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Identification and Characterization of Coronatine-Producing *Pseudomonas syringae* pv. *actinidiae*

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Abstract *Pseudomonas syringae* pv. *actinidiae* strains, which cause canker disease in kiwifruit, were collected from kiwifruit orchards in Korea and identified using biochemical and physiological tests. The nucleotide sequences of the 16S rDNA and 16S-23S internally transcribed spacer of the isolates were found to be identical to those of the pathotype strain, Kw11, of *P. syringae* pv. *actinidiae*. Remarkably, no coding sequence for phaseolotoxin biosynthesis or phaseolotoxin-resistant ornithine carbamoyltransferase was found by PCR amplification in any of the new Korean isolates of *Pseudomonas syringae* pv. *actinidiae*, although this was clearly identified in the control pathotype Kw11 reference strain. In contrast, three primer sets derived from the coronatine biosynthetic gene cluster and DNA from the Korean strains yielded amplified DNA fragments of the expected size. A sequence analysis of the PCR products revealed that *P. syringae* pv. *glycinea* and the Korean strains of pv. *actinidiae* contained coronafacate ligase genes (*cfl*) with identical sequences, whereas their *corR* genes exhibited 91% sequence similarity. The production of coronatine, instead of phaseolotoxin, by the Korean strains of *P. syringae* pv. *actinidiae* was confirmed by a bioassay using reference pathovars known to produce coronatine and phaseolotoxin. The genes for coronatine biosynthesis in the Korean strains of *P. syringae* pv. *actinidiae* were found to be present on plasmids.

Key words: Canker, coronatine, kiwifruit, phaseolotoxin, *Pseudomonas syringae* pv. *actinidiae*

The phytopathogenic bacterium *Pseudomonas syringae* is divided into 57 pathovars according to the pathogenicity towards plants, and exhibits diverse properties [14]. The pathovars of *P. syringae* are traditionally identified on the basis of phenotypic characters, such as their physiological and biochemical traits, pathogenicity towards plants [17], fatty acid [34] and protein profiles [42], phage sensitivity [12], and serological parameters [16]. Genetic fingerprinting methods, such as restriction fragment length polymorphisms (RFLP) [22], random amplified polymorphic DNA (RAPD) [11, 27], and the repetitive extragenic palindromic sequence (Rep)-PCR [26] of the chromosomal DNA also provide an effective way of classifying and identifying bacteria at the pathovar level. Furthermore, PCR-RFLP methods based on restriction endonuclease site differences or a sequence analysis of the ribosomal operons have been used to determine the phylogenetic relationships among microorganisms [8, 20, 28]. Another distinguishing characteristic of *P. syringae* pathovars is the type of phytotoxin they produce. Four distinct classes of phytotoxins, including lipodepsipeptides, such as syringomycin and syringopeptin, coronatine, phaseolotoxin, and tabtoxin, are produced by the plant pathogenic *P. syringae* [6].

Lipodepsipeptide toxins are produced by *P. syringae* pv. *syringae* [15] and pv. *atrofaciens* [43]. These compounds, which are toxic to a wide range of organisms including plants, are inserted into the lipid bilayer of membranes to form pores that are freely permeable to cations [18]. The phytotoxin coronatine is a chlorosis-inducing, non-host-specific phytotoxin, which mimics the action of methyl jasmonate in plants [44]. Several pathovars of *P. syringae*, including pv. *glycinea*, a pathogen of soybean, produce this phytotoxin. Phaseolotoxin is produced by *P. syringae* pv. *phaseolicola* and pv. *actinidiae* [31]. This toxin is active in both plants and microorganisms through the inhibition of enzyme ornithine carbamoyltransferase, which converts ornithine and carbamoylphosphate into citrulline in the urea cycle [6]. Tabtoxin is produced by *P. syringae* pv. *tabaci*, pv. *coronafaciens*, and pv. *garcae* in a dipeptide form, which contains tabtoxinine-β-lactam linked by a peptide bond to threonine. Cleavage of the peptide bond in tabtoxin by aminopeptidases of plant or bacterial origin releases the toxic moiety, tabtoxinine-β-lactam [23, 38].

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Table 1. Bacterial strains used in current study and results of the bioassay.

Strains	Geographic origin	Year isolated	Coronatine production[a]	Phaseolotoxin production[b]
P. syringae pv. *actinidiae*				
CJW7	Bukjeju, Korea	1999	+	-
HNL1	Haenam, Korea	1997	+	-
HNL4	Haenam, Korea	1999	+	-
JDL2	Jindo, Korea	1997	+	-
JYG3	Jindo, Korea	1999	+	-
WGD16	Wando, Korea	1999	+	-
Kw11 (pathotype strain)	Kanagawa, Japan	1984	-	+
P. syringae pv. *glycinea*				
ATCC 8727	ATCC		+	-
P. syringae pv. *phaseolicola*				
ATCC 19304	ATCC		-	+

[a,b]Coronatine and phaseolotoxin production were determined by biological assay, as described in the Materials and Methods.

Recently, the application of molecular genetics has provided new insight on the determinants of the pathogenicity and virulence of *P. syringae* pathovars [10]. PCR amplification procedures have already been applied to detect coronatine and phaseolotoxin biosynthetic gene clusters in the pathovars of *P. syringae* [2, 7, 35]. Similarly, PCR primers based on the sequence of genes encoding phaseolotoxin-resistant ornithine carbamoyltransferase (ROCT), *argK*, have been used for the specific detection of *P. syringae* pathovars that are resistant to phaseolotoxin [31].

P. syringae pv. *actinidiae*, which causes canker disease in kiwifruit (*Actinidia chinensis*), was first described in Japan [36]. Subsequently, the disease was reported in Italy [32] and Korea [19]. Typical symptoms of the disease are characterized by a red-rust exudation, blight of young canes and plants, and dark brown spots on the leaves. In the present study, *P. syringae* pv. *actinidiae* strains were collected from major kiwifruit cultivation areas in Korea. The phytotoxin produced by the Korean strains of *P. syringae* pv. *actinidiae* was identified using a PCR detection method that amplifies the toxin biosynthesis genes and confirmed by a bioassay for toxin production. The location of the phytotoxin gene cluster was determined by pulsed-field gel electrophoresis (PFGE) and Southern blot analysis.

MATERIALS AND METHODS

Isolation of the Pathogen and Pathogenicity Test

To isolate the pathogen, small pieces of diseased twigs and leaves were crushed in sterile mortars containing sterile distilled water. The suspensions were then diluted and streaked on a peptone-sucrose agar (PSA) [32], and incubated for 24–48 h at 27°C. The bacterial colonies were selected and streaked on a nutrient agar to confirm their identity and then stored in glycerol stocks. The taxonomic positions of the collected strains were confirmed by identifying their phenotypic features according to standard methods [17]. For the pathogenicity tests, kiwifruit plants were inoculated with a cell suspension of the test strains using spraying or puncturing methods [36]. The sources of the bacterial strains used in this study are listed in Table 1. For phytotoxin production, the strains were incubated for 96 h on a rotary shaker at 180 rpm and 18°C.

DNA Isolation

Bacterial cultures (100 µl) grown in a PS broth for 24 h at 30°C were centrifuged to remove the culture medium. The cell pellets were then washed twice with sterile water and resuspended in 100 µl of sterile distilled water containing 1% (wt/vol) Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The cell suspensions were boiled for 10 min and centrifuged briefly. The resulting supernatants were used for the PCR assay. The total genomic DNA was extracted, as described by Ausubel *et al.* [3], from the broth cultures (100 ml) of the *P. syringae* strains. The plasmid DNA for PFGE was isolated by the alkaline lysis method and purified by phenol/chloroform extraction and ethanol precipitation [3].

PCR Primers

The sequences of the 16S rDNA and intergenic spacer region located between the 16S and 23S rDNA were amplified for direct sequencing. Template DNAs for sequencing were prepared by amplification of the 16S rRNA genes using bacterial 16S rDNA universal primers [21]: 16S-F, 5'-TNANACATGCAAGTCGAICG-3' corresponding to position 49 to 68 of *Escherichia coli* 16S rRNA [9] and 16S-R, 5'-GGYTACCTTGTTACGACTT-3' corresponding to position 1510 to 1492. The 16S-23S ITS sequences were amplified using the PCR primers designed from the

sequences of the 16S and 23S rRNA genes adjacent to the spacer region. The sequence of primer D21 at the 3' end of 16S rRNA was 5'-AGCCGTAGGGGAACCTGCGG-3'. The primer D22 designed from the sequence at the 5' end of 23S rRNA was 5'-TGACTGCCAAGGCATCCACC-3' [28]. To detect the phaseolotoxin genes, primers previously designed from the sequence of the phaseolotoxin gene cluster were used: HB14F, 5'-CAACTCCGACACCAGC-GACCGAGC-3' and HB14R, 5'-CCGGTCTGCTCGAC-ATCGTGCCAC-3' [2]. The following primers were used to target the ROCT gene (*argK*), which is not inhibited by phaseolotoxin: OCTF, 5'-TATTACCCTGATGAGCTCGA-3' and OCTR, 5'-GATGATCGACCTTGTTGACCTCCCG-3' [31]. Three pairs of oligonucleotide primers derived from the coronatine biosynthetic gene cluster were used to detect the coronatine-producing *P. syringae* strains by PCR. Two primers, CFL-1 and -2, were previously designed from the *cfl* gene encoding coronafacate ligase, which functions in the ligation of CFA and CMA via amide bond formation: CFL-1, 5'-GGCGCTCCCTCGCACTT-3' and CFL-2, 5'-GGTATTGGCGGGGGTGC-3' [7]. Another primer set, CorR-1 and -2, was designed in the current study from the nucleotide sequence of the regulatory gene *corR* located in the coronatine gene cluster [39]: CorR-1, 5'-ATTCCGACATGCATCGTTTT-3' and CorR-2, 5'-GCTGAAGGTCAGGATCAAGC-3'. The CMA primer set was also designed in the current study from the nucleotide sequence of the coronamic acid biosynthetic gene cluster [40]: CMA-1, 5'-CTTTCGTCCCTAGACGCTTG-3' and CMA-2, 5'-CTGAGTTGCGCGACAAAATA-3'.

PCR Amplification

The PCR amplification of the target sequence was carried out in a total volume of 50 µl of the following reaction mixture: 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 µM (each) deoxyribonucleoside triphosphate, 25 pmol of each primer, 2 µl of the template, and 2.5 U of *Taq* DNA polymerase (Takara Shuzo, Otsu, Japan). The PCR was performed in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT, U.S.A.) using the following protocol: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C or 65°C for 30 s, and an extension at 72°C for 30 s, followed by an additional extension at 72°C for 5 min. The annealing temperatures were 58°C for the 16S rDNA and ITS amplification and 65°C for detecting the genes related to phytotoxin biosynthesis.

DNA Sequencing and Analysis

The DNA sequencing was performed using an ABI prism 377XL DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The sequences were compared with databases available at the National Center for Biotechnology Information (NCBI) using the BLAST program [1].

PFGE

PFGE was performed to determine the plasmid profiles of *P. syringae* pv. *actinidiae*. The plasmids were separated by electrophoresis in a 0.5× TBE (Tris-borate-EDTA) buffer at 14°C for 8 h using a CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with pulse times of 0.1 to 0.6 s and a field strength of 6 V/cm. A 0.65% pulse field grade agarose gel (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) was used for the electrophoresis. After the PFGE, the gel was stained with ethidium bromide, and the DNA bands visualized using a UV transilluminator.

Southern Blot Analysis

After the electrophoresis, the DNA was transferred to a positively charged nylon membrane (Roche Molecular Biochemicals, Mannheim, Germany) using the standard capillary method [3]. Southern hybridization was performed using a nonradioactive labeling and detection system (Roche Molecular Biochemicals). A 655-bp PCR product amplified by the CFL primers was cloned into vector pGEM-T using the conditions recommended by the manufacturer (Promega, Madison, WI, U.S.A). The recombinant plasmids were then transformed into competent cells of *E. coli* JM109. The inserted DNA fragments were labeled with DIG-11-dUTP according to the random primed labeling technique. A DIG-labeled probe was used for the hybridization into membrane-blotted DNA at 68°C according to the manufacturer's specifications in a standard hybridization buffer. The probes were then immunodetected with antidigoxigenin conjugated to alkaline phosphatase, and visualized with a chemiluminescence substrate supplied by the manufacturer.

Bioassay of Phaseolotoxin

The bioassay of phaseolotoxin was based on the methods of Staskawicz and Panopoulos [33] and Sawada *et al.* [31]. The test strains of *P. syringae* were grown on M9 minimal media [3] at 18°C, the optimum temperature of toxin production, while *E. coli* DH1 was cultured in M9 at 37°C. Cell-free culture filtrates of the *P. syringae* strains were prepared by filtering 1 ml samples through 0.22 µm membranes. The *E. coli* test plates were then prepared by mixing 10 ml of an M9 medium (1.5% agar) kept at 65°C with 200 µl of log-phase *E. coli* grown in a minimal glucose medium. The phaseolotoxin was assayed by adding 20 µl of the filtrate to wells cut into the agar plates using a cork borer (0.5 cm diameter). After 2 h incubation at 37°C, the inhibition zones formed around the wells were recorded. The reversal of inhibition by citrulline was assayed by adding 20 µl of citrulline solution (2 µg/µl) to an adjacent well containing the test filtrate.

Extraction and Bioassay of Coronatine

Pseudomonas strains were incubated in 20 ml aliquots of a modified Hoitink and Sinden medium optimized for

coronatine production [30] on a rotary shaker at 18°C for 76 h. The coronatine was extracted using the organic acid fractionation method as described elsewhere [5]. The bacterial cultures were centrifuged at 20,000 ×g for 15 min, and the supernatant was decanted. The supernatants were then adjusted to pH 9.0 and extracted twice with 20 ml volumes of ethyl acetate. The aqueous phase was then adjusted to pH 2.0 and extracted three times with 20 ml volumes of ethyl acetate to remove the organic acid fraction. The organic phase was filtered through anhydrous Na_2SO_4 and dried by evaporation. The coronatine was redissolved in sterile, distilled water and applied to the surface of a tomato leaf. The plants were kept in a growth chamber and observed for 5 days for chlorosis surrounding the injection site [13].

Nucleotide Sequence Accession Numbers

The DNA sequences obtained in the current study were submitted to the GenBank under accession numbers AF400251 (*cfl* locus) and AF400252 (*corR* locus).

RESULTS

Identification of the Pathogens

The results of the biochemical tests of the isolates were consistent with the characteristics of a pathotype strain of *P. syringae* pv. *actinidiae*. All the Korean strains tested were Gram-negative rods and did not produce any fluorescent pigment on King's B medium. The strains were also positive in the following tests: catalase, levan, and hydrolysis of casein and Tween 80, yet negative in the following tests: oxidase, tyrosinase, urease, reduction of nitrate, and hydrolysis of esculin and arbutin. All the isolates tested produced pathogenic reactions on kiwifruit. On the leaves, the initial symptoms appeared 5–7 days after inoculation around the wounds as dark and water-soaked lesions. Subsequently, the lesions became red-brown and surrounded by a large yellow halo. The infected canes produced exudate and longitudinal cracks that resembled natural infections. Re-isolation from the inoculated plants yielded a bacterium with the same biochemical characteristics as the inoculated strain.

Confirmation of *P. syringae* pv. *actinidiae* by 16S rDNA and ITS Sequences

The 16S rDNA and 16S-23S internally transcribed spacer (ITS) sequences were amplified from the genomic DNA of six *P. syringae* pv. *actinidiae* strains isolated from Korea, and the PCR products were subjected to direct sequencing. In all cases, a single DNA fragment was amplified and no significant size variation in the amplified ITS was detected between the strains on agarose gel electrophoresis. An almost complete 16S rDNA sequence (1,398 bp) was obtained for the test strains. The lengths of the 16S-23S ITS sequences for all the strains tested were 564 bp. A sequence analysis revealed that the *P. syringae* strains used in the current study displayed homogeneity in the 16S rDNA and 16S-23S ITS sequences. Comparisons of these sequences with the sequences of pathotype strain Kw11, previously submitted to the GenBank under accesion numbers AB001439 and D86357, showed no difference.

Detection of Genes Related to Phaseolotoxin Production

None of the *P. syringae* pv. *actinidiae* strains isolated in Korea and *P. syringae* pv. *glycinea* ATCC 8727 produced the expected DNA band upon amplification with the HB14 primer set. Only the pathotype strain, Kw11, amplified the expected 1,459-bp fragment (Fig. 1A). However, in all strains isolated in Korea, unexpected bands were amplified with the HB14 primer set. Therefore, the nucleotide sequences of the fragments were determined to examine whether the PCR products were in any way related to the phaseolotoxin gene cluster. However, the sequences did not show any homology with the previously reported ones (data not shown). PCR amplification with the OCT primer set flanking the open reading frame of the ROCT gene (*argK*) yielded the same result. As expected, the OCT primers only amplified a 1,098-bp fragment in the pathotype strain of *P. syringae* pv. *actinidiae* (Fig. 1B).

Detection of Coronatine Biosynthesis Genes

Phytotoxin coronatine consists of two moieties, CFA and CMA. The genes required for CFA and CMA biosynthesis are located in two separate transcriptional units designated

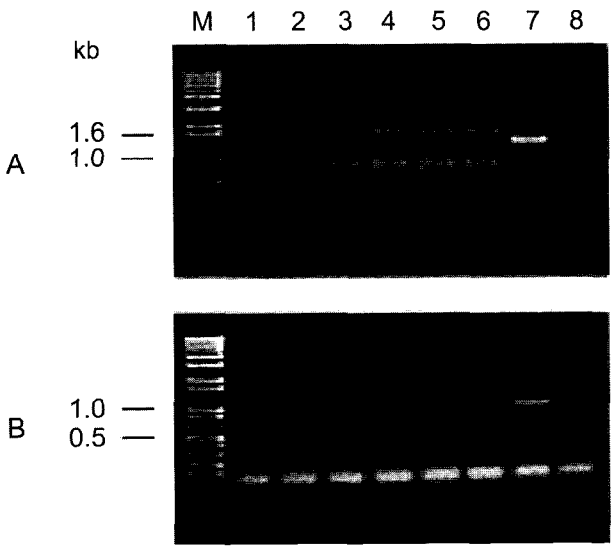

Fig. 1. PCR amplification products using HB14 primers (A) and OCT primers (B).
Lane M, 1 kb Plus DNA ladder (GIBCO BRL.); lanes 1 to 7, *P. syringae* pv. *actinidiae* strains (CJW7, HNL1, HNL4, JDL2, JYG3, WGD16, and Kw11); lane 8, *P. syringae* pv. *glycinea* ATCC 8727.

as the *cfl*/CFA operon and CMA operon, respectively [4, 7, 40]. The two operons are separated by a 3.4-kb regulatory region, which consists of three genes, *corP*, *corS*, and *corR* [39]. Three pairs of primers (CFL, CorR, and CMA) derived from the three regions of the gene cluster were used to examine whether the coronatine biosynthetic gene cluster existed in the *P. syringae* pv. *actinidiae* strains isolated from Korea. The CFL primer set was previously designed [7] to amplify 655-bp DNA fragments from the open reading frame of the *cfl* gene located at the 5' end of the gene cluster encoding CFA. The CorR primer set was derived from the *corR* gene, a positive activator of coronatine gene expression, and was expected to amplify 815-bp fragments, including the entire open reading frame of the gene [39]. The CMA primer set was designed from the sequences of the CMA operon. This primer set was expected to amplify 1,039-bp fragments including the 3' region of *cmaT* and 5' region of *cmaU* [40]. All six Korean strains of *P. syringae* pv. *actinidiae* and *P. syringae* pv. *glycinea* ATCC 8727 amplified PCR products with the predicted size. However, no PCR products were produced, when these primer sets were used with the pathotype strain (Kw11) *P. syringae* pv. *actinidiae* (Fig. 2). When the coronatine primer sets were applied to another 17 Korean strains collected from different cultivation areas, the same results were obtained (data not shown).

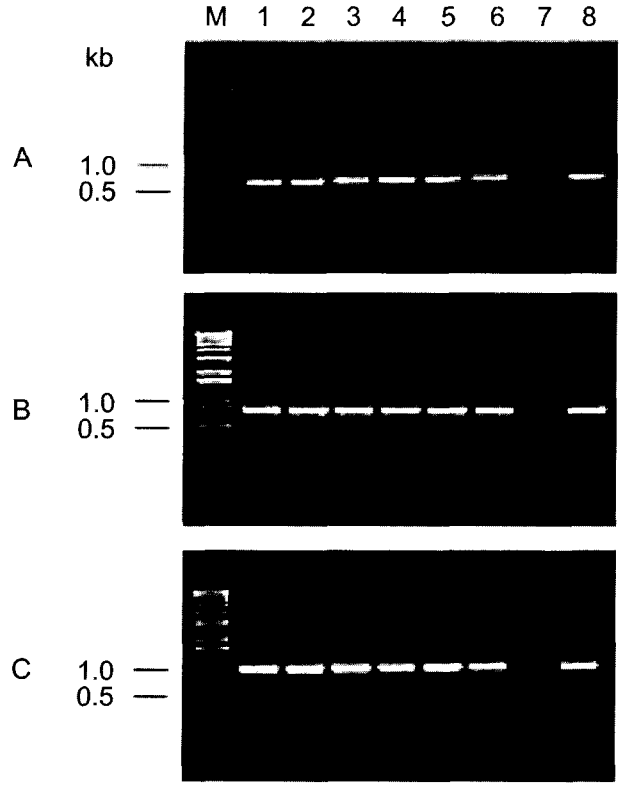

Fig. 2. PCR amplification products using CFL (A), CMA (B), and CorR (C) primers.
Lane M, 1 kb Plus DNA ladder (GIBCO BRL.); lanes 1 to 7, *P. syringae* pv. *actinidiae* strains (CJW7, HNL1, HNL4, JDL2, JYG3, WGD16, and Kw11); lane 8, *P. syringae* pv. *glycinea* ATCC 8727.

Sequence Analysis of PCR Products Amplified from Coronatine Gene Cluster

The *cfl* and *corR* genes of *P. syringae* pv. *actinidiae* were amplified using the CFL and CorR primer sets, and their sequences determined by direct sequencing. The amplified product of the Korean strain WGD16 with the CFL primers had a length of 655 bp. The sequence of the PCR product obtained from WGD16 was identical to the nucleotide sequence of the *cfl* gene from *P. syringae* pv. *glycinea* [7]. However, the PCR product of WGD16 with the CorR primers contained substitutions and deletions at various positions when compared to the *P. syringae* pv. *glycinea* sequences. The 597-bp nucleotide and 198 amino acid sequences corresponding to *P. syringae* pv. *glycinea* are shown in Fig. 3.

Bioassay for Phaseolotoxin and Coronatine

All six Korean strains of *P. syringae* pv. *actinidiae* and *P. syringae* pv. *glycinea*, a known coronatine producer,

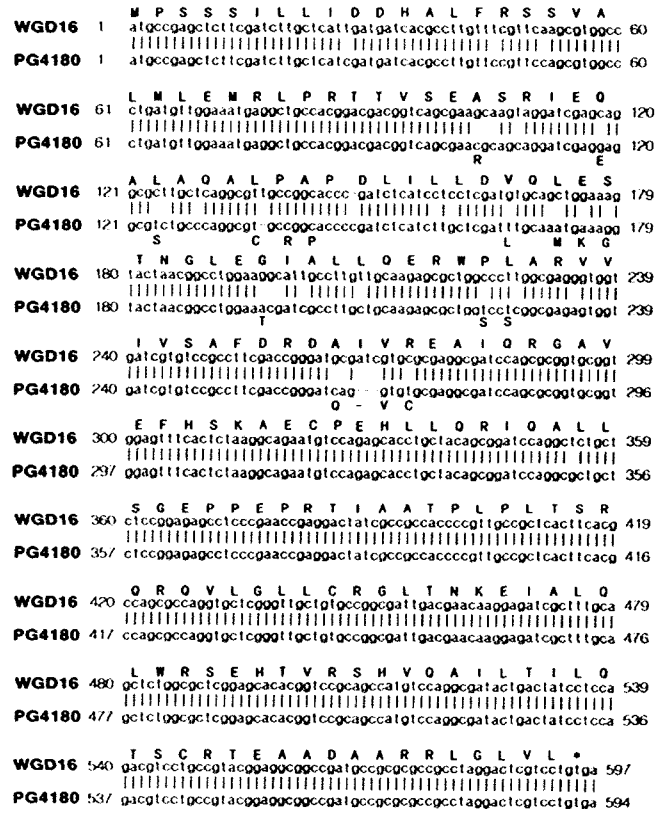

Fig. 3. Comparison of nucleotide sequences and amino acid sequences of the *corR* gene between *P. syringae* pv. *actinidiae* WGD16 and *P. syringae* pv. *glycinea* PG4180 (Bereswill *et al.* 1994). Deletions are indicated by dashes.

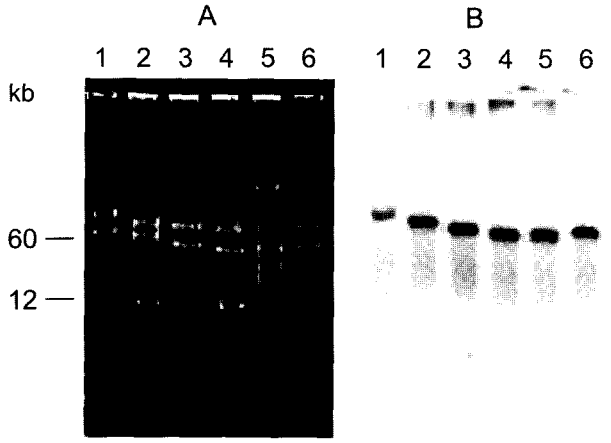

Fig. 4. Pulsed-field gel electrophoresis (A) and Southern blot analysis (B) of plasmid DNA isolated from Korean strains of *P. syringae* pv. *actinidiae* using DIG-labeled *cfl* gene as probe.
The electrophoresis of the plasmid DNA was carried out for 8 h at 6 V in a 0.65% agarose gel. Lanes 1 to 6, *P. syringae* pv. *actinidiae* strains (CJW7, HNL1, HNL4, JDL2, JYG3, WGD16).

caused chlorosis on the tomato leaves, when an ethyl acetate extract of the bacterial culture supernatant was applied. Conversely, the extract from the pathotype strain of *P. syringae* pv. *actinidiae* did not cause any chlorosis. Thus, the Korean strains tested were confirmed to produce coronatine. A bioassay for phaseolotoxin using the *E. coli* test plates only showed that the pathotype strains of *P. syringae* pv. *actinidiae* and *P. syringae* pv. *phaseolicola* inhibited the growth of *E. coli*. The inhibition zone in the *E. coli* caused by phaseolotoxin was reversed by the addition of citrulline. However, the culture filtrate of *P. syringae* pv. *glycinea* and Korean strains of *P. syringae* pv. *actinidiae* did not inhibit the growth of *E. coli* (Table 1).

Plasmid Analysis

The PFGE analysis of the plasmid DNA from the Korean strains of *P. syringae* pv. *actinidiae* yielded two to six plasmids with various sizes. A Southern blot analysis of the plasmid DNAs, using a DIG-labeled *cfl* gene from the coronatine gene cluster as a probe, suggested that the toxin genes of the *P. syringae* pv. *actinidiae* were plasmid-borne (Fig. 4).

DISCUSSION

To identify the pathovar of the strains isolated in Korea, their physiological and biochemical properties were investigated along with the nucleotide sequences of the 16S rDNA and 16S-23S ITS. A 16S rDNA sequence analysis is a powerful tool for elucidating the phylogenetic relationships among prokaryotic organisms, and has been used to determine inter- and intra-specific relationships. Meanwhile, the 16S-23S rDNA ITS sequence is believed to be helpful in studying the relationships between closely related strains, as it has more variable genetic markers for assessing the relationships. In the current study, the 16S rDNA and 16S-23S ITS sequences of the tested strains were found to be identical to those of the pathotype strain of *P. syringae* pv. *actinidiae*. Thus, it was concluded that the *P. syringae* strains isolated from kiwifruit plants in Korea belonged to *P. syringae* pv. *actinidiae*.

Many reports have suggested that phytotoxin production is one of the characteristic traits of phytopathogenic bacterium, even though some pathogens do not produce toxins. *P. syringae* pv. *actinidiae*, along with *P. syringae* pv. *phaseolicola*, which causes halo blight in various legumes, are known as phaseolotoxin producers [29, 31, 37]. They possess genes for phaseolotoxin biosynthesis, an ROCT gene that confers resistance to the toxin they produce, and phaseolotoxin sensitive OCT [31]. However, a PCR conducted with primer sets, designed from phaseolotoxin related genes, did not amplify the expected products in the Korean strains of *P. syringae* pv. *actinidiae*. Only the pathotype strain, Kw11, produced the expected PCR products (Fig. 2). It was also investigated whether the Korean strains of *P. syringae* pv. *actinidiae* included the coronatine biosynthetic gene cluster. When a PCR was conducted with the primer sets derived from the coronatine gene cluster, the Korean strains of *P. syringae* pv. *actinidiae* and *P. syringae* pv. *glycinea* yielded DNA fragments that coincided with the expected size, yet, no amplification was obtained in the pathotype strain of *P. syringae* pv. *actinidiae* (Fig. 1), indicating that the Korean strains of *P. syringae* pv. *actinidiae* included the coronatine biosynthetic gene cluster instead of phaseolotoxin genes. Since the pathotype strain of pv. *actinidiae* was isolated in Japan, the phaseolotoxin and coronatine primer sets were applied to another nine Japanese strains. All the Japanese strains, including the pathotype strain, yielded amplified DNA fragments only with the phaseolotoxin primers (data not shown).

Although coronatine genes were detected in the Korean strains, this does not indicate that the phytotoxin is actually synthesized in these strains. Therefore, to confirm the coronatine production in the Korean strains, a bioassay with tomato plants was performed. Most of the phytotoxins produced by *P. syringae* lack host specificity and cause symptoms on many plants that cannot be infected by the toxin-producing pathogen. As shown in Table 1, all six Korean strains and *P. syringae* pv. *glycinea* produced coronatine. The production of coronatine has already been demonstrated in five pathovars of *P. syringae*, including pv. *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum*, and *tomato*, which infect ryegrass, soybeans, crucifers, *Prunus* spp., and tomatoes, respectively [6]. The phylogenetic relatedness of Korean *P. syringae* pv. *actinidiae* to other

coronatine-producing pathovars was determined by comparing the nucleotide sequences of the PCR products. The nucleotide sequence of the PCR product amplified with the CFL primers was found to be identical to that of *P. syringae* pv. *glycinea*. Thus, when compared to the *cfl* sequence of the Korean strain of *P. syringae* pv. *actinidiae*, the sequences of *P. syringae* pv. *morsprunorum*, pv. *atropurpurea*, pv. *tomato*, and pv. *maculicola*, as in previous findings, contained 3, 8, 17, and 21 substitutions, respectively, at various positions [7], indicating that the Korean strains of *P. syringae* pv. *actinidiae* and *P. syringae* pv. *glycinea* were very closely related. However, the sequence of the *corR* gene of Korean *P. syringae* pv. *actinidiae* was different from that of *P. syringae* pv. *glycinea*, as shown in Fig. 3. CorR is known to be required for the transcription of the *cfl*/CFA operon [25]. Among 597 nucleotides, deletions and substitutions were found at 36 positions in the *corR* genes of *P. syringae* pv. *glycinea* and *P. syringae* pv. *actinidiae*. Thus, the level of sequence similarity between the two pathovars was 91.0%. However, the genetic relatedness between the Korean strains of *P. syringae* pv. *actinidiae* and other coronatine-producing pathovars was difficult to evaluate using this gene, because the nucleotide sequences of the *corR* genes from the other strains are not available at this time. The fact that *P. syringae* pv. *actinidiae* from Korea and *P. syringae* pv. *glycinea* were found to possess an identical *cfl* gene, whereas the *corR* genes showed significant levels of sequence variation, suggests that *cfl* might have been transferred more recently in evolutionary time from its origin to *P. syringae* pv. *glycinea* and *P. syringae* pv. *actinidiae*.

Although coronatine synthesis genes usually reside on a large plasmid in many coronatine-producing *P. syringae* pathovars [6, 24, 41], these genes are sometimes located on the chromosome [13]. The plasmids purified from the Korean *P. syringae* pv. *actinidiae* strains were subjected to PFGE. Each strain was found to carry from two to six indigenous plasmids of variable sizes. A Southern blot analysis, using labeled fragments from the *cfl* gene of *P. syringae* pv. *glycinea* as probes, indicated that the coronatine genes of the Korean strains of *P. syringae* pv. *actinidiae* were on plasmids (Fig. 4).

Coronatine and syringomycin are produced by multiple pathovars of *P. syringae*, whereas the production of phaseolotoxin is thought to be restricted to *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *actinidiae*. However, the results of the current work suggest that strains belonging to the same pathovar from different geographic origins can produce different phytotoxins. The knowledge of pathogens and the genes related to phytotoxins is important not only in the detection of pathogens, but also for understanding plant-pathogen interactions. Accordingly, further studies are necessary to clarify the evolutionary relatedness between the kinds of phytotoxins produced by different strains.

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