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Detection of *Xanthomonas arboricola* pv. *pruni* by PCR Using Primers Based on DNA Sequences Related to the *hrp* Genes

So Yeon Park[1], Young Sun Lee[1], Young Jin Koh[2], Jae-Sun Hur[3], and Jae Sung Jung[1]*

[1]Department of Biology, [2]Department of Plant medicine, and [3]Department of Environmental Education, Sunchon National University, Suncheon 540-742, Republic of Korea

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Efficient control of *Xanthomonas arboricola* pv. *pruni*, the causal agent of bacterial spot on stone fruit, requires a sensitive and reliable diagnostic tool. A PCR detection method that utilizes primers to target the *hrp* gene cluster region was developed in this study. The nucleotide sequence of the PCR product amplified with primers specific for the *hrp* region of the xanthomonads and genomic DNA of *X. arboricola* pv. *pruni* was determined, and the sequence was aligned with that of *X. campestris* pv. *campestris*, which was obtained from the GenBank database. On the basis of the sequence of the amplified *hrp* region, a PCR primer set of XapF/R specific to *X. arboricola* pv. *pruni* was designed. This primer set yielded a 243-bp product from the genomic DNA of *X. aboricola* pv. *pruni* strains, but no products from other 21 strains of *Xanthomonas* or from two epiphytic bacterial species. Southern blot hybridization with the probe derived from the PCR product with the primer set and *X. aboricola* pv. *pruni* DNA confirmed the PCR results. The Xap primer system was successfully applied to detect the pathogen from infected peach fruits. When it was applied in field samples, the primer set was proved as a reliable diagnostic tool for specific detection of *X. aboricola* pv. *pruni* from peach orchards.

***Keywords*:** bacterial spot, detection, PCR, *X. arboricola* pv. *pruni*

Xanthomonas arboricola pv. *pruni* (sin. *Xanthomonas campestris* pv. *pruni*) (Vauterin *et al*., 1995) is a Gram-negative bacterium, which is the causal agent of bacterial spot in several fruit plants belonging to the genus *Prunus*, including the peach, apricot, and plum. The pathogen produces necrotic angular lesions on leaves, cankers on twigs and sunken lesions on fruits and induces significant crop losses. Although new peach cultivars exhibiting high levels of resistance to the disease have been previously developed via both conventional breeding and somaclone selection, none of these variants are sufficiently resistant to the pathogens (Hammerschlag, 2000).

Since its first description on the Japanese plum in the United States in 1903, this disease has been observed all over the world (Smith, 1903; Battilani *et al*., 1999; Jami *et al.*, 2005). As the *X. arboricola* pv. *pruni* populations from different continents evidence a relatively low level of diversity, it has been suggested that the pathogen originated in the United States has subsequently been disseminated to other regions (Boudon *et al*., 2005).

The most practical method currently available for the control of this disease is spraying with copper compounds or tetracycline early in the growing season (Ritchie, 1995). For the efficient control of the disease, however, a reliable and sensitive diagnostic procedure is required for allowing for the detection of latent infections or low levels of epiphytic pathogenic populations. In addition, *X. arboricola* pv. *pruni* is included in the list of European quarantine pathogen agents (EPPO/CABI, 1997).

* For correspondence. E-mail: jjung@sunchon.ac.kr; Tel: +82-61-750-3616; Fax: +82-61-750-5469

The diagnosis of *X. arboricola* pv. *pruni* infection currently entails the isolation of the pathogen with a selective medium (Civerolo *et al*., 1982), followed by biochemical and biophysical tests and 16S rDNA sequence analysis. This identification scheme requires one to two weeks before final confirmation can be obtained. However, the polymerase chain reaction (PCR) allows for the rapid, sensitive, and specific detection of DNA sequences, and thus may prove a suitable method for the rapid detection of the plant pathogens.

The principle objective of this study was to develop specific primers for amplification of the *hrp* region of *X. arboricola* pv. *pruni*, and to evaluate the feasibility of their application in the detection of the pathogen in natural samples.

Materials and Methods

Bacterial strains, media, and DNA extraction

A list of bacterial strains used in this study is given in Table 1. *Xanthomonas* strains were cultured on YDC medium (20 g glucose, 20 g $CaCO_3$, 10 g yeast extract, 15 g agar per L) and other bacteria on nutrient agar at 28°C. All strains were grown in liquid peptone sucrose media (PS; 20 g peptone, 20 g sucrose per L) on a rotary shaker (150 rpm). Total DNA from each bacterial strain was extracted using the genomic DNA Extraction kit (Bioneer, Korea).

Primer design

Primers RST21 (5′-GCACGCTCCAGATCAGCATCGAGG-3′) and RST22 (5′-GGCATCTGCATGCGTGCTCTCCGA-3′) designed from the nucleotide sequence of the *hrp* region of *X. capestris* pv. *vesicatoria* (Leite *et al*., 1994) were used to amplify a 1,074-bp DNA fragment from *X. aboricolar* pv. *pruni* LMG852, a pathovar reference strain.

Table 1. List of the bacterial strains used in this study

No.	Species, pathovar, or subspecies	Strains[a]	Geographic origin
1	*Xanthomonas arboricola* pv. *pruni*	LMG 852	New Zealand
2	*Xanthomonas arboricola* pv. *pruni*	MAFF 310420	Japan
3	*Xanthomonas arboricola* pv. *celebensis*	KACC 12135	New Zealand
4	*Xanthomonas arboricola* pv. *poinsettiicola*	LMG 5403	New Zealand
5	*Xanthomonas camperstri* pv. *camperstri*	KACC 10913	UK
6	*Xanthomonas camperstri* pv. *glycines*	KACC 10491	Korea
7	*Xanthomonas oryzea* pv. *oryzea*	KACC 10208	Korea
8	*Xanthomonas citri* subsp. *citri*	KACC 11271	Japan
9	*Xanthomonas citri* subsp. *malvacearum*	LMG 761	Sudan
10	*Xanthomonas axonopodis* pv. *begoniae*	LMG 551	UK
11	*Xanthomonas axonopodis* pv. *alfalfea*	KACC 11119	India
12	*Xanthomonas axonopodis* pv. *dieffenbachiae*	LMG 695	Brazil
13	*Xanthomonas axonopodis* pv. *phaseoli*	LMG 7455	USA
14	*Xanthomonas axonopodis* pv. *phyllanthi*	LMG 844	Sudan
15	*Xanthomonas axonopodis* pv. *vasculorum*	LMG 901	Mauritius
16	*Xanthomonas axonopodis* pv. *vesicatoria*	LMG 905	Unknown
17	*Xanthomonas cassavae*	LMG 673	Malawi
18	*Xanthomonas cucurbitae*	LMG 8662	New Zealand
19	*Xanthomonas pisi*	LMG 847	Japan
20	*Xanthomonas theicola*	LMG 8684	Japan
21	*Xanthomonas translucens* pv. *cerealis*	LMG 679	USA
22	*Xanthomonas translucens* pv. *hordei*	LMG 882	Canada
23	*Xanthomonas translucens* pv. *phleipratensis*	LMG 843	USA
24	*Panthoea agglomerans*	KACC 10054	Japan
25	*Pseudomonas syringea* pv. *morsprunorum*	KACC 10397	Korea

[a] Abbreviations : LMG, Bacterial Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; MAFF, GenBank, National Institute of Agrobiological Sciences, Japan; KACC, Korea Agricultural Culture Collection, National Institute of Agricultural Biotechnology, Korea

Amplified fragments were cloned into a pGEM-T Easy vector (Promega, USA) following the manufacturer's instructions. Recombinant plasmid DNA containing the PCR-amplified fragment was verified by restriction enzyme digestion with *Eco*RI. The nucleotide sequence of the inserted DNA was determined by SolGent Co. (Korea) using the standard primers SP6 and T7. To search for homologies, the nucleotide sequences were compared with sequences in the GenBank databases by using BLAST. By the sequence alignment of the PCR product and the *hrp* region of *X. campestris* pv. *campestris*, a pair of PCR primers, XapF and XapR, was designed for specific detection of *X. aboricolar* pv. *pruni*.

PCR amplification

DNA was amplified in a total volume of 50 μl with a PCR Thermal Cycler (TaKaRa Shozo, Japan). The reaction mixture contained 5 μl of 10× buffer (100 mM Tris-HCl; pH 8.0, 500 mM KCl, 25 mM $MgCl_2$), 200 μM each deoxynucleoside triphosphate, 20 pmol of each forward and reverse primer, 2.0 U of *Taq* polymerase (Bioneer, Korea), and 50 ng of purified genomic DNA as template. The PCR was carried out as follows. After an initial denaturation step for 5 min at 94°C, amplifycation was performed by 30 cycles. Cycles consisted of a 30 sec denaturation at 94°C, 30 sec of primer annealing at 52°C for the RST primers and at 55°C for Xap primers, and a 30 sec of primer extension at 72°C, followed by final step at 72°C for 7 min. PCR products were analyzed by electrophoresis in 1.2% agarose gels and ethidium bromide staining.

Southern blot hybridization

Probe was generated from PCR product amplified with Xap primer pair and genomic DNA of *X. arboricola* pv. *pruni* LMG852. PCR product was excised from the agarose gel after electrophoresis and labeled with digoxigenin. Probe labeling, hybridization, and detection were performed with DIG High Prime DNA Labeling and Detection Starter kit II (Roche Diagnostics, Germany) as recommended by the manufacturer.

Sensitivity of detection

To estimate the sensitivity, serial dilutions of the genomic DNA of *X. arboricola* pv. *pruni* LMG852 were prepared and 10 μl aliquots from each dilution series, ranging 3 ng to 3 fg, were added to the PCR mixture containing primer pair XapF/XapR. After amplification stage, 20 μl aliquots of PCR product underwent electrophoresis on agarose gel as described earlier.

Isolation of DNA from fruit and soil samples

For the direct detection of *X. arboricola* pv. *pruni* from peach tissues, fruits with or without bacterial spot symptoms were collected from the field. Fruit tissue samples of 1.0-1.5 g were ground into a fine powder in liquid nitrogen and transferred to 1.5 ml tubes. DNA was extracted using the DNeasy Plant Mini kit (QIAGEN, USA) following manufacturer's instruction. For the isolation of bacteria from fruit lesions showing *X. arboricola* pv. *pruni* infection, homogenized tissue was diluted with sterile water and plated on YDC medium. DNA from soil

was extracted from 0.5 g of samples using UltraClean Soil DNA kit (Mo Bio Laboratories, USA) following manufacturer's instruction.

Bio-PCR assay of plant materials

For the PCR assay, soil and plant samples were collected in April from peach orchards. The samples were collected from trees of both orchards where bacterial spot had and had not occurred last year. Woody tissues (500-700 mg) were removed from the different parts of tree, including trunk, branch, second branch, twig, sucker, and pruned twig. Tissues were soaked in 5 ml of PS broth and cultured for 18 h in a shaking incubator at 28°C. The resulting 1 ml of culture suspension was transferred to a fresh tube and centrifuged at 8,000 rpm for 5 min and total DNA was extracted with genomic DNA extraction kit for PCR assay. The remaining 100 μl of suspension was diluted with sterile distilled water and plated on YDC to isolate the pathogen.

Nucleotide sequence accession number

The DNA sequence of PCR product amplified with RST21/RST22 primer set and strain LMG852 DNA has been deposited in the GenBank database under accession number FJ753284.

Results

Specificity of PCR primer

An expected DNA fragment of 1,074-bp was amplified from the genomic DNA of *X. arboricola* pv. *pruni* LMG852 using primers RST21 and RST22. This fragment, which corresponded to the *hrpC-hrpD* region of the *hrp* gene cluster of *X. campestris* pv. *vesicatoria*, was cloned and subsequently sequenced (data not shown). Via alignment of the nucleotide sequence of the amplified fragment of *X. arboricola* pv. *pruni* with that of *X. campestris* pv. *campestris* obtained from the GenBank database, a pair of PCR primers was designed: XapF (5′-GAAGCTCG ATGCCACATCGT-3′) and XapR (5′-GTGCAGATCCTCAG CATGTC-3′). The expected product size was 243-bp. In order to verify the specificity of the XapF/XapR primer set, the strains listed in Table 1 were subjected to PCR amplification. When tested with total genomic DNA of the 25 bacterial strains including several pathovars of *X. arboricola* and 19 *Xanthomonas* spp., the primer pair was observed to direct the amplification of the expected 243-bp target DNA fragment only from two strains of *X. arboricola* pv. *pruni*. Conversely, no PCR products were detected from the 23 bacterial strains tested. Southern blot analysis of the agarose gel using PCR product as a probe also revealed that the expected band was amplified only using the XapF/XapR primer set with *X. arboricola* pv. *pruni* DNA. The DNA from the other 23 tested bacteria failed to hybridize to the *hrp*-specific fragment (Fig. 1). Sequencing of the PCR product amplified with the primer set and *X. arboricola* pv. *pruni* DNA verified that the DNA fragment had originated from the *hrp* gene region. The primer set, XapF and XapR, also resulted in a PCR product of 243-bp for fourteen *X. arboricola* pv. *pruni* strains isolated from peach orchards in Korea (data not shown).

Sensitivity of amplification

The XapF/XapR primer set was evaluated for sensitivity on the basis of the detection limit of the amplification of the target fragment from serial dilutions of the genomic DNA of *X. arboricola* pv. *pruni* LMG 852. The smallest quantity of template from which the target fragment could be amplified was 3 pg of total genomic DNA per reaction (Fig. 2). This DNA concentration corresponds to approximately 300 bacteria cells.

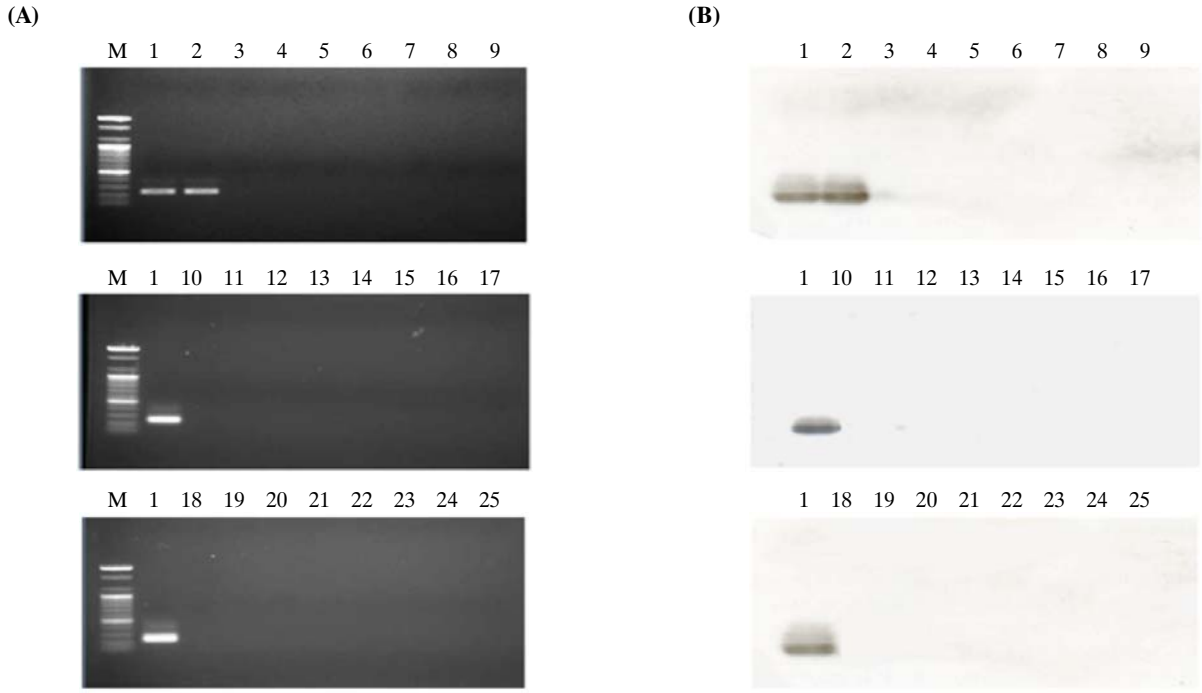

Fig. 1. Agarose gel (A) and corresponding Southern blot (B) of PCR product amplified using primers XapF and XapR. Lanes: M, size marker (100 bp DNA ladder, Bioneer); 1-25, correspond to organisms listed in Table 1.

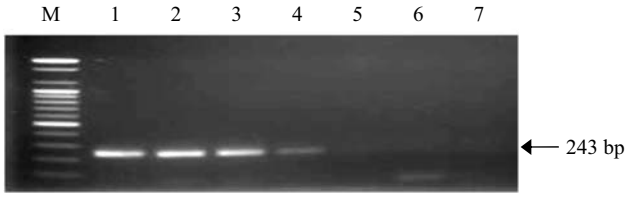

Fig. 2. Limits of PCR detection of *X. arboricola* pv. *pruni* starting with purified DNA and primer set XapF/XapR. Aliquots of 20 µl from each PCR reaction were separated by electrophoresis on 1.2% agarose gel. Lanes: M, size marker (100 bp DNA ladder; Bioneer); 1-7, 3 ng, 300 pg, 30 pg, 3 pg, 300 fg, 30 fg, 3 fg of genomic DNA as the template, respectively.

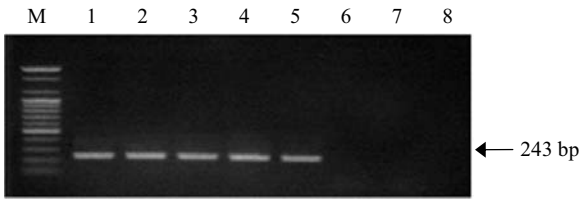

Fig. 3. Detection of *X. arboricola* pv. *pruni* using a PCR method in naturally infected peach tissue. The 243-bp PCR product amplified using primer pairs of XapF/XapR was visible on the ethidium bromide-stained agarose gel. Lanes: M, size marker (100-bp DNA ladder; Bioneer); 1, positive control (DNA of *X. arboricola* pv. *pruni*); 2-5, DNA from symptomatic fruit tissue of bacterial spot; 6-8, DNA from asymptomatic fruit tissue.

Detection of *X. arboricola* pv. *pruni* in peach fruits

Symptomatic and asymptomatic tissues of bacterial spot on peach fruits were used to detect *X. arboricola* pv. *pruni* by PCR using primers XapF and XapR. The unique 243-bp amplicon produced by the primer pair was obtained from peach fruits samples showing typical symptoms of bacterial spot. No amplification was achieved with the healthy peach tissues (Fig. 3). Viable *X. arboricola* pv. *pruni* strains were recovered from these lesions, and each strain also produced expected DNA fragment by PCR with the primer pair (data not shown).

PCR efficiency in plant material

When *X. arboricola* pv. *pruni* was enriched prior to PCR by incubating plant tissues for 18 h in PS medium, populations of the target bacterium reached detectable levels. The expected PCR product was amplified from the tree samples collected from orchard that had symptoms of bacterial spot last year (Fig. 4). Especially, *X. arboricola* pv. *pruni* was detected from newly growing part of the tree as sucker and twig, and from pruned branch which was discarded to the ground. The pathogen was isolated when the bacterial suspension was plated on YDC medium. However, no DNA fragment was amplified from tree samples of asymptomatic orchard.

Discussion

X. arboricola pv. *pruni* is a highly destructive pathogen, which is listed by the European Union as a quarantine organism (EPPO/CABI, 1997). The pathogen attacks only *Prunus* spp. and causes defoliation, resulting in weakened trees and severe damage to the fruit surface. In this paper, we described the development of a specific method for the detection of *X. arboricola* pv. *pruni* using a PCR primer set based on the *hrp* gene sequences.

The *hrp* genes are involved in the induction of the hypersensitivity response in resistant or non-host plants, and pathogenicity in susceptible hosts. The majority of the *hrp* genes is clustered in the chromosome and is highly conserved among phytopathogenic bacteria, where they encode for the type III secretion system. This system is crucial to the deliver of bacterial proteins relevant to pathogenecity and hypersensitivity response into plant cells (Alfano and Collmer, 1997). One of the advantage of using *hrp* sequences as a tool for the specific detection of phytopathogenic bacteria is their marked lack of homology with DNA from non-phytopathogenic bacteria. For the specific detection of *Xanthomonas* spp. by PCR, therefore, *hrp* gene sequences have been chosen in previous studies as a useful target for primer design (Leite *et al*., 1994; Park *et al*., 2004, 2006; Berg *et al*., 2005, 2006).

The *hrp* gene cluster of *X. campestris* pv. *vesicatoria* harbors six transcription units, which are designated *hrpA* to *hrpF* (Bonas *et al*., 1991). In a previous work, three pairs of oligonucleotide primers were designed from the *hrp* region of *X. campestris* pv. *vesicatoria* and assessed for their efficacy in the detection and identification of phytopathogenic xanthomonads (Leite *et al*., 1994). The results of which DNA fragments amplified with a particular primer set were of

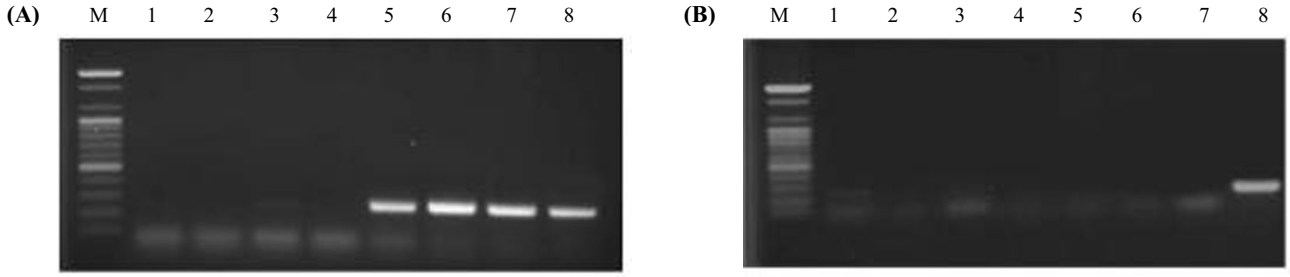

Fig. 4. PCR detection of *X. arboricola* pv. *pruni* from environment samples collected from the orchard infected with bacterial spot (A) and the healthy orchard (B). Lanes: M, size marker (100-bp DNA ladder; Bioneer); 1, soil; 2, trunk; 3, branch; 4, second branch; 5, twig; 6, sucker; 7, pruned branch; 8, positive control.

identical size from each of the xanthomonads indicated that the *hrp* DNA sequences are conserved among the tested strains. However, sequence polymorphisms were also noted, on the basis of observed variations in the restriction patterns of amplified DNA fragments.

A comparison of the cloned *X. campestris* pv. *pruni* sequence with the corresponding sequences from other *Xanthomonas* species obtained from the GenBank database showed that the closest match was with the *X. campestris* pv. *campestris* sequence (accession no. NC010688.1), with an 88% similarity, followed by pv. *vesicatoria* (accession no. NC007508.1), which exhibited a similarity of 76%. On the basis of the nucleotide sequences of pv. *campestris* and pv. *pruni*, a pair of PCR primers, XapF/XapR, was synthesized for the specific amplification of the *hrp* region in *X. arboricola* pv. *pruni*. Using these primers, a single 243-bp fragment was amplified with DNA extracted from symptomatic fruit tissue and from *X. arboricola* pv. *pruni* cells. Nonspecific bands are frequently amplified by PCR when DNA extracted from tissue is used as a template. However, the results shown in Fig. 3 clearly demonstrate the usefulness of the XapF/XapR primer set in the detection of *X. arboricola* pv. *pruni* in fruit tissues. Although the detection limit of this primer set is insufficient for application to field samples such as leaves and twigs, the enrichment of target bacteria via incubation in general liquid medium may increase the detection sensitivity, by increasing the number of the target cells. As shown in Fig. 4, it was possible to detect the pathogen on the infected orchard with the Bio-PCR method before the symptoms appeared. The infection of orchard which showed positive PCR results with XapF/XapR primers on April was confirmed by observing the typical symptoms of bacterial spot in the harvesting season.

In conclusion, PCR methods employing the specific primers designed herein will be proved useful in the detection and identification of *X. arboricola* pv. *pruni* in cases requiring sensitivity and reliability, most notably for disease diagnosis and plant quarantine programs.

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