Molecular Characteristics of *Pseudomonas syringae* pv. *actinidiae* Strains Isolated in Korea and a Multiplex PCR Assay for Haplotype Differentiation

Hyun Seok Koh¹, Gyoung Hee Kim², Young Sun Lee¹, Young Jin Koh² and Jae Sung Jung^{1*}

¹Department of Biology; ²Department of Plant Medicine, Sunchon National University, Suncheon 540-950, Korea

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The molecular features of *Pseudomonas syringae* pv. *actinidiae* strains isolated in Korea were compared with strains isolated in Japan and Italy. Sequencing of eight *P. syringae* pv. *actinidiae* and three *P. syringae* pv. *theae* strains revealed a total of 44 single nucleotide polymorphisms across 4,818 bp of the concatenated alignment of nine genes. A multiplex PCR assay was developed for the detection of *P. syringae* pv. *actinidiae* and for the specific detection of recent haplotype strains other than strains isolated since the 1980s in Korea. The primer pair, designated as TacF and TacR, specifically amplified a 545-bp fragment with the genomic DNA of new haplotype of *P. syringae* pv. *actinidiae* strains. A multiplex PCR conducted with the TacF/TacR primer pair and the universal primer pair for all *P. syringae* pv. *actinidiae* strains can be simultaneously applied for the detection of *P. syringae* pv. *actinidiae* and for the differentiation of new haplotype strains.

Keywords : bacterial canker, kiwifruit, multiplex PCR, PCR detection, *Pseudomonas syringae* pv. *actinidiae*

Pseudomonas syringae pv. *actinidiae*, the causal agent of bacterial canker of kiwifruit, was first described in Japan in 1989 (Takikawa et al., 1989) and subsequently reported in Korea (Koh et al., 1994). The bacterial canker was highly destructive, devastating kiwifruit orchards and causing economic losses in both countries. Although this pathogen was isolated in Italy in 1992, it had a limited economic impact with a low incidence on kiwifruit plants until 2008 (Scotichini, 1994). However, severe epidemics have been reported in central Italy since 2008, first in *Actinidia chinensis* and then in *A. deliciosa* (Balestra et

al., 2009; Ferrante and Scortichini, 2009). The bacterial canker disease found in central Italy appeared to have been caused by a different population from that reported in 1992 (Ferrante and Scortichini, 2010). A severe form of *P. syringae* pv. *actinidiae* was then isolated in other European countries, including Portugal (Balestra et al., 2010), Spain (Balestra et al., 2011), France (Vanneste et al., 2011), and Turkey (Bastas and Karakaya, 2012). This pathogen was also found in both New Zealand and Chile in 2010 (Everett et al., 2011; Anonymous, 2011). Indeed, the worldwide kiwifruit industry is seriously threatened by this new pathogen.

P. syringae pv. *actinidiae* was suspected of being introduced into Korea from Japan through imported seedlings of kiwifruit, because bacterial canker was first observed in the late 1980s in Korea (Koh et al., 1994), several years after its first occurrence in Japan. However, the Korean strains did not have the coding sequences for phaseolotoxin, which is present in the Japanese strains; instead they have the coronatine biosynthesis genes (Han et al., 2003). The genes for coronatine biosynthesis were not found in *P. syringae* pv. *actinidiae* isolates from Japan, Italy, New Zealand, and Chile (Chapman et al., 2012). To date, the coronatine biosynthesis genes have only been detected from the Korean strains. Lee et al. (2005) distinguished Korean strains from Japan strains using the random amplified polymorphic DNA (RAPD) analyses and suggested that Korean and Japanese strains of *P. syringae* pv. *actinidiae* may have different phylogenetic origins.

The *P. syringae* pv. *actinidiae* population consists of different haplotypes that show different levels of virulence toward kiwifruit and their ability to rapidly spread. *P. syringae* pv. *actinidiae* strains recently isolated in Europe, New Zealand, and Chile belong to a single genetic lineage and are considered more virulent than the Italian strains isolated in 1992 (Chapman et al., 2012; Mazzaglia et al., 2012). In 2011 *P. syringae* pv. *actinidiae* strains showing the rep-PCR pattern similar to those from recent Italian

*Corresponding author.

Phone) +82-61-750-3616, FAX) +82-61-750-5469

E-mail) jjung@sunchon.ac.kr

outbreaks were isolated from a kiwifruit orchard in Korea (Koh et al., 2012). These strains do not possess gene clusters coding for the toxins coronatine or phaseolotoxin, which were retained by the previous Korean and Japanese strains, respectively (Han et al., 2003). The effector genes, *hopA1* and *hopH1*, present in the strains from recent outbreaks in Italy (Ferrante and Scortichini, 2010) were detected from these isolates. These strains were therefore considered new type of *P. syringae* pv. *actinidiae* closely related to those recently isolated from Italy (we will refer to these strains as “new haplotype strains” throughout this article).

In this work, two Korean and two Japanese strains (obtained in 1999 and 1989, respectively), two Italian strains (isolated in 2011), two “new haplotype” of Korean strains (isolated in 2011), and three *P. syringae* pv. *theae* strains were used for multilocus sequence analysis (MLSA). DNA fragments of six housekeeping genes, *cts*, *gyrB*, *acnB*, *gapA*, *pfk*, and *pgi*, and three effector genes, *hrpL*, *hrpS*, and *hrpZ*, were amplified and sequenced (Table 1). The nucleotide sequences of PCR primers and annealing temperature used for MLSA are listed in Table 2. Forward and reverse sequences of PCR products were obtained by direct sequencing method using the primers for each locus.

Sequencing of eight *P. syringae* pv. *actinidiae* and three *P. syringae* pv. *theae* strains revealed a total of 44 single nucleotide polymorphisms (SNPs) across 4,818 bp of the concatenated alignment of nine genes (Table 1). Two Italian and two “new haplotype” of Korean strains were identical in sequences at nine loci determined. Consequently, these strains may belong to the same genetic lineage.

The numbers of SNPs between the new haplotype strains (SYS1, SYS4) and Korean (CJW3, JYG6) and Japanese strains (Kw11, PaB1) previously isolated were 11 and 4, respectively. These data indicate that the new haplotype strains are more closely related to the Japanese strains than the Korean strains. This result also confirmed previous report that the strains from the recent European outbreaks belong to separate branch in phylogenetic tree with the Korean and the Japanese strains (Mazzaglia et al., 2011).

In addition, the number of SNPs between Korean (CJW3, JYG6) and Japanese strains (Kw11, PaB1) was 13, suggesting these two populations belong to separate genetic lineages. Of these 13 nucleotide variations, eleven sites were in the housekeeping genes, *cts*, *acnB*, and *gapA*, which are essential for survival and have a low probability of horizontal genetic exchange. Mazzaglia et al. (2012) estimated a divergence time of the Korean and Japanese strains from their common ancestor on the order of hundreds

of years.

Chapman et al. (2012) also elucidated the phylogenetic relationships between *P. syringae* pv. *actinidiae* isolates worldwide using MLSA of housekeeping, type III effector, and phytotoxin genes. They showed that at least four MLSA groups, Psa1-4, are present in *P. syringae* pv. *actinidiae* populations globally. The Japanese strains were genetically identical to Italian strains isolated in 1992 and designated as Psa1. The *P. syringae* pv. *actinidiae* population present in Korea was genetically very similar to Japanese strains but formed a distinct genetic lineage named Psa2. The Psa3 group represented isolates from Italy (2008–2009 outbreaks), New Zealand, and Chile. The Psa4 strains have been isolated from kiwifruit plants in New Zealand and Australia that showed no bacterial canker symptoms. MLSA results of this work suggested that the new haplotype of Korean strains isolated in 2011 also belong to the Psa3 MLSA group.

The number of SNPs between type strains of *P. syringae* pv. *actinidiae* (Kw11) and *P. syringae* pv. *theae* (LMG 5092) was 36, while that of Kw11 and two other *P. syringae* pv. *theae* strains (MAFF 302851 and 302852) was 31. A total of nine SNPs were identified between type strain LMG 5092 and two other *P. syringae* pv. *theae* strains, illustrating genetic variability at the intra-pathovar level (Table 1). *P. syringae* pv. *theae* and *P. syringae* pv. *actinidiae* both belong to genomospecies 8 *sensu* Gardan et al. (1999) and are very closely related (Scortichini et al., 2002; Ferrante and Scortichini, 2011). In fact, PCR primers designed to be complementary to a portion of the 16S–23S rRNA intertranscribed spacer regions are unable to distinguish *P. syringae* pv. *actinidiae* from *P. syringae* pv. *theae* (Rees-George et al., 2010). For the further characterization of strains belonging to the two pathovars, the 16S rRNA gene sequences were compared. PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Lee et al., 2012). The 16S rRNA gene sequence of a type strain of *P. syringae* pv. *theae* (LMG 5092) was identical to the new haplotype strains but showed three base differences with the pathotype strain of *P. syringae* pv. *actinidiae* (Kw11). However, the 16S rRNA gene sequences of three *P. syringae* pv. *theae* strains determined in this work disclosed some differences. The nucleotides “ATC” were found for pathovar type strain LMG 5092, while the nucleotides “GAT” were found for the other two strains at position 471–473 of *Escherichia coli* numbering (Brosius et al., 1978). Although the 16S rRNA gene sequence in a type strain of *P. syringae* pv. *theae* revealed 100% similarity with that of the new haplotype of *P. syringae*

Table 1. Single nucleotide polymorphisms (SNPs) that distinctively recognize *Pseudomonas syringae* pv. *actinidiae* and *P. syringae* pv. *theae* strains

No.	<i>P. syringae</i> pathovar	strain	origin	year	<i>cts</i>		<i>gapA</i>								<i>hrpS</i>	
					^a 252	432	27	171	237	255	513	514	624	3	120	
1	pv. <i>actinidiae</i>	SYS1	Korea	2011	C ^b	C	T	C	T	C	T	C	T	G	A	
2		SYS4		2011	.	.	.	.	.	.	.	.	.	.	.	
3		IHL1	Italy	2011	.	.	.	.	.	.	.	.	.	.	.	
4		IKB4		2011	.	.	.	.	.	.	.	.	.	.	.	
5		CJW3	Korea	1999	.	.	C	T	C	T	C	T	.	.	.	
6		JYG6		1999	.	.	C	T	C	T	C	T	.	.	.	
7		Kw11	Japan	1989	T	A	.	.	.	.	.	.	.	.	.	
8		PaB1		1989	T	A	.	.	.	.	.	.	.	.	.	
9	pv. <i>theae</i>	LMG5092	Japan	1970	.	.	.	.	.	.	.	C	C	A	G	
10		MAFF302851		1993	.	.	.	.	.	.	C	C	.	G	.	
11		MAFF302852		1993	.	.	.	.	.	.	C	C	.	G	.	

No.	<i>hrpL</i>					<i>hrpZ</i>												
	33	48	54	153	207	1244	1262	1265	1271	1274	1277	1293	1294	1379	1936	1988	2015	2051
1	A	A	G	C	T	G	C	C	A	C	C	G	G	C	G	A	T	C
2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
7	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
8	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
9	C	.	C	T	C	.	T	G	C	T	T	T	C	G	.	G	C	T
10	C	C	.	T	C	.	T	G	C	T	T	T	C	G	T	G	C	T
11	C	C	.	T	C	.	T	G	C	T	T	T	C	G	T	G	C	T

No.	<i>gyrB</i>						<i>acnB</i>			<i>pgi</i>				<i>pflk</i>	16S rRNA gene			
	144	174	198	213	234	475	358	369	372	291	339	348	393	429	24	471	472	473
1	A	C	T	T	T	T	C	T	G	T	C	A	T	A	G	A	T	C
2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5	.	.	.	.	.	.	T	G	A	.	.	.	.	.	A	G	A	T
6	.	.	.	.	.	.	T	G	A	.	.	.	.	.	A	G	A	T
7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	A	T
8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	A	T
9	G	T	G	C	C	C	T	G	A	C	T	G	C	G	A	.	.	.
10	G	T	G	C	C	C	.	G	.	C	T	.	.	.	A	G	A	T
11	G	T	G	C	C	C	.	G	.	C	T	.	.	.	A	G	A	T

^aPositions of SNPs are from the corresponding nucleotide sequences found in GenBank^bDots indicate identical base composition

Table 2. PCR primers used in this study

Primer name	Sequence (5'→3')	Annealing temp. (°C)	Target gene	Amplicon size	Reference
cts-F	AGTTGATCATCGAGGGCGCWGCC	56	cts	480bp	Sarker & Guttman, 2004
cts-R	TGATCGGTTTGATCTCGCACGG				
gyrB-F	MGGCGGYAAGTTTCGATGACAAYTC	63	gyrB	620bp	Sarker & Guttman, 2004
gyrB-R	TRATBKAGTCARACCTTCRCGSGC				
acn-F	ACATCCCGCTGCACGCYCTGGCC	60	acn	332bp	Sarker & Guttman, 2004
acn-R	GTGGTGTCTTGGAACCGACGGTG				
gapA-F	CGCCATYCGCAACCCG	62	gapA	700bp	Sarker & Guttman, 2004
gapA-R	CCCAYTCGTTGTCGTACCA				
pfl-F	ACCMTGAACCKKCGCTGGA	55	pfl	850bp	Sarker & Guttman, 2004
pfl-R	ATRCCGAAVCCGAHCTGGGT				
pgi-F	TTCAGCATGCGCAAGCG	55	pgi	448bp	Sarker & Guttman, 2004
pgi-R	TGCGCCAGGTACCAGG				
hrpL-F	TTTTGGCTGGCAYGGTTATCGCTATA	60	hrpL	555bp	Sawada et al., 1999
hrpL-R	TGTGGTTTTGCGTGCGAGTTGGTTCC				
hrpS-F	CTSCAGGCCAAGCTGCTGAGGGTGC	60	hrpS	240bp	Sawada et al., 1999
hrpS-R	TTGAGCTCRCGGATATTGCCGGGCC				
hrpZ-F	GCTGTGATCGATCAGCTGGT	60	hrpZ	993bp	Inoue & Takikawa, 2006
hrpZ-R	TCAGGCCACAGCCTGGTTAG				
27F	AGAGTTTGATCCTGGCTCAG	55	16S rRNA	1,535bp	Lane, 1991
1525R	AAAGGAGGTGATCCAGCC				
P.s.a-F	CAGAGGCGCTAACGAGGAAA	65	hopZ3	311bp	Balestra et al., 2013
P.s.a-R	CGAGCATAACATCAACAGGTCA				
Tac-F	CGGGCTAGACAGTACGCTGT	65	–	545bp	This study
Tac-R	CAGGCCCTTCTACCGCTAC				

pv. *actinidiae*, the other two pv. *theae* strains had identical sequences with Korean and Japanese strains of *P. syringae* pv. *actinidiae* previously isolated. As the type strain of *P. syringae* pv. *theae* LMG 5092 formed distinct clusters with other strains in ribotyping analysis in a previous study (Gardan et al., 1999), Bull et al. (2010) suggested that this strain is not suitable as a pathotype strain. Although the number of strains examined in this study is not enough to make a definite conclusion, the MLSA and the 16S rDNA sequence results also revealed that strain LMG 5092 could not represent *P. syringae* pv. *theae* as a pathotype strain.

The new haplotype strains isolated in a Korean orchard in 2011 have not spread to other orchards to date. Instead, *P. syringae* pv. *actinidiae* strains recently isolated from other orchards in Korea were identical to those first

reported in 1994 (Koh et al., 1994). It is interesting to note that although the new haplotype strain found in Italy had rapidly spread, that from Korea was limited to the orchard where it first occurred. To monitor the spread of the new haplotype strain in Korea, a multiplex PCR method which can detect *P. syringae* pv. *actinidiae* and differentiate the new haplotype strains, was developed in this study.

The first primer pair was previously designed to specifically detect *P. syringae* pv. *actinidiae* (Balestra et al., 2013). This primer pair was based on the sequence of the *hopZ3* gene which is conserved among all *P. syringae* pv. *actinidiae* strains determined but different from other *P. syringae* pathovars including pv. *theae*. The primer pair “*P. syringae* pv. *actinidiae* F/R” amplifies a 311-bp product from *P. syringae* pv. *actinidiae* strains. However, three additional

Fig. 1. Agarose gel electrophoresis patterns of PCR products amplified by the multiplex PCR assay. Lanes 1–8 were *Pseudomonas syringae* pv. *actinidiae* strains and lanes 9–11 were *P. syringae* pv. *theae* strains. Lane M, 100 bp marker (Bioneer); lane 1, SYS1; lane 2, SYS4; lane 3, IHL1; lane 4, IKB4; lane 5, CJW3; lane 6, JYG6; lane 7, Kw11; lane 8, PaB1; lane 9, LMG 5092; lane 10, MAFF 302851; lane 11, MAFF 302852.

primer pairs which were designed by Balestra et al. (2013) to distinguish European, Chinese, and Japanese/Korean *P. syringae* pv. *actinidiae* strains did not produce the expected size of amplicons with DNA of new haplotype strain isolated in Korea (data not shown). The second primer set was designed based on a RAPD marker specific to the new haplotype strain. Random primer OPA-01 (Operon Biotechnologies, USA) was used as a single primer. A specific RAPD product was excised from the agarose gel and the DNA was purified from the gel using an AccuPrep gel purification kit (Bioneer, Korea). The purified DNA was ligated into a pGEM-T Easy vector (Promega, USA) following the manufacturer's instructions. The nucleotide sequence of the inserted fragment was determined by SolGent Co. (Korea) and was deposited in GenBank under accession number KF772879. A RAPD marker was converted into a sequence characterized amplified region (SCAR) marker using sequence information. The SCAR primer pair designated as Tac F/R was expected to amplify a 545-bp DNA fragment with genomic DNA of the new haplotype strains. Nucleotide sequences of PCR primers used for multiplex PCR are listed in Table 2.

The multiplex PCR was performed with a DNA Thermal Cycler (Takara Shozo, Japan) under the following conditions: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 7 min. Each reaction mixture contained a 1x PCR buffer (10 mM Tris-HCl, 40 mM KCl, 1.5 mM MgCl₂, pH 9.0), 0.2 mM dNTPs, 10 pmol of each primers, 1.25 U of *Top* DNA polymerase (Bioneer, Korea), and 10 ng of

template DNA in a volume of 50 µl. Results employing two primer pairs in a multiplex PCR with eight *P. syringae* pv. *actinidiae* and three *P. syringae* pv. *theae* are shown in Fig. 1.

The expected fragments of 311 bp were amplified from the all eight *P. syringae* pv. *actinidiae* strains (lanes 1 to 8) but the three *P. syringae* pv. *theae* strains did not produce any amplicon (lanes 9 to 11). The four new haplotype strains, including two strains isolated in Korea (SYS1, SYS4) and two obtained from Italy (IHL1, IKB4), produced an expected 545-bp amplicon (lanes 1 to 4), while the two Korean (CJW3, JYG6) and two Japanese strains (Kw11, PaB1) did not amplify this DNA segment with primer pair Tac F/R (lanes 5 to 8). The multiplex PCR method with two primer pairs, *P. syringae* pv. *actinidiae* F/R and Tac F/R, can be used simultaneously to detect *P. syringae* pv. *actinidiae* and to distinguish the new haplotype strains from other haplotype strains which have been isolated in Japan and Korea since the 1980s. Therefore, the multiplex PCR assay designed in this study could be a valuable tool for monitoring the spread of the new haplotype strain in Korea.

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