Development of Molecular Marker through Genome Realignment for Specific Detection of *Xanthomonas campestris* pv. *campestris* Race 5, a Pathogen of Black Rot Disease^S

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Black rot caused by *Xanthomonas campestris* pv. *campestris* (Xcc) is the most damaging disease in *Brassica* crops around the world. In this study, we developed a molecular marker specific to Xcc race 5. To do this, the available whole genome sequences of Xcc races/strains and Xc subspecies were aligned and identified a highly variable genomic region (XccR5-89.2). Subsequently, a primer set covering the 'XccR5-89.2' region was designed and tested against the genomic DNA of Xcc races/strains, Xc subspecies and other plant-infecting bacterial strains (*Pseudomonas syringae* pv. *maculicola* and *Erwinia carotovora* subsp. *carotovora*). The results showed that the 'XccR5-89.2' primer pair amplified a 2,172-bp fragment specific to Xcc race 5. Moreover, they also amplified a 1,515-bp fragment for Xcc race 1 and an over 3,000-bp fragment for Xcc race 3. However, they did not amplify any fragments from the remaining Xcc races/strains, subspecies or other bacterial strains. The 'XccR5-89.2' primer pair was further PCR amplified from race-unknown Xcc strains and ICMP8 was identified as race 5 among nine race-unknown Xcc strains. Further cloning and sequencing of the bands amplified from race 5 and ICMP8 with 'XccR5-89.2' primers revealed both carrying identical sequences. The results showed that the 'XccR5-89.2' marker can effectively and proficiently detect, and identify Xcc race 5 from Xcc races/strains, subspecies and other plant-infecting bacteria. To our knowledge, this is the first report for an Xcc race 5-specific molecular marker.

Keywords: *Xanthomonas campestris* pv. *campestris*, race 5, black rot, molecular marker, cabbage

Introduction

Xanthomonas campestris pv. *campestris* (Pam.) Dowson (Xcc) is one of the most important pathogens for *Brassica* vegetable growing regions of the world. Xcc is a gram-negative, aerobic and rod-shaped bacterium that causes black rot. Black rot is a devastating disease of cabbage and other *Brassica* crops, including cauliflower, broccoli, kohlrabi, Brussels sprout, kale, mustard, turnip and turnip greens [1, 2]. This pathogen can also infect other cruciferous vegetables, including radish and *Arabidopsis* as well as weeds and ornamentals [2].

The primary sources of inoculation of Xcc are infected seeds and plant residues; however, it can also survive in cruciferous weeds and ornamentals [1]. Besides infected

seeds and plants, Xcc can also spread via, rain, wind, irrigation, agricultural implements, international seed trade, cruciferous weeds and non-cruciferous crops [3]. Xcc is a vascular pathogen that can enter into plants via wounds, leaf hydathodes and insects [4]. Subsequently, infection proceeds to the vascular tissue where it multiplies and develops typical V-shaped yellow lesions at the leaf borders along with necrotic and darkened veins [4–7].

Black rot disease drastically reduces the economic value of cruciferous crops. It has been reported that up to 100% of cabbage yield can be reduced by Xcc under favorable environmental conditions [4, 8]. In the Republic of Korea, heading cabbage (*Brassica oleracea* ssp. *capitata*) is the third most important *Brassica* vegetable and one in which black rot is also recognized as a major constraint for production [9].

It is very hard to control black rot with traditional disease management practices that include the use of disease-free seeds, removal of crop residues, practicing crop rotation, and the use of resistant cultivars and agrochemicals. To date, several black rot-resistant cultivars/F1/lines have been reported in different *Brassica* crops [1, 10–13]. However, these genotypes have not shown resistance against all the *Xcc* races. At present, a total of eleven physiological races of *Xcc* (races 1–11) has been reported by different researchers. Initially, Kamoun *et al.* [10] identified five different physiological races (0–4). Thereafter, Vicente *et al.* [1] reported six *Xcc* races (1–6) of *Xcc*. Further, three additional races (7, 8, and 9) were reported by Jensen *et al.* [14], Fargier and Manceau [11]. Recently, two new races (10–11) were reported by Cruz *et al.* [12]. However, these races were identified with traditional methods based on the reactions of differential *Brassica* species with resistance genes with pathogens carrying avirulence genes. Among 11 races, races 1 and 4 have been reported as the most virulent throughout the world due to severe infections in *B. oleracea* subspecies, while race 6 is common in *B. rapa* [3, 15]. Moreover, the occurrence of races 5 and 6 was reported in black rot-infected fields in Nepal [14] and northwest Spain [3]. Recent studies have demonstrated that the type III secretion system (T3SS) is an essential secretion system for the virulence of the bacterial pathogens of plants and animals [16, 17]. In *Xcc*, the *hrp* (*hypersensitive response and pathogenicity*) gene cluster encodes a T3SS which is highly conserved and involved in pathogenicity [15]. Therefore, Zaccardelli *et al.* [18] developed a PCR-based molecular marker using T3SS gene *hrcC* for detection and identification of *Xcc* from other *Xc* subspecies. Recent studies have revealed that type III effector genes are required for the virulence of *Xcc* races. For example, Jiang *et al.* [19] have shown that *XopN* is essential for full virulence of *Xcc* race 9 (strain 8004), while Ignatov *et al.* [20] showed that avirulence gene *avrRxc1/3* (homologue of *avrBs2*) determines the *Xcc* races.

Therefore, proper and quick race determination is essential to develop race-specific resistant cultivars against black rot. The PCR-based race detection technique has been proven as a very powerful, rapid and reliable method for *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Xcc* [21–23]. Thus, one of our research goals is to identify and determine the predominant *Xcc* races in Korea. Our research group has already developed race-specific molecular markers for *Xcc* race 1, 3, and 4 [22, 23] and some others are under development. Therefore, the objective of this study was to also develop a marker for rapid detection of *Xcc* race 5.

Materials and Methods

Bacterial Strains and Media

A total of 24 bacterial strains including standard reference races (race 1–8) of *Xanthomonas campestris* pv. *campestris* (*Xcc*), *Xcc* strains (race undetermined), *Xc* subspecies, strains of other bacteria was used (Table 1). These bacterial strains were grown on 'King's Medium B' at 30°C (48 h) [24].

Extraction of Genomic DNA

The genomic DNA (gDNA) was extracted from all the bacteria with DNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's guidelines. The concentration and purity of the gDNA were ascertained by a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA) and kept in the freezer at –20°C until analyzed.

Identification of Variable Genomic Region

The whole genome sequences of *Xcc* races (race 1, 3, 4, and 9), *Xcc* strains (ICMP 4013, ICMP 21080 and *Xcc* strain 17) and *Xc* subspecies (*Xci* strain 'CFBP 1606R', *Xcr* '756C', *Xcv* strain '85-10' were downloaded from the NCBI (<https://www.ncbi.nlm.nih.gov/genome/microbes/>) (Table 2). Successively, these available whole genomes were re-aligned with the progressiveMauve tool [25]. Finally, the highly variable genomic regions were recognized by using Geneious software (trial version, www.geneious.com).

PCR Analysis

The extremely variable genomic fragment 'XccR5-89.2' was subjected to primer design (Table 3) with the help of PCR Primer Stats (http://www.bioinformatics.org/sms2/pcr_primer_stats.html). Thereafter, the specificity of the primers was tested with *in silico* PCR (<http://insilico.ehu.es/PCR/index.php?mo=Xanthomonas>). The PCR amplification was carried out using gDNA samples extracted from bacterial strains (Table 1). The PCR reaction was performed in 20 µl volume, which contained 30 ng of gDNA, 10 pmol of each forward and reverse primers, 0.25 mM dNTP mix, 5× GoTaq buffer, 1 unit of GoTaq DNA Polymerase (Promega, WI, USA). The reaction condition was adjusted with a preliminary denaturation at 95°C for 5 min followed by 22 cycles (95°C for 30 sec, 63°C for 40 sec, and 72°C for 45 sec) with a final extension at 72°C for 5 min. The amplified PCR products were analyzed by 1.2% agarose gel electrophoresis and the bands were visualized using a gel documentation system.

Cloning and Sequencing

A genomic fragment of about 2.2 kb specifically amplified from *Xcc* race 5 with 'XccR5-89.2' primers was extracted from the agarose gel and purified by the Wizard SV Gel and PCR Cleanup system (Promega, USA). Thereafter, the purified PCR product was cloned by using TOP Cloner Blunt Kit (Enzynomics, Korea) following the manufacturer's protocol. The selected positive clones were further cultured in liquid LB media for overnight and

Table 1. List of bacterial strains with their host used in this study.

Sl. No.	Bacterial species/strains	Race	Host	Origin	Source
1	<i>Xcc</i> (HRIW-3811)	Race 1	<i>B. oleracea</i>	United States of America	HRI-W
2	<i>Xcc</i> (HRIW-3849A)	Race 2	Cauliflower	United States of America	
3	<i>Xcc</i> (HRIW-5212)	Race 3	Brussels sprout	United Kingdom	
4	<i>Xcc</i> (HRIW-1279A)	Race 4	Cabbage	United Kingdom	
5	<i>Xcc</i> (HRIW-3880)	Race 5	Cabbage	Australia	MBG-CSIC
6	<i>Xcc</i> (HRIW-6181)	Race 6	Chinese cabbage	Portugal (Sardoal)	
7	<i>Xcc</i> (HRIW-8450A)	Race 7	Cabbage	United Kingdom	
8	<i>Xcc</i> (MBG-145.3)	Race 8	Turnip greens	Northwestern Spain	
9	<i>Xci</i> (HRI-W-6377)	-	Hoary stock	United Kingdom	HRI-W
10	<i>Xcr</i> (WHRI-8305)	-	Japanese mustard spinach	United Kingdom	ICMP
11	<i>Psm</i> (ICMP13051)	-	Cabbage	New Zealand	
12	<i>Ecc</i> (ICMP12464)	-	Cabbage	New Zealand	
13	<i>Xcc</i> strain (ICMP 8)	-	Cabbage	New Zealand	
14	<i>Xcc</i> strain (KACC19132)	-	Chinese cabbage	South Korea	KACC
15	<i>Xcc</i> strain (KACC19133)	-	Chinese cabbage	South Korea	
16	<i>Xcc</i> strain (KACC19134)	-	Chinese cabbage	South Korea	
17	<i>Xcc</i> strain (KACC19135)	-	-	South Korea	
18	<i>Xcc</i> strain (KACC19136)	-	-	South Korea	
19	<i>Xcc</i> strain (KACC17966)	-	-	South Korea	
20	<i>Xcc</i> strain (KACC10377)	-	-	South Korea	
21	<i>Xcv</i> (KACC 11153)	-	-	South Korea	
22	<i>Xag</i> (KACC10491)	-	Soybean	South Korea	
23	<i>Xad</i> (KACC17821)	-	Anthurium	South Korea	
24	<i>Xcz</i> (KACC17126)	-	Zinnia	South Korea	

HRI-W, Horticulture Research International, Wellesbourne, United Kingdom; ICMP, International Collection of Microorganisms from Plants, New Zealand; KACC, Korean Agricultural Culture Collection, South Korea; MBG-CSIC, Misión Biológica de Galicia, Pontevedra, Spain. *Xcc*, *Xanthomonas campestris* pv. *campestris*; *Xcz*, *X. campestris* pv. *zinnia*; *Xad*, *X. axonopodis* pv. *dieffenbachiae*; *Xag*, *X. axonopodis* pv. *glycines*; *Xcv*, *X. campestris* pv. *vesicatoria*; *Psm*, *Pseudomonas syringae* pv. *maculicola*; *Ecc*, *Erwinia carotovora* subsp. *carotovora*.

plasmid DNA was purified using a commercial kit (GeneAll, Korea). The purified plasmid DNA samples were sequenced by ABI 3730XL DNA Sequencer (Macrogen Inc., Korea) with universal

primers. The sequence alignment was performed using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

Table 2. The available whole genome sequences of *Xanthomonas campestris* pv. *campestris* (*Xcc*) races, *Xcc* strains and *Xc* subspecies, and their accession number.

Sl. No.	Bacterial strains	Genebank accession	Races	Genome size (bp)	References
1	B100 (<i>Xcc</i>)	AM920689	1	5,079,002	[31]
2	CFBP1869 (<i>Xcc</i>)	NZ_CM002545	1	5,011,687	[32]
3	Xca5 (<i>Xcc</i>)	NZ_HF570389	1	4,916,437	[33]
4	ATCC 33913 (<i>Xcc</i>)	AE008922	3	5,076,187	[34]
5	CFBP 5817	NZ_CM002673	4	4,918,955	[32]
6	Strain 8004 (<i>Xcc</i>)	NC_007086	9	5,148,708	[35]
7	ICMP 4013	CP012146	-	4,908,383	[36]
8	ICMP 21080 (<i>Xcc</i>)	CP012145	-	4,911,121	[36]
9	Strain 17 (<i>Xcc</i>)	CP011946	-	4,994,426	[37]
10	CFBP 1606R (<i>Xci</i>)	NZ_CM002635	-	4,967,288	[38]
11	Strain 756C (<i>Xcr</i>)	NC_017271	-	4,941,214	[39]
12	Strain 85-10 (<i>Xcv</i>)	NC_007508	-	5,178,466	[40]

Xci, *Xanthomonas campestris* pv. *incanae*; *Xcr*, *Xanthomonas campestris* pv. *raphani*; *Xcv*, *Xanthomonas campestris* pv. *vesicatoria*.

Table 3. List of primers used in this study.

Primer name	Nucleotide sequence (5' to 3')	Length (bp)
XccR5-89.2F	GCGAGCGATTTCACCTTCAT	1515
XccR5-89.2R	AATTCGGACAACAGCGCAAT	

Bio-PCR Assay

The leaves of 3-week-old cabbage plants of a black rot susceptible inbred line (SCNU-C-3328) were inoculated with *Xcc* races (races 1-8) following the method as previously described by Afrin *et al.* [23] by clipping secondary veins followed by dipping into a bacterial suspension (10^9 CFU/ml) and maintaining high humidity [1]. About 1-cm infected leaves were collected when

black rot symptoms were visible and cut into small pieces and soaked in sterile water (250 μ l) for 40 min at room temperature. Thereafter, 10- μ l exudates were directly used as a template for the bio-PCR amplification.

Results

Development of *Xcc* Race 5-Specific Molecular Markers

Up until now, the whole genome sequences of only four *Xcc* races (races 1, 3, 4, and 9), some *Xcc* strains (race-unspecified), and a few *Xc* subspecies have been reported (Table 2). Realignment of these available whole genomes permits us to find similarities (boxes have the same colors)

Fig. 1. Mauve alignment of the available whole genome sequences of *Xanthomonas campestris* pv. *campestris* (*Xcc*) races (races 1, 3, 4, and 9), strains and other subspecies of *Xanthomonas campestris*. The syntenic blocks are connected among the *Xcc* races/strains/*Xc* subspecies. The highly variable regions among the *Xcc* races/strains/*Xc* subspecies are shown with the line diagram at the bottom of the alignment. The detailed descriptions and genebank accessions are provided in Table 1. Genomic blocks are shown as colored boxes.

Fig. 2. Agarose gel electrophoresis of the Xcc race 5-specific XccR5-89.2 PCR products (2,172 bp) amplified from genomic DNA of *Xanthomonas campestris* pv. *campestris* (Xcc) races and other subspecies of *Xanthomonas campestris*.

Lane M: 100 bp plus DNA ladder; lanes 1-8: Xcc races 1-8; lane Xci: *X. campestris* pv. *incane* (Xci); lane Xcr: *X. campestris* pv. *raphani* (Xcr); lane Xcv: *X. campestris* pv. *vesicatoria* (Xcv); lane Xag: *X. axonopodis* pv. *glycines* (Xag); lane Xad: *X. axonopodis* pv. *dieffenbachiae* (Xad); lane Xcz: *X. campestris* pv. *zinniae* (Xcz); lane Psm: *Pseudomonas syringae* pv. *maculicola* (Psm); lane Ecc: *Erwinia carotovora* subsp. *carotovora* (Ecc).

and variabilities among the aligned genomes (Fig. 1). Since the genome of Xcc race 5 was not yet sequenced, hence, to develop a race 5-specific molecular marker, we looked for the highly variable genomic regions among the 12 re-aligned genomes and identified the variable genomic fragment 'XccR5-89.2' (Fig. 1). Thereafter, primers were designed based on the 'XccR5-89.2' fragment from Xcc race 1 (strain B100) sequence. The PCR results showed that the 'XccR5-89.2' primers amplified an approximately 2,200-bp fragment specific to Xcc race 5 as well as a 1,515-bp and an over 3,000-bp fragment for race 1 and race 3, respectively (Fig. 2). Even though very faint bands were detected with Xcc race 2 and race 6, the band was stronger when race 5 was present. However, there was no amplicon detected for the remaining Xcc races, subspecies or other-plant infecting bacteria tested in this study. Furthermore, the fragment amplified from Xcc race 5 was cloned and sequenced. Since

the size of the cloned fragment was around 2.2-kb, hence, with the universal primers, we found only a part of both the 5' and 3' ends of the sequence. Consequently, we performed primer walking to cover the full sequence (Fig. S1). The sequence analysis revealed that fragment amplified from race 5 was 2,172-bp (Fig. S2).

The 2,172-bp nucleotide sequence obtained through cloning and sequencing of the 'XccR5-89.2' fragment from Xcc race 5 was subjected to ORF prediction using the ORF Finder in the NCBI (<https://www.ncbi.nlm.nih.gov/orffinder/>) (Fig. 4). The result showed that the longest ORF (negative strand) was 990-bp (329-aa residues) (Fig. S3). Furthermore, the BLASTP was accomplished against the NCBI and found that it has significant homology with the protein encoding an IS4 family transposase of *Xanthomonas campestris* (WP_011038666.1).

Race Determination for Race Unknown Xcc Strains

The 'XccR5-89.2' primer pair was further used to find the race information of eight race-unknown Xcc strains (Table 2). The PCR result showed that this primer pair specifically amplified a 2,200-bp fragment similar to Xcc race 5 from Xcc strain ICMP 8 (Fig. 3). As regards Xcc strain KACC10377, it amplified two fragments (about 1,500-bp and 3,000-bp) while the remaining six unknown strains did not show any amplification.

Detection of Xcc Race 5 from Infected Cabbage Leaves

The potentiality of race 5-specific primers was determined by bio-PCR with artificially inoculated cabbage leaves with different Xcc races (races 1-8). The results revealed that only cabbage samples inoculated with race 5 were amplified while no visible bands were detected in the remaining 21 samples inoculated with other Xcc races (Fig. 6).

Fig. 3. PCR amplification *Xanthomonas campestris* pv. *campestris* (Xcc) race 5 specific XccR5-89.2 primers from race-unknown Xcc strains for race determination.

Lane M: 100 bp plus DNA ladder; lane 1: Xcc race 5 (HRIW-3880) as positive control; lanes 2-9: Xcc race unknown strains (KACC19132, KACC19133, ICMP 8, KACC17966, KACC10377, KACC19134, KACC19135 and KACC19136); lane -ve: negative control.

AATTCGGACAACAGCGCAATAACCTCCTCATCCCGATCGGGACGGCGCCGATAACGTGCTGT 62
 CGAGCGAGCCACGCCAACCCAGCCACAAAGCCCGCGCTCGCTCCAGCCATGCTGCTCGATGA 124
 GCCAGGCAAGAAGCGGGCGCTTATGCGCCGGGTCTACAGCTTTTTTTCGATGACATCCTTCA 186
 ATGCATGGTTTTTCATCGCGAGATCGGCGTACATACGTTTGAGCTTGTGTGCTCAGTCTCC 248
 AGGTCGCGGAGGCGTTGCACGTAGCGGCCCTCCATGCCGCCGTACTTGGACTTCCAGACGTA 310
 GTACGTCGCATCGGAAATGCCAGCTCGCGACAGACATCCTTGACCTGGCGACCGCCCTCGA 372
 CCTGCTTCAGCGTGGCGACGATCTGGCTCTCGGTGAACTTGCTCTTGCAGATTGGTGGTCTC 434
 CTTGGTGGCTAGTGTGCCCGGAGACCTCTAGTTATGGATGGATCAATTTTACGGGGAGTCGA 496
 CACCAAGACCCCGCGCTTTGCGGGCATCGGGCCCGCGCACCGGCCACGCAAGCCGAGCGCCA 558
 TCGACTAATAAGTGAACCTGACCTGAGGTTTCCCAACATTTCTCTGGCAAATCAATCAC 620
 TTGCGACGTAATTGCCTGGAAGAAAGTGCAGCATGGCGCAATCTTCAAGGTGACTAAGCCA 682
 M R A S E V L Q K C L P N S L
 GAGAAGGTTGACGACCATGCGCGCCAGCGAAGTATTGCAGAAGTGCTGCCCAACTCACTGT 744
 S G M H A L R E R A L L H A V E A L L H G
 CCGGGATGCATGCGTTCGCGAAGCGCGTGTGCTGCATGCGTTCGAGGCGTTGTTGCACGGA 806
 R R L T L M D I A R S W P S A L R V R A P
 CGCAGGCTGACACTGATGACATCGCACGTTCTGTTGGCCGAGCGCACTGCGGGTGCAGCGGCC 868
 L K A V D R L L S N R N L Q V E R S V I
 GCTCAAGGCAGTTGATCGCCTGTTGAGTAATCGCAATTTGCAGGTCGAGCGATCAGTGATCG 930
 D H E M A H W L L R G A Q P V I V I D W S
 ACCACGAGATGGCGCACTGGCTGCTGCGCGCGCGCAGCCGTAATCGTCATCGACTGGAGC 992
 D L K P D K S W C L L R A A V P V G G R T
 GATCTGAAGCCAGACAAATCGTGGTGTCTGCTGCGCGCAGCGGTGCCGGTGGGCGGCCGAC 1054
 L T L L D M V V P G K Q Q G S P G A E R
 GCTTACCTTGCTGGACATGGTAGTGCCAGGAAAGCAGCAGGGATCGCTGGTGCAGAAAGAC 1116
 R F L Q Q L R A L V P D D V R P I L V T D
 GCTTCTTGCAACAACTGAGGGCACTGGTTCCGGACGATGTTGCCCGGATCCTGGTCACTGAC 1178
 A G F R T P W F R A V S A M G W C W V G R
 GCCGGCTTCCGGACACCGTGGTTCCGCGCTGATCGGCCATGGGCTGGTGTGGGTTGGGCG 1240
 L R G R T Q V K P Q D V R D E A D Q W I
 ACTGCGCGGGCGCACGCAAGTCAAGCCGACGACGTGCGGGATGAAGCAGATCAGTGGATCG 1302
 D S R K L H V L A S N R A C E L P P M Q A
 ACAGCCGGAAGTGCATGTGTTGGCGTCCAATCGTGCATGCGAGTTACCGCCGATGCAGGCC 1364
 N R S D P L D C R L V I Y A K A R Q G R K
 AATCGCAGCGATCCACTCGATTGCAGGTTGGTGATTACGCCAAGGCACGGCAAGGGCGCAA 1426
 Q C N R R S P A K V S R A S S S L K A A
 ACAGTGAATCGCCGCTCACCCGCCAAAGTCTCGCGTGCATCGAGTCTGAAGGCCGACG 1488
 A R E R E P W L I V A S P Q L Q A P S A K
 CGCGCAGAGCGAGCCTTGGCTCATCGTTGCATCCCCGACGTGCAGGCGCCAGCGCAAG 1550
 Q L V N V Y A R R M Q I E L A F R D L K S
 CAGTTGGTTAATGTGTACGCACGACGGATGCAGATCGAGCTTGCAATTTCGCGATCTGAAATC 1612
 H R Y G Q A L E D S L T R R G E R L D T
 ACATCGTACGCGCCAGGCGCTGGAAGACAGTTTGACCCGACGCGCGAGCGACTTGATACGC 1674
 P R V P *
 CCAGGGTTCCGTAGACACTCAAGACCCTCGTTGCGCGTAGCGCGTTCAAACTCTACAGGTG 1736
 ACAGGTCGCCAGTTGAACCGTGGCGCGGTTGGGGTTGTAGAATCTCGATGTAGTCAAT 1798
 ACCTCGGCGGAGCGCGTCTTGGTGGGATAGGTCCCGCCCTGATCCGCTCGCGTTTGG 1860
 CAGGCCGAAGAAGCTCTCCACGGGTGCGTTGTGTTGGCAGTTGCCACGCCGACTCATGCTGC 1922
 ACACCAAGCCATGGGACGCCAGGAACTGCGCCAGTTCATCGTGGTGTAGACAGACCCCTGG 1984
 TCCGAATGAACCAAGCAACAGCGTTGGGTTTGCGCCGCCACCCGAGACAACACCGCTG 2046
 CACGACCAACTCGGTGTGCGCCCGATCGCGCATCGCCAGCCGACGACCTGCCTGGAAAACA 2108
 GATCGATCACAACAGCCAAATACATCCAGCCTTCATGCGTGCAGATGAAGGTGAAATCGCTC 2170
 GC 2172

Fig. 4. Nucleotide and amino acid sequences of the XccR5-89.2 fragment cloned and sequenced from *Xanthomonas campestris* pv. *campestris* race 5.

A 990-bp ORF is shown in the grey highlighted area. The deduced amino acid sequence (329 aa) is shown in the above of the nucleotide sequence. The nucleotide sequence is shown as reverse complement.

Discussion

Black rot disease is one of the major constraints of *Brassica* vegetables including cabbage which causes substantial

yield loss throughout the world. So far, a total of eleven physiological races of *Xcc*, the pathogen of black rot disease have been reported [1, 10–12]. Moreover, Afrin et al. [13] have reported some black rot disease-resistant cabbage

lines, nonetheless, they showed *Xcc* race-specific resistance. Thus, proper detection of *Xcc* races is crucial to manage this disease and to understand its occurrence and evolution. Recently, race-specific molecular markers for detection of *Xcc* race 1, race 3 and race 4 have been reported [22, 23]. The complete genome sequence of some *Xcc* races/strains and subspecies opened the avenue for comparative genome analysis which facilitates us in finding out highly specific and variable genomic regions (Table 2 and Fig. 1).

We developed a molecular marker for rapid, reliable, specific and effective detection of *Xcc* race 5. This marker can specifically and effectively detect and distinguish *Xcc* race 5 among the *Xcc* races/strains, *Xc* subspecies and other plant-infecting bacteria (Figs. 2 and 3). This molecular marker based-PCR detection method is simple, inexpensive, reliable and rapid as compared to the traditional race determination techniques which are very expensive and time consuming. The molecular marker-based pathogen detection technique has successfully been used to identify plant-infecting bacterial and fungal pathogens. Rubel *et al.* [22] reported molecular markers (InDel and SCAR) for detection of *Xcc* race 1 and race 4, the pathogen of black rot disease. Later, Afrin *et al.* [23] also reported molecular markers (InDel and SCAR) for detection of *Xcc* race 3. These molecular markers can quickly and effectively detect *Xcc* races and even from directly infected plants with bio-PCR. Song *et al.* [21] developed a SCAR marker for specific detection of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) race K3a, the causal agent of bacterial blight of rice. A SCAR marker has been reported by Luongo *et al.* [26] to detect the fungal pathogen, *Fusarium oxysporum* f. sp. *melonis* race 2, which causes vascular wilt of cucurbits. Similarly, Lin *et al.* [27] demonstrated PCR-based molecular marker 'Foc-1/Foc-2' which can easily identify *Fusarium oxysporum* f. sp. *cubense* race 4, the pathogen of the panama disease of banana. Two SCAR markers have also been reported for detecting

Fig. 5. Limits of detection of *Xcc* race 5 from genomic DNA by XccR5-89.2 primers.

Lane M: DNA ladder; lane 1, 50 ng/μl gDNA; lane 2, 5 ng/μl; lane 3, 0.5 ng/μl; lane 4, 0.05 ng/μl; lane 5, 0.005 ng/μl; lane 6, negative control. For the PCR amplification, 1 μl DNA of *Xcc* race 5 from each dilutions was used as a template.

Chinese races (CYR32 and CYR33) *Puccinia striiformis* f. sp. *tritici*, the pathogen of stripe rust disease in wheat [28].

The 'XccR5-89.2' marker amplified a similar specific fragment as *Xcc* race 5 from the *Xcc* race-unknown strain ICMP 8 suggesting that it might belong to race 5 (Fig. 3). Moreover, we confirmed this via cloning and sequencing of the band amplified from ICMP8 with the 'XccR589.2' primers. The sequence analysis revealed that the amplified band from ICMP8 was 100% identical to the 'XccR5-89.2' genomic fragment of *Xcc* race 5 (Fig. S2). Rubel *et al.* [22] developed 'Xcc_47R1' and 'Xcc_85R1' markers with a similar technique and demonstrated that those markers can distinguish race 1 from race-unknown *Xcc* strains.

It has been reported that insertion sequences (IS) and transposons are playing a major role in bacterial pathogenicity and evolution including *Xanthomonads* via the genome rearrangement [29]. The sequence analysis of the 'XccR5-89.2' fragment revealed that it has a 990-bp ORF which showed 100% identity with *IS4* family *transposase* of *Xanthomonas campestris*. Further, conserved domain analysis of the protein sequence (329-aa) identified the existence of

Fig. 6. Detection of *Xcc* race 5 by bio-PCR with artificially *Xcc* (races 1–8, R1–R8) inoculated cabbage leaves with XccR5-89.2 primers.

Lanes 1–24, artificially inoculated leaf samples with *Xcc* races (lanes 1–3, race 1; lanes 4–6, race 2; lanes 7–9, race 3; lanes 10–12, race 4; lanes 13–15, race 5; lanes 16–18, race 6; lanes 19–21, race 7; +ve, gDNA of *Xcc* race 5 as a positive control; -ve, negative control; M, DNA ladder).

transposase DDE domain (DDE_Tnp_1) (Fig. S3). This DDE_Tnp_1 domain is essential for effective DNA transposition [30]. This result suggests that this *IS4* family transposase might have a role in the race diversity of *Xcc*.

The 'XccR5-89.2' primers can detect *Xcc* race 5 from a very small quantity of gDNA by PCR amplification indicating the higher sensitivity of the marker (Fig. 5). The novel 'XccR5-89.2' marker developed through this study provides an effective, specific, rapid, easy and powerful tool for detection of race 5 from other *Xcc* races/strains throughout the world.

Recently, a PCR-based technique named as bio-PCR has been shown as an effective method of detecting and identifying plant pathogens, including *Xcc* and *Xoo* directly from infected samples [21–23]. We also applied a similar bio-PCR assay in our study and race 5-specific 'XccR5-89.2' primers simply and efficiently detected and recognized *Xcc* race 5 directly from diseased cabbage leaves (Fig. 6). Therefore, this marker might be convenient for quick detection of *Xcc* race 5 from black rot infected cabbage leaves directly from farmers' fields.

In this study, we developed a molecular marker (XccR5-89.2) specific to *Xcc* race 5 through the realignment of whole genome sequences of 12 bacterial strains including *Xcc* races/strains and subspecies. This novel 'XccR5-89.2' marker detected *Xcc* race 5 from other *Xcc* races/strains/subspecies by PCR quickly with high reliability and sensitivity. To the best of our knowledge, this is the first report for an *Xcc* race 5-specific marker that could be useful for simple, rapid and effective detection of black rot disease at an earlier stage of its development throughout the world.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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