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Multiplex Polymerase Chain Reaction for Simultaneous Detection of *Xanthomonas campestris* pv. *campestris* and *X. campestris* pv. *raphani*

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ABSTRACT

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Black rot of crucifers caused by *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson (Xcc) is an important disease worldwide. In recent years, the bacterial leaf spot of *Brassica* spp. caused by *X. campestris* pv. *raphani* (White) Dye (Xcr) has been reported and has caused serious economic losses in several countries. The primary inoculum sources of these two pathogens of crucifers are mainly from the infected or contaminated seeds or seedlings. For disease diagnosis and quarantine purpose, a specific and rapid detection technique for Xcc and Xcr is needed. In this study, the genomic suppression subtractive hybridization (SSH) method was used to obtain specific DNA fragments for Xcc or Xcr. Six and 9 clones were randomly selected from Xcc-Xcr and Xcr-Xcc SSH library, respectively. The insert DNA of these clones were sequenced and blasted to the NCBI and JCVI database. Two candidates of specific DNA fragments, Xcc 34-2 and XLS 2-14 were selected. According to the sequences of specific DNAs fragments in clones of Xcc 34-2 and XLS 2-14, primer pairs Xcc 2f/2r and Xcr 14f/14r were designed for the detection and identification of Xcc and Xcr. The DNA fragments of 200 bp and 277 bp were specifically amplified from strains of Xcc and Xcr by multiplex PCR with these two primer pairs. The detection sensitivity of primer pairs Xcc 2f/2r and Xcr 14f/14r was 10 pg and 100 pg DNA for Xcc and Xcr, respectively. When the black rot and bacterial spot naturally infected leaf tissues or seeds of crucifers were examined, Xcc and Xcr could be detected and identified specifically and simultaneously by multiplex PCR. The detection technique developed in this study could be used to differentiate the diseases caused by Xcc and Xcr, and it could also be used to detect Xcc and Xcr from cruciferous seeds.

Keywords: *Xanthomonas campestris* pv. *campestris*, *Xanthomonas campestris* pv. *raphani*, multiplex PCR

INTRODUCTION

Black rot of crucifers caused by *Xanthomonas campestris* pv. *campestris* (Xcc) (Pammel) Dowson, is a major disease worldwide. It often causes severe economic losses [35]. The typical symptoms of black rot are vein blackening, V-shaped chlorotic and necrotic lesions extending from leaf margins along vines on the leaves. The bacterial leaf spot of brassica caused by *X. campestris* pv. *raphani* (Xcr) (White) Dye (synonym *X. c.* pv. *armoraciae* (McCulloch) Dye) [2], has been reported in many countries including United States [37], Japan [30], Brazil [7], China , Turkey and India [7]. The disease has recently occurred in Taiwan [36]. *Xanthomonas campestris* pv. *raphani* and Xcc are similar in cultural, biochemical and physiological characteristics [2, 19, 33]. However, they can be distinguished by the pathogenicity assay on tomato (*Solanum esculentum*) and pepper (*Capsicum annuum*) [13].

Xanthomonas campestris pv. *campestris* and Xcr are important seedborne pathogens of crucifers. The primary source of Xcc and Xcr is mainly from the infected or contaminated seeds. The initial inoculum carried by infected seeds is the critical factor for determining the severity of black rot and bacterial spot diseases of crucifers [22, 25, 27]. To prevent the occurrence of these two diseases, zero tolerance of Xcc and Xcr is often required for seedling production [27]. An accurate, sensitive and rapid detection technique for Xcc and Xcr is needed to prevent the introduction of the pathogens into the fields. Conventional methods for identification and detection of pathogens in seeds or plants mainly rely on the grow-out test [26], selective media [8, 10, 15, 28] and serological techniques [3, 14]. However, three or more days are often needed to detect these pathogens on selective media [20, 26]. The serological methods such as Enzyme-linked immunosorbent assay for detection of seed-borne bacteria are also relatively time-consuming and insensitive compared to the polymerase chain reaction (PCR) [9, 20]. PCR has been widely used to identify and detect phytopathogenic bacteria [1]. Although PCR detection techniques have been developed for detection of *X. campestris*, the primer pairs used were often designed according to the sequences of *hrp* (hypersensitive response and pathogeniciy) F [5, 20]. Since the sequence of *hrp*F is highly conserved in Xcc and Xcr, these primers can not differentiate Xcc from other pathovars of *X. campestris* [5].

Suppression subtractive hybridization (SSH) is a powerful tool to obtain the differential DNA fragments from two closely related bacterial strains [6, 16, 29, 31]. In SSH the differential DNA fragments between two closely related bacteria are selectively amplified by the primers with different adaptor sequences. The similar DNA fragments can be simultaneously suppressed by hybridization. In this study, the SSH was used to obtain the specific DNA fragments of Xcc or Xcr. Based on the sequences of these specific DNA fragments, primer pairs Xcc 2f/2r and Xcr 14f/14r were designed. These two primer pairs can specifically detect and identify Xcc and Xcr simultaneously by multiplex PCR from naturally infected leaf tissues and seeds.

MATERIALS AND METHODS

Bacterial strains and DNA extraction

Strains of *X. campestris* pv. *campestris*, Xcr and the other bacteria used in this study are listed in Table 1. All strains of Xcc were isolated from various cruciferous crops in Taiwan, and the strains of Xcr used in this study were originally provided by A. M. Alvarez, Department of Plant Pathology, University of Hawaii, Honolulu, U. S. A. All cultures were stored at -70℃ in Luria-Bertani (LB) broth containing 15% glycerol. Before use, all strains of xanthomonads and *Enterobacter cloacae* were subcultured on potato dextrose agar (PDA) medium, fluorescent pseudomonads and *Acidovorax avenae* subsp. *citrulli* were subcultured onto King's medium B [17], *Ralstonia solanacearum* was subcultured onto TTC medium [11], *Escherichia coli* were subcultured onto LB agar medium [24], and other bacterial strains were subcultured onto nutrient agar medium. All strains were grown at 30℃ except *E. coli* which was grown at 37℃. For DNA extraction, bacterial cultures were grown at 30℃ for 24 hr, and cells from 5 ml of LB broth were used. Extraction of total DNA was performed as described by Lazo *et al.* [18].

Suppression subtractive hybridization (SSH)

Suppression subtractive hybridization was performed by using the PCR Select Bacterial Genome Subtraction Kit (Clontech Inc., CA. USA). To obtain specific DNA fragments from Xcc, Xcc strain Xcc 34 was used as the

tester and Xcr strain XLS 2 as the driver; whereas to obtain specific DNA fragments from Xcr, Xcr strain XLS 2 was used as the tester and Xcc strain Xcc 34 as the driver. Genomic DNA (2 μg) from the tester (Xcc 34 or XLS 2) was briefly digested with *RsaI* and separated into two portions, each of which was subjected to a ligation reaction to attach to a different set of PCR adaptors. The two portions were then separately hybridized to excess *Rsa*I-digested driver DNA (XLS 2 or Xcc 34) at 63℃ for 1.5 h. All sequences which are common in the two strains were hybridized, and leaving enriched tester specific single-stranded DNA. The two preparations were then mixed together and hybridized at 63℃ for 20 h. Only sequences specific for the tester strain which had different adaptors on each strand were amplified in the subsequent PCRs as described in the Clontech subtraction protocol. The mixture of PCR products was purified by Gel Elution Kit (GeneMark Technology, Taiwan), ligated to the T-A cloning vector pGEM-T (Promega, WI, USA), and transformed into *E. coli* DH 10B.

Sequence analysis and primer designed

The DNA fragments from Xcc and Xcr acquired from SSH were randomly selected for sequencing. DNA sequencing was performed by Mission Biotech Co., Ltd. (R.O.C.). Sequence of each SSH clones was blasted by National Center for Biotechnology Information (NCBI) BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and Comprehensive Microbial Resource (CMR) BLAST program (http://tigrblast.tigr.org/ufmg/) of J. Craig Venter Institute. Two clones Xcc 34-2 and XLS 2-14 were selected to design specific primer pairs for Xcc and Xcr by the San Diego Supercomputer Center (SDSC) biological workbench Primer 3 program.

PCR test of the specific primer pair for Xcc and Xcr

The primer pairs designed for detection of Xcc and Xcr were tested by PCR. All amplifications had a final volume of 20 μl containing 0.25 mM dNTP, 1X GenTaq reaction buffer, 0.25 μM of each primer pair, 100 ng of total DNA and 0.8U GenTaq polymerase (GeneMark Technology, Taiwan). Reactions were run in a thermal cycler (PTC-200 MJ Research cycler, Bio-rad, USA) for 35 cycles, each consisting of 30 sec at 95℃, 30 sec at 60℃ and 30 sec at 72℃ with initial denaturation of 5 min at 95℃ and final extension 10 min for 72℃. A 10 μl of each amplified PCR products was electrophoresed on a 1.5% agarose gel. The gel was further stained with ethidium bromide, and visualized under a UV transilluminator.

Specificity and sensitivity of primer pairs

Specificity test

To determine the specificity of primer pairs Xcc 2f/2r and Xcr 14f/14r, total DNA from strains of Xcc, Xcr and other plant pathogenic bacteria (Table 1) were tested by PCR with single primer pair. The PCR tests were performed as described above.

Sensitivity test

To determine the sensitivity of primer pairs Xcc 2f/2r and Xcr 14f/14r for detection of Xcc and Xcr, serial dilution of the total DNA from Xcc strain Xcc 74 and Xcr strain XLS 10 were prepared ranging from 100 ng to 1 fg. The conditions for PCR test were done as described above.

Multiplex PCR for detection of Xcc and Xcr

Fifteen strains of Xcc and seven strains of Xcr were tested by multiplex PCR with primer pairs Xcc 2f/2r and Xcr 14f/14r. The multiplex PCR reaction mixture contains 0.25 mM dNTP, 1X PCR GenTaq reaction buffer, 0.25 μM of each primer (Xcc 2f/2r and Xcr 14f/14r), 100 ng of each total DNA of Xcc Xcr and 0.8U GenTaq polymerase (GeneMark Technology, Taiwan). The amplification program of multiplex PCR assays was done as PCR test described above.

Simultaneous detection of Xcc and Xcr from naturally infected plant tissues and seed

To detect the Xcc and Xcr in natural infected tissues by PCR. The black rot symptomatic cabbage leaves and seeds naturally infected by Xcc were obtained from Tainan, Taiwan. The leaves of cabbages with bacterial leaf spot symptoms were collected from Changhwa, Taiwan. The DNA from diseased leaf tissues was extracted by a procedure modified from Audy *et al.*[4]. The diseased leaves were weight for one gram and placed in 1.5 ml eppendorf. A 200 μl of the extraction buffer (0.5N NaOH containing 0.5% polyvinylpyrrolidone) were added in to an eppendorf tube. The leaf tissues were then ground with a 200 μl

Table 1. Bacterial strains used in this study

Spiecies	Strain designation
Acidovorax avenae subsp. *citrulli*	Aac 19
Enterobacter cloacae	SM 1
Pectobacterium carotovorum subsp. *carotovorum*	ZL 1
P. chrysanthemi	CAS 7
Pseudomonas aeruginosa	WFP 33
P. putida	YFLP 56
P. syringae pv. *averrhoi*	PA 5
P. syringae pv. *syringae*	Pss 226
P. syringae pv. *tabaci*	ATCC 11528
Ralstonia solanacearum	G 21
Xanthomonas axonopodis pv. *citri*	Xw 19
X. axonopodis pv. *glycines*	Xcg 68
X. axonopodis pv. *vesicatoria*	XVT 88
X. campestris pv. *campestris*	Xcc 4, Xcc 7, Xcc 34, Xcc 40, Xcc 45, Xcc 61, Xcc 62, Xcc 68, Xcc 70, Xcc73, Xcc 78, Xcc 86, Br 1, Br 6, Xsp 17
X. campestris pv. *mangiferaeindicae*	Xcm 90
X. campestris pv. *raphani*	A342, XLS 2, XLS 6, XLS 10, Xca 417, Xca 756, G3-27
X. oryzae pv. *oryzae*	Xoo 84
X. vesicatoria	Xv 124

pipette tip. The residues of leaf tissue were separated from DNA extraction by centrifugation with 12,000 rpm for 15 min. The supernatant were collected as the stock DNA. The stock DNA was diluted 10- and 100-fold with distilled water and used in multiplex PCR assay as described above. The pretreatment processes for detection Xcc in naturally infected cabbage seeds were described as follows. The cabbage seeds (1000 seeds about 3 g) were placed in a 300 ml flask containing 100 ml saline (0.85% NaCl and 0.02% Tween 20). The flask was placed at 4℃ for 1 hr, then shook with 200 rpm at 30℃ for 30 min in a rotary shaker. The washing solution was collected and centrifuged with 15,000 rpm at 4℃ for 15 min. The supernatant was discarded and the pellet was resupsended with 500 μl distilled water. The seed extract was diluted 10- and 100-fold with distilled water and used in multiplex PCR assay as described above.

RESULTS

DNA fragments specific for Xcc and Xcr obtained by suppression subtractive hybridization

In suppression subtractive hybridization library, six clones of Xcc-Xcr SSH library and nine clones of Xcr-Xcc SSH library were randomly selected for sequencing and blasted in NCBI Genebank and the Comprehensive Microbial Resource (CMR) of J. Craig Venter Institute. Among the six SSH clones of Xcc 34, the sequence of Xcc 34-2 clone was conserved to a hypothetical protein gene of Xcc strain ATCC 33913. This hypothetical protein gene is not in the genome of Xcr 756C. The sequence of XLS 2-14 from the nine Xcr-Xcc SSH clones was conserved to the 3 genes of Xcr 756C with 99% identity, which contain 2 conserved hypothetical protein genes (XCAORF_1460 and 1462) and one IS5 transposase gene (XCAORF_1461) (Table 2). These two conserved hypothetical protein genes are specific for Xcr. Since the sequences of SSH clone Xcc 34-2 and XLS 2-14 were highly specific for Xcc and Xcr, respectively. Based on these two sequences, specified primer pairs Xcc 2f (5'- TGGGTTTTCGCCTATCAAAC -3') /2r (5'- TGCAACTATTCCTAGCACCG-3') or Xcr 14f (5'- CGTTAGCCAGG TAGAAAGCG-3') /14r (5'- TCGCTATTTCCATCTACCCG-3') for Xcc and Xcr were designed with the Primer 3 program in San Diego Supercomputer Center (SDSC) biological workbench.

Specificity and sensitivity of primer pairs for Xcc and Xcr

Specific DNA fragment of Xcc (200 bp) and Xcr (277 bp) was amplified by PCR with primer pairs Xcc 2f /2r and

Xcr 14f /14r, respectively. The specificity tests revealed that primer pairs Xcc 2f/2r and Xcr 14f/14r were highly specific. The specific DNA fragment was only amplified from Xcc or Xcr, but not from other phytopathogenic bacteria strains tested (Fig. 1). The minimal DNA concentration for detection of Xcc strain Xcc 74 by PCR with primer pair Xcc 2f/2r was 10 pg, while for Xcr strain XLS 10 by PCR with primer pair Xcr 14f/14r was 100 pg (Fig. 2).

Multiplex PCR for detection of Xcc and Xcr

To test whether Multiplex PCR with primer pairs of Xcc 2f/2r and Xcr 14f/14r could specifically detect and identify Xcc and Xcr, respectively. The results showed that DNA fragments specific for Xcc and Xcr were amplified from fifteen strains of Xcc and seven strains of Xcr listed in Table 1. No interference between these two primer pairs was observed (Fig. 3)

Table 2. Summary of BLAST of sequences from selected suppression subtractive hybridization (SSH) clones of *X. campestris* pv. *campestris* strain Xcc 34 and *X. campestris* pv. *raphani* strain XLS 2

SSH clone	Insert size (bp)	Organism, accession no. and BLASTN E value in NCBI , Predicted function or property	Organism, accession no. and BLASTN E value in JCVI CMR, Predicted function or property
Xcc 34-2	343	*X. campestris* pv. *campestris* strain ATCC 33913, AAM41191.1, 1e-145, hypothetical protein	*X. campestris* pv. *campestris* strain ATCC33913, XCC_1902, 2.4e-60, hypothetical protein
XLS 2-14	561	*X. campestris pv. campestris* strain ATCC33913, AE008922.1, 9.1e-13, IS1479 transposase	*X. campestris* pv. *raphani* strain 756C, XCAORF_1460, 1.6e-22, hypothetical protein XCAORF_1461, 4.4e-19, IS5 transposase XCAORF_1462, 1.5e-16, hypothetical protein

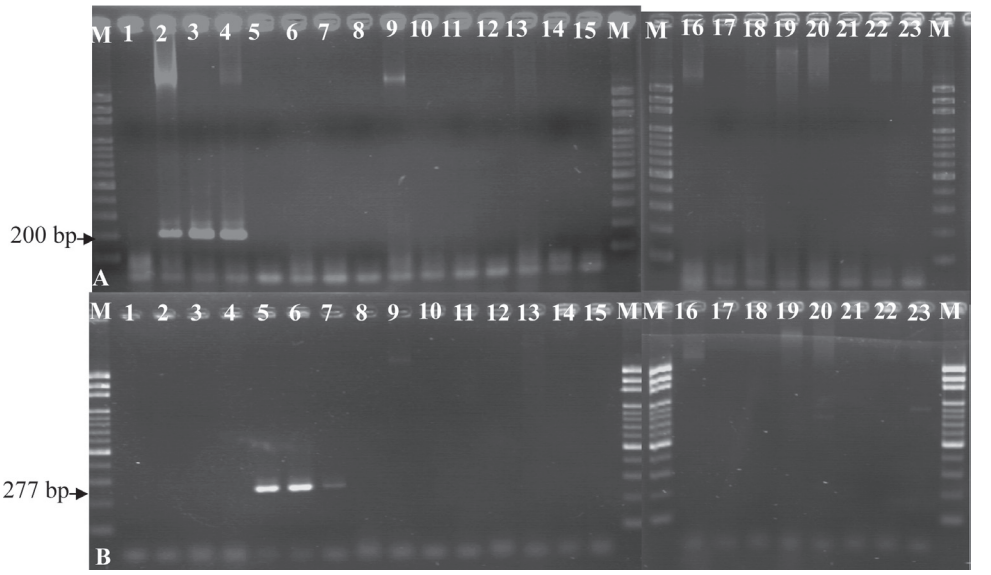

Fig. 1. Specificity test for *Xanthomonas campestris* pv. *campestris*, *X. campestris* pv. *raphani* and other plant pathologenic bacteria by polymerase chain reaction with primer pairs Xcc 2f/2r (A) or Xcr 14f/14r (B). From left, lane M, Bio 100 DNA ladder (PROtech Technology, R.O.C.); lane 1, negative control; lanes 2~4, *X. campestris* pv. *campestris* strains Xcc 34, Xcc 70, Xcc 74; lanes 5~7, *X. campestris* pv. *raphani* Xca 417, Xca 756, A342; lane 8, *Acidovorax avenae* subsp. *citrulli* Aac19; lane 9, *Pectobacterium carotovorum* subsp. *carotovorum* ZL 1; lane 10, *Pectobacterium chrysanthemi* Cas 7; lane 11, *Enterobacter cloacae* SM1; lane 12, *Pseudomonas aeruginosa* WFP 33; lane 13, *Pseudomonas putida* YFLP 56; lane 14, *Pseudomonas syringae* pv. *averrhoi* PA 5; lane 15, *Pseudomonas syringae* pv. *syringae* Pss 226 ; lane 16, *Pseudomonas syringae* pv. *tabaci* ATCC 11528 ; lane 17, *Ralstonia solanacearum* G 21; lane 18, *X. axonopodis* pv. *citri* Xw 19; lane 19, *X. campestris* pv. *mangiferaeindicae* Xcm 90; lane 20, *X. axonopodis* pv. *glycine* Xcg 68; lane 21, *X. axonopodis* pv. *vesicatoria* XVT 88; lane 22, *X. vesicatoria* XV 124 and lane 23, *X. oryzae* pv. *oryzae* Xoo 84.

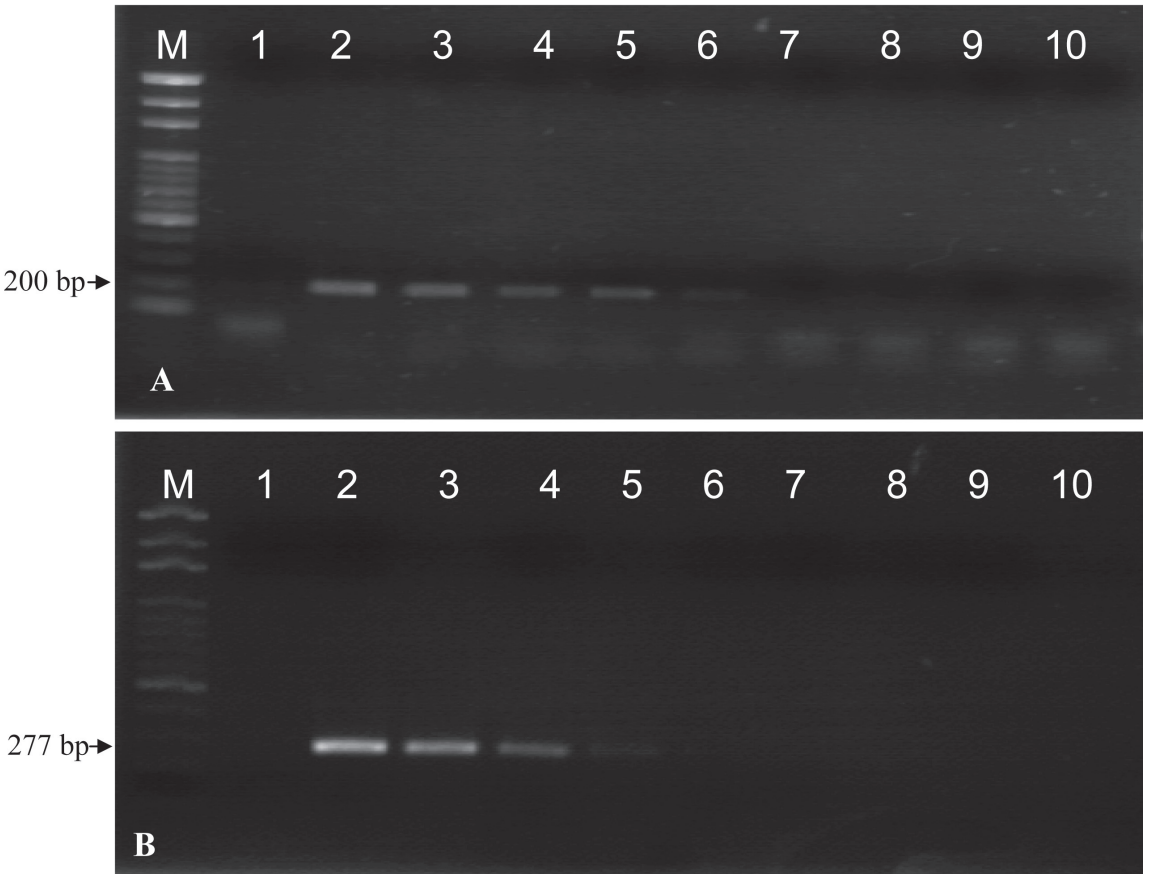

Fig. 2. Sensitivity of detection of DNA of *Xanthomonas campestris* pv. *campestris* strain Xcc 74 and *X. campestris* pv. *raphani* XLS 10 by PCR with primer pairs Xcc 2f/2r (A) or Xcr 14f/14r (B). From left, Lane M, Bio100 DNA Ladder (PROtech Technology, R.O.C.); lane 1, sterilized distilled water; lanes 2 ~10, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100fg,10 fg, and 1 fg DNA, respectively.

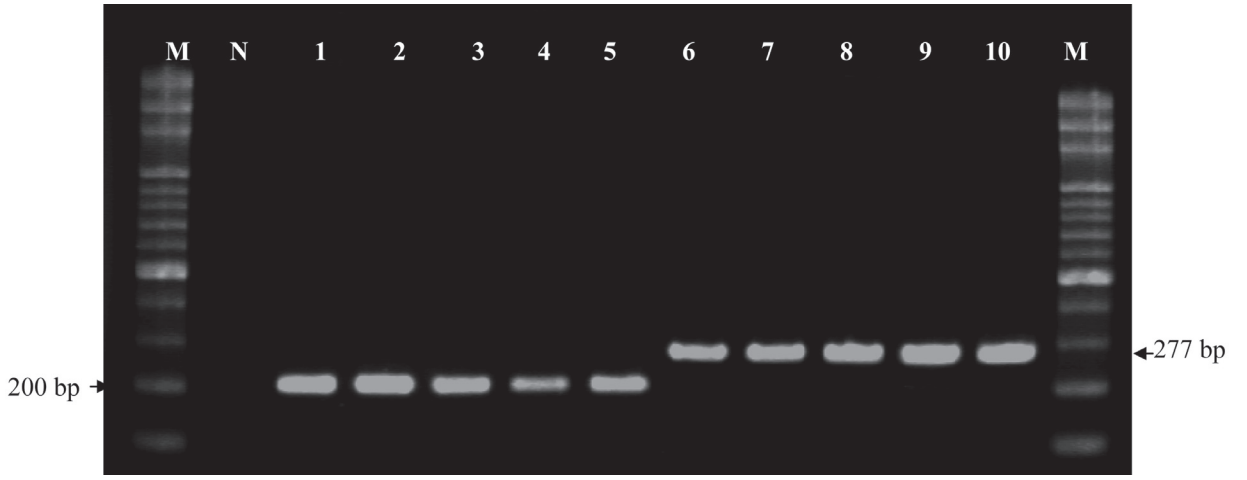

Fig. 3. Detection of *Xanthomonas campestris* pv. *campestris* and *X. campestris* pv. *raphani* strains by multiplex PCR with primer pairs Xcc 2f/2r and Xcr 14f/14r. From left: lane M, Bio 100 DNA ladder (PROtech Technology, R.O.C.); lane N, sterilized distilled water; lanes 1~5, *X. campestris* pv. *campestris* strain Xcc 34, Xcc 40, Xcc 70, Xcc 78, Br 1 and lanes 6~10, *X. campestris* pv. *raphani* strain Xca 417, Xca 756,A342, G3-27, XLS 2.

Simultaneous detection of Xcc and Xcr from naturally infected plant tissues and seeds by multiplex PCR

The extracts prepared from the leaf tissues of cabbage with black rot symptoms were examined by multiplex PCR with the primer pairs Xcc 2f/2r and Xcr 14f/14r. The DNA fragment specific for Xcc was amplified, but no other DNA fragment was amplified (Fig. 4). The specific products of Xcc was consistently amplified from the 10 fold diluted extracts, while the products were inconsistently amplified from undiluted extracts. Leaf tissues of cabbage with symptoms of bacterial leaf spot were collected and detected by multiplex PCR with primer pairs Xcc 2f/2r and Xcr 14f/14r. The DNA fragments specific for both Xcc and Xcr were amplified from undiluted leaf tissue extractions. Among the ten samples, there was only one sample (lane 4) without any amplification product (Fig. 5). When cabbage seeds naturally infected by Xcc were examined by multiplex PCR with primer pairs Xcc 2f/2r and Xcr 14f/14r. DNA fragment specific for Xcc was amplified from the 10 fold and 100 fold diluted extracts, but no specific product was amplified from undiluted seed extracts (Fig. 6).

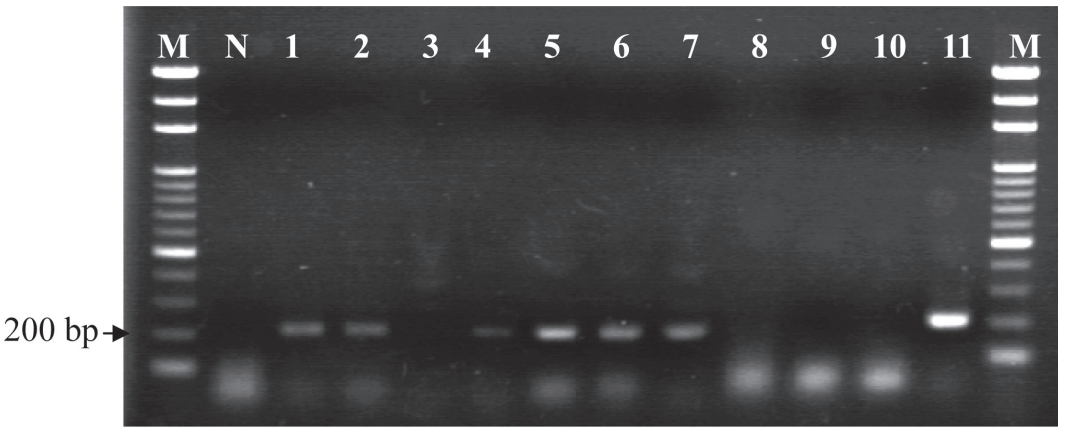

Fig. 4. Detection of *Xanthomonas campestris* pv. *campestris* and *X. campestris* pv. *raphani* from leaf tissues of cabbage with black rot symptoms by multiplex PCR with primer pairs Xcc 2f/2r and Xcr 14f/14r. From left, lane M, Bio 100 DNA ladder (PROtech Technology, R.O.C.); lane N, sterilized distilled water; lanes 1~3, diseased tissue extract; lane 4, healthy tissue extract + total DNA of Xcc 73 (100 ng); lanes 5~7, 10 fold dilution of diseased tissue extract; lanes 8~10, 100 fold dilution of diseased tissue extract and lane 11, 100 fold dilution of healthy tissue extract + total DNA of Xcc 73 (100 ng).

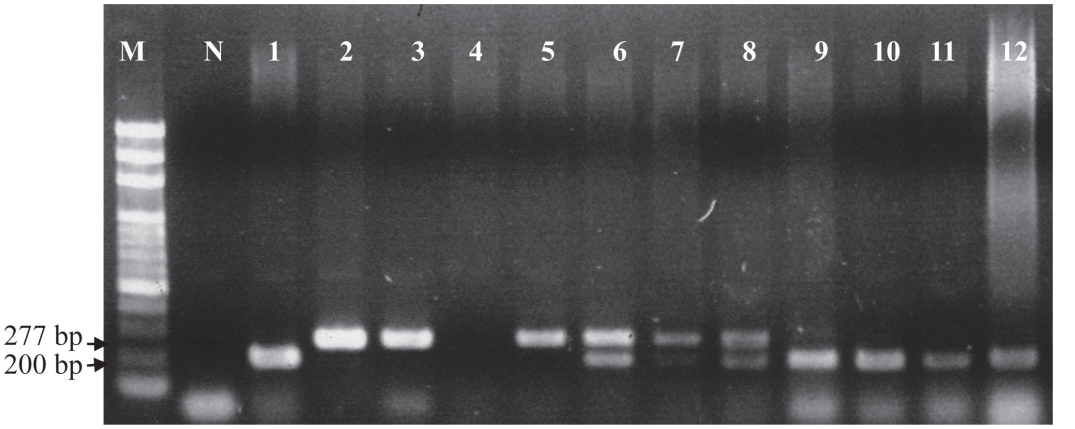

Fig. 5. Detection of *Xanthomonas campestris* pv. *campestris* and *X. campestris* pv. *raphani* from leaf tissues of cabbage with bacterial leaf spot symptoms collected from nursery in Changhwa by multiplex PCR with primer pairs Xcc 2f/2r and Xcr 14f/14r. From left, lane M, Bio 100 DNA ladder (PROtech Technology, R.O.C.); lane N, sterilized distilled water; lane 1, total DNA of *Xanthomonas campestris* pv. *campestris* Xcc 73 (100 ng); lane 2, total DNA of *X. campestris* pv. *raphani* XLS 10 (100 ng); lanes 3~12, diseased leaf tissues.

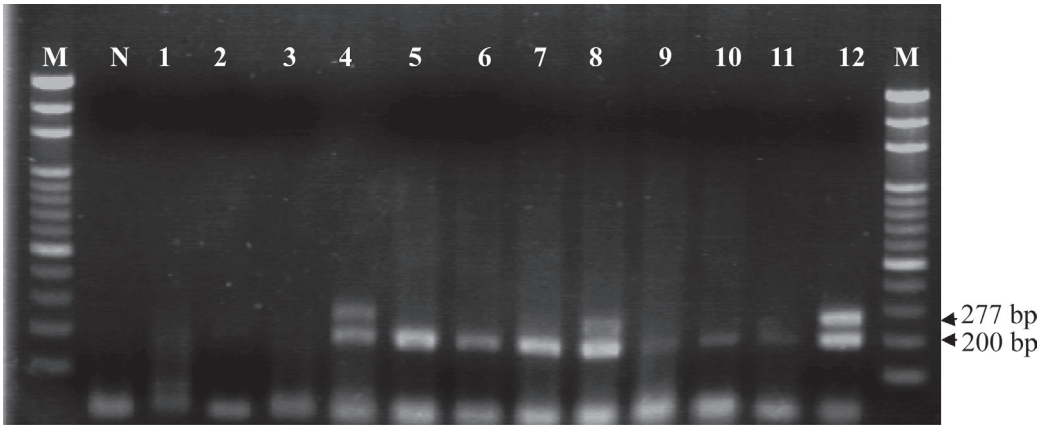

Fig. 6. Detection of *Xanthomonas campestris* pv. *campestris* in naturally infected cabbage seeds by multiplex PCR with primer pairs Xcc 2f/2r and Xcr 14f/14r. From left, lane M, Bio 100 DNA ladder (PROtech Technology, R.O.C.); lane N, sterilized distilled water; lanes 1~3, undiluted seed extract; lane 4, undiluted seed extract + total DNA of Xcc 73 (100 ng) and *X. campestris* pv. *raphani* XLS 10 (100 ng); lanes 5~7, 10 fold dilution of seed extract; lane 8, 10 fold dilution of seed extract + total DNA of Xcc 73 (100 ng) and *X. campestris* pv. *raphani* XLS 10 (100 ng); lanes 9~11, 100 fold dilution of seed extract and lane 12, 100 fold dilution of seed extract + total DNA of Xcc 73 (100 ng) and *X. campestris* pv. *raphani* XLS 10 (100 ng).

DISCUSSION

In the reclassification of genus *Xanthomonas* based on DNA-DNA hybridization, the species *X. campestris* includes pathovars *campestris* (Xcc), *aberrans, armoraciae*, *barbareae*, *incanae*, and *raphani* [32]. However, *X. campestris* pathovars of *armoraciae* and *raphani* have been considered as synonyms by Alvarez *et al.* [2]. Based on pathogenicity and repetitive-DNA PCR based fingerprinting assay, the *X. campestris* pathovars that cause nonvascular leaf spot disease on *Brassica* have been identified as pv. *raphani*, but not pv. *armoraciae* [34]. Further study based on the pathogenicity assay also indicated that *X. campestris* should be restricted into three pathovars: (i.) *X. campestris* pv. *campestris* which causes black rot diseases on at least one cruciferous plant, (ii.) *X. campestris* pv. *raphani* which causes leaf spot disease on hosts of Brassicacae and Solanaceae, and (iii) *X. campestris* pv. *incanae* which causes bacterial blight of garden stock [13].

In this study, specific DNA fragments were cloned and sequenced from Xcc and Xcr by suppression subtractive hybridization. Among them, the DNA fragment in the Xcc 34-2 clone is a conserved hypothetical protein gene (XCC1902) of Xcc strain ATCC 33913. While in the clone of XLS 2-14, it contains two conserved hypothetical protein genes (XCAORF_1460 and_ 1462) of Xcr strain 756C. This DNA fragment is located in the downstream of *hrp*F gene. Comparing the downstream sequences of *hrp*F gene in Xcc and Xcr, it revealed that the Xanthomonas out protein F1 gene (Xcc 1218) is adjacent to the *hrp*F of Xcc, while there are 10 genes between *hrp*F and Xanthomonas out protein F1 gene in Xcr. These genes include the DNA fragment XLS 2-14 (XCAORF_1460~1462), *skwp4* (XCAORF_1464), *hpa3* (XCAORF_1469) etc. Since these genes were found in the *hrp* (hypersensitive response and pathogenicity) gene cluster of *X. campestris* pv. *raphani*, they might play important roles in pathogenicity of Xcr.

Multiplex PCR consists of multiple primer pairs added into one reaction tube, and these primer pairs can only amplify their specific DNA fragments [12]. In this study, the multiplex PCR with primer pairs Xcc 2f/2r and Xcr 14f/14r, the specific DNA fragments for Xcc (200 bp) and Xcr (277 bp) were amplified. It reveals no interference between these two primer sets. In this study, both Xcc and Xcr can be detected from the sample of leaf spot tissue of cabbage by multiplex PCR. Zhao *et al.* [37] reported that two pathovars of *X. campestris* were isolated from leaf spot of crucifers in eight fields in Oklahoma. In this study, yellow colonies of *Xanthomonas campestris* were isolated from the leaf spot diseased tissues on the NA plates. *Xanthomonas campestris* pv. *campestris* can not differentiated from Xcr based on their colony morphology; however, they can be rapidly and specifically identified by multiplex PCR with primer pairs Xcc 2f/2r and Xcr 14f/14r.

Xanthomonas campestris pv. *campestris* can be detected from naturally infected cabbage seeds by multiplex PCR with primer pairs of Xcc 2f/2r and Xcr 14f/14r. However, the DNA fragment specific for Xcc was consistently amplified from 10 fold dilutions of seed extract, but was inconsistently from the undiluted original extract. The results indicated that there were PCR inhibitors presented in cabbage seed extraction. Since the seed washings of *Brassica* seeds contain other microflora and inhibitory compounds, *X. campestris* colonies formed on selective medium was often inhibited [21]. These microflora and inhibitors are problems for both the plating and PCR based detection methods [5]. To prevent the effect of PCR inhibitors, the seed washings often need to dilute 10 or 100 folds for PCR detection or isolation of *X. campestris* colonies [5]. However, there are some PCR based techniques can be used to overcome the inhibitor such as real-time PCR or immunomagnetic separation PCR (IMS-PCR) [1].

The methods recommended for detection of Xcc by International Seed Test Association (ISTA) are based on the selective medium and pathogenicity tests. The protocol to detect Xcc advises that any colony able to hydrolyse starch on mCS20ABN or FS medium needed to be further analyzed by pathogenicity testing on seedlings; it often takes two weeks to do these tests [23]. The mixture of Xcc/ Xcr- specific monoclonal antibodies has been used for detection of the black rot/leaf spot pathogens [2]. In this study, Xcc and Xcr can be simultaneously and rapidly detected from diseased leaf tissues and in infected seeds by multiplex PCR with Xcc 2f/2r and Xcr 14f/14r. It only took around 6 hours to perform the detection processes. In summary, the detection technique developed in this study could be used to differentiate the diseases caused by Xcc or Xcr, and it could also be used to detect Xcc and Xcr simultaneously from crucifer seeds.

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摘　要

呂昀陞[1, 2]、鄧文玲[1]、楊雯馨[1]、吳雅芳[3]、鄭安秀[3]、徐世典[1]、曾國欽[1, 4]. 2010. 應用多引子聚合酶連鎖反應技術同時檢測十字花科黑腐病菌與細菌性葉斑病菌. 植病會刊 19: 137-147. ([1]台中市國立中興大學植物病理學系；[2]台中縣 行政院農業委員會農業試驗所植物病理組；[3]行政院農業委員會台南區農業改良場；[4]聯絡作者，電子郵件：kctzeng@nchu.edu.tw；傳眞：+886-4-2285-4633)

Xanthomonas campestris pv. *campestris* (Xcc) 所引起之黑腐病爲十字花科作物重要之病害，近年來 *X. camestris* pv. *raphani* (Xcr) 所引起之十字花科細菌性葉斑病已在許多國家發生，並造成嚴重之損失。感染或污染此兩種病原細菌之種子或種苗爲其最初感染源，因此開發此兩種植物病原細菌之準確、靈敏且快速之檢測技術，爲病害診斷與植物檢疫所亟需。本研究應用基因體減扣法分別篩選出 Xcc 與 Xcr 特異性核酸片段，依其核酸序列設計出 Xcc 與 Xcr 之專一性引子對 Xcc 2f/2r 與 Xcr 14f/14r，測試此兩組引子對之專一性與靈敏度，顯示此兩組引子對可分別對Xcc 與 Xcr 增幅出 200 bp 與 277 bp 之核酸片段，其相對應之靈敏度分別爲 10 pg 與 100 pg。利用此兩組引子進行多引子聚合酶連鎖反應測試，顯示此技術可同時檢測與區別此兩種病原細菌，此技術可應用於十字花科黑腐病與細菌性葉斑病之診斷與帶菌種子之檢測。

關鍵詞：十字花科黑腐病菌、十字花科細菌性葉斑病菌、多引子聚合酶連鎖反應