PCR-based Assay for the Specific Detection of *Pseudomonas syringae* pv. *tagetis* using an AFLP-derived Marker

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A PCR method has been developed for the pathovar-specific detection of *Pseudomonas syringae* pv. *tagetis*, which is the causal agent of bacterial leaf spots and apical chlorosis of several species within the Compositae family. One primer set, PSTF and PSTR, was designed using a genomic locus derived from an amplified fragment length polymorphism (AFLP) fragment produced a 554-bp amplicon from 4 isolates of *P. syringae* pv. *tagetis*. In DNA dot-blot analysis with the PCR product as probe, a positive signal was identified in only 4 isolates of *P. syringae* pv. *tagetis*. These results suggest that this PCR-based assay will be a useful method for the detection and identification of *P. syringae* pv. *tagetis*.

**Keywords**: AFLP, Detection, PCR, *Pseudomonas syringae* pv. *tagetis*

**Introduction**

*Pseudomonas syringae* pv. *tagetis* was first described in Denmark as a pathogen that affects marigold production (Hellmers, 1955). It is now known as a phytopathogenic bacterium that is the causal agent of bacterial leaf spots and apical chlorosis in several species within the family Compositae: African marigold (*Tagetes erecta* L.), sunflower (*Helianthus annuus* L.), common ragweed (*Ambrosia artemisiifolia* L.), Jerusalem artichoke (*Helianthus tuberosus* L.), dandelion (*Taraxacum officinale* Weber), compass plant (*Silphium perfoliatum* L) and another sunflower species (*Helianthus salicifolius* A. Diter) (Gulya et al., 1981; Hellmers, 1955; Rhodeshamel and Durbin, 1985; Rhodeshamel and Durbin, 1989a; Shane and Baumer, 1984; Syer and Durbin, 1982).

*P. syringae* pv. *tagetis* produces a toxin (tagetitoxin) in host leaves that is then translocated to emerging leaves, where it inhibits RNA polymerase III, thereby preventing chloroplast biogenesis and resulting in apical chlorosis (Mathews and Durbin, 1990; Steinberg et al., 1990). The pathogens are divided into three classes based on their capability to produce tagetitoxin: class 1 and 2 strains produce tagetitoxin in plants; class 3 strains do not produce the toxin (Rhodehamel and Durbin, 1989b).

There are many reports that specific detection methods for phyto toxin-producing *P. syringae* pathovars have been developed based on genes required for their production (Bereswill et al., 1994; Lydon and Patterson, 2001; Schaad et al., 1995; Sorensen et al., 1998). Recently, a PCR protocol to distinguish *P. syringae* pv. *tagetis* from other *P. syringae* pathovars and closely related species was developed based on genes required for tagetitoxin production (Kong et al., 2004). However, this approach is unable to distinguish the bacterium from other *Pseudomonas* isolates at the pathovar level. Furthermore, *Pseudomonas* species other than *P. syringae* pv. *tagetis* have been reported to induce apical chlorosis in Canada thistle and pea (Suzuki et al., 2003; Zhang et al., 2002). Therefore, a PCR-based assay that is able to unambiguously distinguish *P. syringae* pv. *tagetis* from *P. syringae* pv. *helianthi* and other apical chlorosis-inducing *Pseudomonas* species is needed.

In this study, we report the development of a pathovar-specific marker derived from the AFLP technique for detecting and distinguishing *P. syringae* pv. *tagetis* from other pathovars and species of *Pseudomonas* and *Xanthomonas*. The specificity of the PCR-based assay using pathovar-specific primers was validated by testing 47 isolates collected from various geographical
regions and host plants.

Material and Methods

Bacterial strains and DNA isolation. The bacterial strains that are listed in Table 1 were obtained from the Korean Agricultural Culture Collection (KACC) in Suwon, Korea, and the Belgian Co-ordinated Collections of Micro-organisms (BCCM) in Brussels, Belgium. The genomic DNA was isolated as described previously (Song et al., 2014).

AFLP PCR analysis. The AFLP assay was performed using a previously described method (Song et al., 2014), with minor modification. Genomic DNA (approximately 300 ng) was

Table 1. List of bacterial strains used in this study

| No. | Species | Source | Geographical origin | Hosts |
|-----|---------|--------|---------------------|-------|
| 1   | Pseudomonas syringae pv. tagetis | LMG 5090 | Zimbabwe | Tagetes erecta |
| 2   | Pseudomonas syringae pv. tagetis | LMG 5684 | Australia | Tagetes erecta |
| 3   | Pseudomonas syringae pv. tagetis | LMG 5685 | Australia | Tagetes erecta |
| 4   | Pseudomonas syringae pv. tagetis | LMG 5686 | USA | Tagetes sp. |
| 5   | Pseudomonas syringae pv. helianthi | LMG 2198 | Zambia | Helianthus annuus |
| 6   | Pseudomonas syringae pv. helianthi | LMG 5067 | Mexico | Helianthus annuus |
| 7   | Pseudomonas syringae pv. helianthi | LMG 5556 | Canada | Helianthus annuus |
| 8   | Pseudomonas syringae pv. helianthi | LMG 5557 | Germany | Helianthus annuus |
| 9   | Pseudomonas syringae pv. helianthi | LMG 5558 | New Zealand | Helianthus annuus |
| 10  | Pseudomonas syringae pv. syringae | LMG 1274 | UK | Zea mays |
| 11  | Pseudomonas syringae pv. syringae | LMG 5082 | UK | Zea mays |
| 12  | Pseudomonas syringae pv. syringae | LMG 5494 | Hungary | Prunus avium |
| 13  | Pseudomonas syringae pv. actinidiae | KACC10772 | | |
| 14  | Pseudomonas syringae pv. aptata | LMG 5059 | USA | Beta vulgaris |
| 15  | Pseudomonas syringae pv. atrofaciens | LMG 5095 | New Zealand | Triticum aestivum |
| 16  | Pseudomonas syringae pv. atrofaciens | LMG 5000 | UK | Thacher wheat |
| 17  | Pseudomonas syringae pv. japonica | LMG 5068 | Japan | Hordeum vulgare |
| 18  | Pseudomonas syringae pv. tomato | LMG 5093 | UK | Lycopersicon esculentum |
| 19  | Pseudomonas syringae pv. tabaci | LMG 5393 | Hungary | Nicotiana tabacum |
| 20  | Pseudomonas syringae pv. mori | LMG 5074 | Hungary | Morus alba |
| 21  | Pseudomonas syringae pv. antirrhini | LMG 5057 | UK | Antirhinum majus |
| 22  | Pseudomonas syringae pv. glycinia | LMG 5066 | New Zealand | |
| 23  | Pseudomonas syringae pv. delphinii | LMG 5381 | New Zealand | Delphinium sp. |
| 24  | Pseudomonas syringae pv. ericobryae | LMG 2184 | USA | Erichobrya japonica |
| 25  | Pseudomonas syringae pv. lachrymans | LMG 5070 | USA | Cucumis sativus |
| 26  | Pseudomonas syringae pv. morsprunorum | LMG 5075 | UK | Prunus domestica |
| 27  | Pseudomonas syringae pv. morsprunorum | LMG 2222 | UK | Prunus avium cv. Napoleon |
| 28  | Pseudomonas syringae pv. garcae | LMG 5064 | Brazil | Coffea arabica |
| 29  | Pseudomonas syringae pv. delphinii | LMG 2177 | UK | Delphinium elatum |
| 30  | Pseudomonas syringae pv. pisi | LMG 5383 | Canada | Pisum sativum |
| 31  | Pseudomonas syringae pv. pisi | LMG 5384 | Italy | Pisum sativum |
| 32  | Pseudomonas syringae pv. sesami | LMG 2289 | Yugoslavia | |
| 33  | Pseudomonas azotofornans | KACC10302 | UK | |
| 34  | Pseudomonas fuscovaginae | LMG 2158 | Japan | Oryza sativa |
| 35  | Pseudomonas coronafaciens | LMG 5060 | UK | Avena sativa |
| 36  | Pseudomonas citrusellolis | LMG 18378 | USA | soil collected under pine trees |
| 37  | Pseudomonas oryzaehabitans | LMG 7040 | Japan | rice paddy |
| 38  | Pseudomonas mucidaensis | LMG 2223 | USA | |
| 39  | Pseudomonas graminis | LMG 21661 | Germany | grasses |
| 40  | Pseudomonas jessenii | LMG 21605 | France | |
| 41  | Pseudomonas libanensis | LMG 21606 | Lebanon | |
| 42  | Pseudomonas lundensis | LMG 13517 | USA | |
| 43  | Pseudomonas taetrolens | LMG 2336 | USA | |
| 44  | Xanthomonas oryzae pv. oryzae | KACC10331 | Korea | |
| 45  | Xanthomonas campestris pv. citri | KACC10444 | Korea | |
| 46  | Xanthomonas campestris pv. glycines | KACC10445 | Zambia | |
| 47  | Xanthomonas campestris pv. vesicatoria | LMG 905 | Japan | |

*KACC, Korean Agricultural Culture Collection, Korea (http://www.genebank.go.kr/); LMG, The Belgian Co-ordinated Collections of Micro-organisms (BCCM), Belgium; ‘-’ unknown.
digested with EcoRI and MseI enzymes and was then ligated to the ends of the restricted DNA fragments with EcoRI adaptor and MseI adaptor (Table 2). A pre-selective PCR reaction was performed with the AccuPower PCR Premix (Bioneer, Daejeon, Korea) in a 25 μl reaction mixture containing 1 μl of DNA (50 ng/μl), 10 pmol of Eco0 (5'-GACTGCGTACCAATTC-3'), and 10 pmol of Ms0 (5'-GATGAGTCCTGAGTAA-3'). The pre-selective PCR and AFLP PCR amplification were conducted as described previously (Song et al., 2014). The amplified products were resolved in a 1.2% agarose gel with a 1-kb DNA ladder (TNT Research, Seoul, Korea) as a reference, stained with ethidium bromide, and visualized on a UV transilluminator.

**Primer design and PCR amplification.** The specific DNA fragment was eluted as described previously (Song et al., 2014). DNA was directly used in the ligation reaction with a pGEM-T Easy Vector (Promega, Madison, WI, USA) and was then transferred into competent DH5α (RBC Bioscience, Taipei, Taiwan) cells according to the supplier's instructions. The sequencing reaction was performed with an ABI Prism 3730 DNA Sequencer (Life Technologies, Carlsbad, CA, USA). After trimming the vector sequence, one pair of primers was designed based on the obtained sequence.

The specificity of the designed primers was evaluated against *P. syringae* pv. *tagetis* and other *Pseudomonas* and *Xanthomonas* species. The PCR reaction was performed with premixed polymerase (Taq PreMix; TNT Research, Seoul, Korea) in a 20 μl reaction mixture containing 1 μl of DNA (50 ng/μl), 10 pmol of PSTF (5'-AATGAGCTGAAATTCAACGG-3'), and 10 pmol of PSTR (5'-CGACCTGGATATAAGTTGCC-3'). The PCR amplification was performed with a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: initial denaturation (5 min at 96°C), 25 cycles (15 s at 96°C; 15 s at 62°C; and 30 s at 72°C), and a final extension (5 min at 72°C). Subsequently, 5 μl of each reaction mixture was resolved in a 1.2% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

**DNA dot-blot analysis.** A DNA dot-blot analysis was performed using a previously described method (Kang et al., 2007), with some modifications. A total volume of 5 μl of genomic

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**Table 2. Oligonucleotide adaptors and primers used for AFLP analysis**

| No. | Primer name  | Sequence (5’ - 3’) | No. | Primer name  | Sequence (5’ - 3’) |
|-----|--------------|--------------------|-----|--------------|--------------------|
| 1   | EcoR-Adaptor | Forward CTCTAGAAGCTGACC | 1   | Mse-Adaptor | Forward TACTCAGGACTCAT |
| 2   | EcoR + 0 | Reverse AATTGAGCTTACGCTTAC | 2   | Reverse GACGATGACTCTGAG |
| 3   | EcoR + 3 | Eco0 GACTGCGTACCAATTC | 3   | Mse + 0 | Ms0 GATGAGTCTGAGTAA |
| 4   | Eco1 | Eco1 GACTGCGTACCAATTCAG | 4   | Ms1 | Ms2 GATGAGTCTGAGTAAAC |
| 5   | Eco2 | Eco2 GACTGCGTACCAATTCAGC | 5   | Ms2 | Ms3 GATGAGTCTGAGTAAAC |
| 6   | Eco3 | Eco3 GACTGCGTACCAATTCAC | 6   | Ms3 | Ms4 GATGAGTCTGAGTAAC |
| 7   | Eco4 | Eco4 GACTGCGTACCAATTCACA | 7   | Ms4 | Ms5 GATGAGTCTGAGTAAAC |
| 8   | Eco5 | Eco5 GACTGCGTACCAATTCACC | 8   | Ms5 | Ms6 GATGAGTCTGAGTAAAC |
| 9   | Eco6 | Eco6 GACTGCGTACCAATTCAGC | 9   | Ms6 | Ms7 GATGAGTCTGAGTAAC |
| 10  | Eco7 | Eco7 GACTGCGTACCAATTCACT | 10  | Ms7 | Ms8 GATGAGTCTGAGTAAC |
| 11  | Eco8 | Eco8 GACTGCGTACCAATTCAG | 11  | Ms8 | Ms9 GATGAGTCTGAGTAAAC |
| 12  | Eco9 | Eco9 GACTGCGTACCAATTCAGA | 12  | Ms9 | Ms10 GATGAGTCTGAGTAAAC |
| 13  | Eco10 | Eco10 GACTGCGTACCAATTCAGT | 13  | Ms10 | GATGAGTCTGAGTAAAC |

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**Fig. 1.** Agarose gel electrophoresis of PCR amplicons amplified from *Pseudomonas syringae* pv. *tagetis* isolates using the pathovar-specific PSTF/PSTR primer set. Lane M: size marker (1-kb ladder); lanes 1–47: *Pseudomonas* and *Xanthomonas* isolates (numbers 1–47, respectively, in Table 1).
DNA (approximately 250 ng) was spotted onto an Amersham Hybond-N+ nylon membrane (GE Healthcare, Little Chalfont, UK), which was then air-dried and baked at 80°C for 2 h. The PCR product from *P. syringae* pv. *tagetis* LMG 5090 was labeled with \([a-32P]\) dCTP using a random primer method according to the manufacturer’s instructions (Ladderman Labeling Kit, Takara Bio, Otsu, Japan). The pre-hybridization and hybridization were conducted as described by Sambrook and Russell (2001). The membrane was exposed to an imaging screen (Fuji, Tokyo, Japan) for 3 h, and captured radiation was visualized using a Personal Molecular Imager system (Bio-Rad).

**Fig. 2.** DNA dot-blot analysis using PCR amplicon with PSTF and PSTR from *Pseudomonas syringae* pv. *tagetis* LMG 5090. Lanes 1–4: *P. syringae* pv. *tagetis*; lanes 5–47: corresponding to isolates numbered in Table 1.

**Discussion**

The plant pathogen *P. syringae* pv. *tagetis* causes apical chlorosis and bacterial leaf spots in various Asteraceae, including the weeds common ragweed and dandelion (Gulya et al., 1981; Hellmers, 1955; Rhodehamel and Durbin, 1985; Rhodehamel and Durbin, 1989a; Shane and Baumer, 1984; Styer and Durbin, 1982). Since the isolation of this pathogen from weeds displaying apical chlorosis, it has been evaluated as a biological agent to control Canada thistle in soybean and woollyleaf bursage in cotton (Gronwald et al., 2002; Sheikh et al., 2001). Apical chlorosis-inducing *Pseudomonas* species other than this pathogen have also been reported in Canada thistle and pea (Suzuki et al., 2003; Zhang et al., 2002). However, pathovar-specific primers, which could be used for identifying a particular *P. syringae* pv. *tagetis*, are still lacking. Therefore, we utilized the AFLP technique to identify a specific polymorphic amplicons for *P. syringae* pv. *tagetis*. Polymorphic band produced only from this pathogen was cloned and sequenced. The sequence was used...
to design pathovar-specific primers that precisely distinguished \( P. syringae \) pv. \( tagetis \) from other pathovars and species of \( Pseudomonas \) and \( Xanthomonas \) (Fig. 1).

Previously, Kong \textit{et al.} (2004) described a PCR method for the identification of \( P. syringae \) pv. \( tagetis \) based on genes required for tagetitoxin production, but was unable to differentiate between \( P. syringae \) pv. \( tagetis \) and \( P. syringae \) pv. \( helianthi \) or \( P. syringae \) pv. \( atrophaciens \). In contrast, the PCR technique described in this study was able to unambiguously differentiated 4 isolates of \( P. syringae \) pv. \( tagetis \) from among other \( Pseudomonas \) and \( Xanthomonas \) isolates, including both \( P. syringae \) pathovars (Fig. 1). Furthermore, a DNA dot-blot analysis using the PCR product as a probe showed a positive signal for all the \( P. syringae \) pv. \( tagetis \) (Fig. 2), confirming that the entire 554-bp amplicon was highly conserved in this pathogen. This fragment was analyzed by a BLASTN search and showed no significant matches with known nucleotide sequences. BLASTX results revealed that the sequences showed relatively low similarity (32%) to the hypothetical protein from \( Paenibacillus \) sp. WLY78. These results suggest that the specificity of the primers for \( P. syringae \) pv. \( tagetis \) described in the present study is due to the uniqueness of the DNA sequence within the amplified region.

In conclusion, the results presented herein indicate that this PCR-based assay could be a reliable and useful method for the specific detection of \( P. syringae \) pv. \( tagetis \) strains.

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