agar and incubated at 25 °C for 2 days.

2.2. DNA extraction

For pure isolates, cultures were grown overnight in LB medium at 25 °C. The genomic DNA was extracted using TIANamp Bacteria DNA Kit (TIANGEN Biotech Co., Ltd., Beijing, China) and quantified using a Nanodrop 2000 spectrophotometer (Gene Company Limited, Hong Kong), which was then adjusted to a final concentration of 50 ng/μL with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at −20 °C. For artificially and naturally infected plant tissues, genomic DNA was extracted using Plant Genomic DNA Kit (TIANGEN Biotech Co., Ltd., Beijing, China) and quantified using Nanodrop 2000 spectrophotometer and then adjusted to a final concentration of 50 ng/μL with TE buffer.

2.3. M209 identification

The biochemical properties of M209 were determined as described by Lelliott et al. (1966) and Schaad et al. (2001) with *Psa* strain M7 as the control. Levan production, presence of oxidase, soft rot activity on potato disks, presence of arginine dehydrolase, hypersensitivity reaction in tobacco leaves (LOPAT tests), gelatine hydrolysis and glycerol utilization were included. M209 was detected by the *P. syringae* pathovar-specific primers listed in Table 1. PCR products were separated in 1.5% agarose gels and visualized using a gel imaging system with GeneSnap/GeneTool software (Syngene, UK). Pathogenicity assays were conducted on both the excised woody canes and leaves of three-year-potted *A. chinensis* cv. Hongyang described by Zhao et al. (2013).

The 16S rRNA, ITS (16S–23S ribosomal RNA intergenic spacer), *dnaA*, *acnB*, *gltA*, *gyrB*, *pgi* and *rpoD* sequences were amplified and sequenced using primers listed in Table 1. Sequences were compared with those available in GenBank. A series of individual trees from the eight partial gene sequences were generated by MEGA 6.06 (Tamura et al., 2013). A concatenated tree was created with individual alignments in an order of gyrB-rpoD-16S in MEGA 6.06 as well. The length and nucleotide positions reference the *Psa* strain 3739^{PT}: from 427 to 896 for the *gyrB* gene, from 409 to 920 for the *rpoD* gene, and from 98 to 1392 for the 16S rRNA gene. Concatenated sequences of three genes have a length of 2277 nucleotides. For individual and concatenated trees, the methods of maximum parsimony (MP) using the Subtree-Pruning-Regrafting algorithm, neighbor-joining (NJ) using the Kimura 2-parameter model, and maximum likelihood (ML) using the estimated best models were used and compared, and the branch robustness was estimated by 1000 bootstrap replicates.

2.4. Development of a multiplex PCR for the detection of Psa, Pss and M209

Three pairs of primers, PsaF/R, PssB1/B2, and M209F/R, were used in the multiplex PCR protocol (Table 1). The primers were synthesized by Invitrogen. The basic multiplex PCR reaction consisted of a 25 μL total volume mixture containing 2.5 μL of 10 × *Taq* buffer, 1.5 mM $MgCl_2$, 1 U of *Taq* DNA Polymerase (Fermentas), 0.2 mM dNTP mix, 50 ng of template DNA, 10 pmol of each primer, and sterile distilled water (SDW) to a final volume. Amplifications were performed in a S1000 thermal cycler (Bio-Rad Laboratories, Shanghai, China) under the following conditions: 1 cycle at 95 °C for 10 min; 36 cycles at 94 °C for 30 s, 60 °C for 35 s, and 72 °C for 90 s and a final extension cycle at 72 °C for 5 min. A water control was included in each PCR batch. Aliquots (5 μL) of the amplification products were electrophoresed on 1.5% TAE agarose gels at room temperature at 5 V/cm for 1 h and visualized using a gel imaging system with GeneSnap/GeneTool software (Syngene, UK) after staining with ethidium bromide. At least three replicate PCRs were