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Development of PCR-Based Molecular Marker for Detection of *Xanthomonas campestris* pv. *campestris* Race 6, the Causative Agent of Black Rot of Brassicas

Khandker Shazia Afrin[1†], Md Abdur Rahim[1,2†], Mehede Hassan Rubel[1], Jong-In Park[1*], Hee-Jeong Jung[1], Hoy-Taek Kim[1], and Ill-Sup Nou [ID][1*]

[1]*Department of Horticulture, Sunchon National University, Suncheon 57922, Korea*
[2]*Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh*

Xanthomonas campestris **pv.** *campestris* **(***Xcc***), the pathogen of black rot which is the most destructive disease of Brassica vegetables throughout the world. Here, we reported two novel sequence-characterized amplified region (SCAR) markers (i.e., XccR6-60 and XccR6-67) for the detection of *Xcc* race 6 via re-alignment of the complete genome sequences of *Xcc* races/strains/pathovars. The specificity of SCAR primer sets was verified by mean of PCR amplification using the genomic DNA template of *Xcc* races/strains/pathovars and two other plant infecting bacterial strains. The PCR result revealed that the XccR6-60 and XccR6-67 primer sets amplified 692-bp and 917-bp DNA fragments, respectively, specifically from race 6, while no visible amplification was detected in other samples. In addition, the SCAR primers were highly sensitive and can detect from a very low concentration of genomic DNA of *Xcc* race 6. However, the complete genome sequence of *Xcc* race 6 is not yet publicly available. Therefore, the cloning and sequencing of XccR6-60 and XccR6-67 fragments from race 6 provide more evidence of the specificity of these markers. These results indicated that the newly developed SCAR markers can successfully, effectively and rapidly detect *Xcc* race 6 from other *Xcc* races/strains/pathovars as well as other plant pathogenic bacteria. This is the first report for race-specific molecular markers for *Xcc* race 6.**

Keywords **:** black rot, cabbage, molecular marker, race 6, *Xanthomonas campestris* pv. *campestris*

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[†]These authors contributed equally to this work.
*Co-corresponding authors.
Jong-In Park
Phone) +82-61-750-3241, FAX) +82-61-750-5389
E-mail) jipark@sunchon.ac.kr
Ill-Sup Nou
Phone) +82-61-750-3249, FAX) +82-61-750-5389
E-mail) nis@sunchon.ac.kr
ORCID
Ill-Sup Nou
https://orcid.org/0000-0001-6768-0520

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Xanthomonas campestris pv. *campestris* (*Xcc*) is a gram negative rod-shaped proteobacterium which causes black rot disease, a global threat to Brassica crops. It can infect from the cultivated species (such as cabbage, cauliflower, broccoli, kohlrabi, Brussels sprouts, kale, mustard, radish, turnip and turnip greens) to the model plant *Arabidopsis*, ornamentals and weeds (An et al., 2011; Soengas et al., 2007; Vicente et al., 2001).

Xcc is a seed born, vascular pathogen which enters the plant through the hydathodes, wounds and insect transmission, and develops typical V-shaped lesions, as well as necrotic and darkened veins (Cook et al., 1952; Williams, 1980). This pathogen can spread through plant debris, rain drops, irrigation, agricultural implements, seed trade and weeds (Arias et al., 2000; Grimm and Vogelsanger, 1989; Lema et al., 2012; Schaad and Alvarez, 1993; Yuen et al., 1987). Black rot significantly reduces the yield (50-65%) and quality of cruciferous vegetables (Saha et al., 2016). Also, under a favorable condition, up to 100% yield loss has been reported in cabbage (Massomo et al., 2003; Williams, 1980). Traditionally, cabbage is one the most important (ranked third) Brassica vegetables in Republic

of Korea and black rot is also identified as a major barrier for cabbage production (Kim, 1986; Park, 2006).

Xcc strains have been classified into different physiological races based on the interactions of pathogens with differential cultivars of *Brassica* species. To date, eleven physiological *Xcc* races have been noticed (Fargier and Manceau, 2007; Kamoun et al., 1992; Vicente et al., 2001). Vicente et al. (2001) isolated six *Xcc* races (race 1, 2, 3, 4, 5, and 6) in the United Kingdom. A total of seven races (race 1, 4, 5, 6, 7, 8, and 9) have been isolated from *Brassica* species in northwestern Spain (Lema et al. 2012). Five races (race 1, 4, 5, 6, and 7) were reported in Nepal while race 1, 4, and 6 were most common (Jensen et al., 2010). Four races (race 1, 3, 4, and 6) have been isolated in South Africa (Chidamba and Bezuidenhout, 2012). In 2017, two new *Xcc* races (race 10 and 11) have been reported in Portugal (Cruz et al., 2017). Among the *Xcc* races, race 1 and 4 are predominant in *Brassica oleracea* subspecies whereas race 6 is predominant in *B. rapa* (Lema et al., 2012; Vicente and Holub, 2013). Recently, race-specific molecular markers for *Xcc* race 1, 3, 4, and 5 have already been reported by our research group (Afrin et al., 2018, 2019; Rubel et al., 2017). In this study, we aimed to develop molecular markers specific to *Xcc* race 6.

Materials and Methods

Bacterial strains and culture media. Eight reference strains of *Xcc* races (races 1-8), eight race-unknown *Xcc* strains, six *Xc* pathovars, and two additional plant infecting-bacteria including *Pseudomonas syringae* pv. *maculicola* and *Pectobacterium carotovora* subsp. *carotovora* (described in our previous study by Afrin et al., 2019) were used in this study. The bacterial strains were cultured on King's medium B at 30°C (48 h) (King et al., 1954).

Isolation of genomic DNA (gDNA). The gDNA of the bacterial strains was isolated with a commercial kit (DNeasy Plant Mini Kit, Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The intensity and purity of the gDNA were determined by using a 'NanoDrop spectrophotometer' (NanoDrop Technologies, Wilmington, DE, USA). Subsequently, the gDNA samples were stored at −20°C refrigerator until use.

Detection of variable genomic regions. The available complete genome sequences of *Xcc* races (races 1, 3, 4, and 9), race-unknown *Xcc* strains and *Xc* pathovars were

Table 1. Publicly available complete genome sequences of *Xcc* races, *Xcc* strains, and *Xc* subspecies, and their accession numbers

Sl. No.	Bacterial strains	GenBank accession	Races	Origin	Host	Disease	Genome size (bp)	Gene	Protein	References
1	B100 (*Xcc*)	AM920689	1	Italy	*Brassica oleracea* var. *botrytis*	Black rot	5,079,002	4,542	4,236	Vorhölter et al. (2008)
2	CFBP1869 (*Xcc*)	NZ_CM002545	1	Côte d'Ivoire	*Brassica oleracea*	Black rot	5,011,687	4,489	4,166	Bolot et al. (2015)
3	Xca5 (*Xcc*)	NZ_HF570389	1	America	Brassicaceae	Black rot	4,916,437	4,486	4,169	Bolot et al. (2013a)
4	ATCC 33913 (*Xcc*)	AE008922	3	-	Cabbage	Black rot	5,076,187	4,240	4,179	da Silva et al. (2002)
5	CFBP 5817(*Xcc*)	NZ_CM002673	4	Chile	*Brassica oleracea* var. *botrytis*	Black rot	4,918,955	4,447	4,122	Bolot et al. (2015)
6	Strain 8004 (*Xcc*)	NC_007086	9	Sussex, UK	Cauliflower	Black rot	5,148,708	4,584	4,297	Qian et al.(2005)
7	ICMP 4013 (*Xcc*)	CP012146	-	New Zealand	Crucifers	Black rot	4,908,383	4,389	4,040	Desai et al. (2015)
8	ICMP 21080 (*Xcc*)	CP012145	-	New Zealand	Crucifers	Black rot	4,911,121	4,389	4,039	Desai et al. (2015)
9	CN14 (*Xcc*)	CM001871	-	Guilin, China	*B. juncea, B. rapa*	Black rot	4,908,255	4,445	4,127	Bolot et al. (2013b)
10	CN15 (*Xcc*)	NZ_CM001890	-	Guilin, China	*B. juncea, B. rapa*	Black rot	4,920,230	4,496	4,162	Bolot et al. (2013b)
11	CN16 (*Xcc*)	NZ_CM001897	-	Guilin, China	*B. juncea, B. rapa*	Black rot	4,928,563	4,496	4,162	Bolot et al. (2013b)
12	Strain 17 (*Xcc*)	CP011946	-	Taiwan	Crucifers	Black rot	4,994,426	4,398	4,086	Liu et al. (2015)
13	CFBP 1606R (*Xci*)	NZ_CM002635	-	France	*Matthiola incana*	Leaf spot	4,967,288	4,423	4,071	Roux et al. (2015)
14	Strain 756C (*Xcr*)	NC_017271	-	-	*Raphanus sativus*	Bacterial spot	4,941,214	4,372	4,055	Bogdanove et al. (2011)
15	Strain 85-10 (*Xcv*)	NC_007508	-	Florida, USA	Pepper	Bacterial spot	5,178,466	4,900	4,799	Thieme et al. (2005)

Xcc, Xanthomonas campestris pv. *campestris*; *Xci, Xanthomonas campestris* pv. *incanae*; *Xcr, Xanthomonas campestris* pv. *raphani*; *Xcv, Xanthomonas campestris* pv. *vesicatoria*.
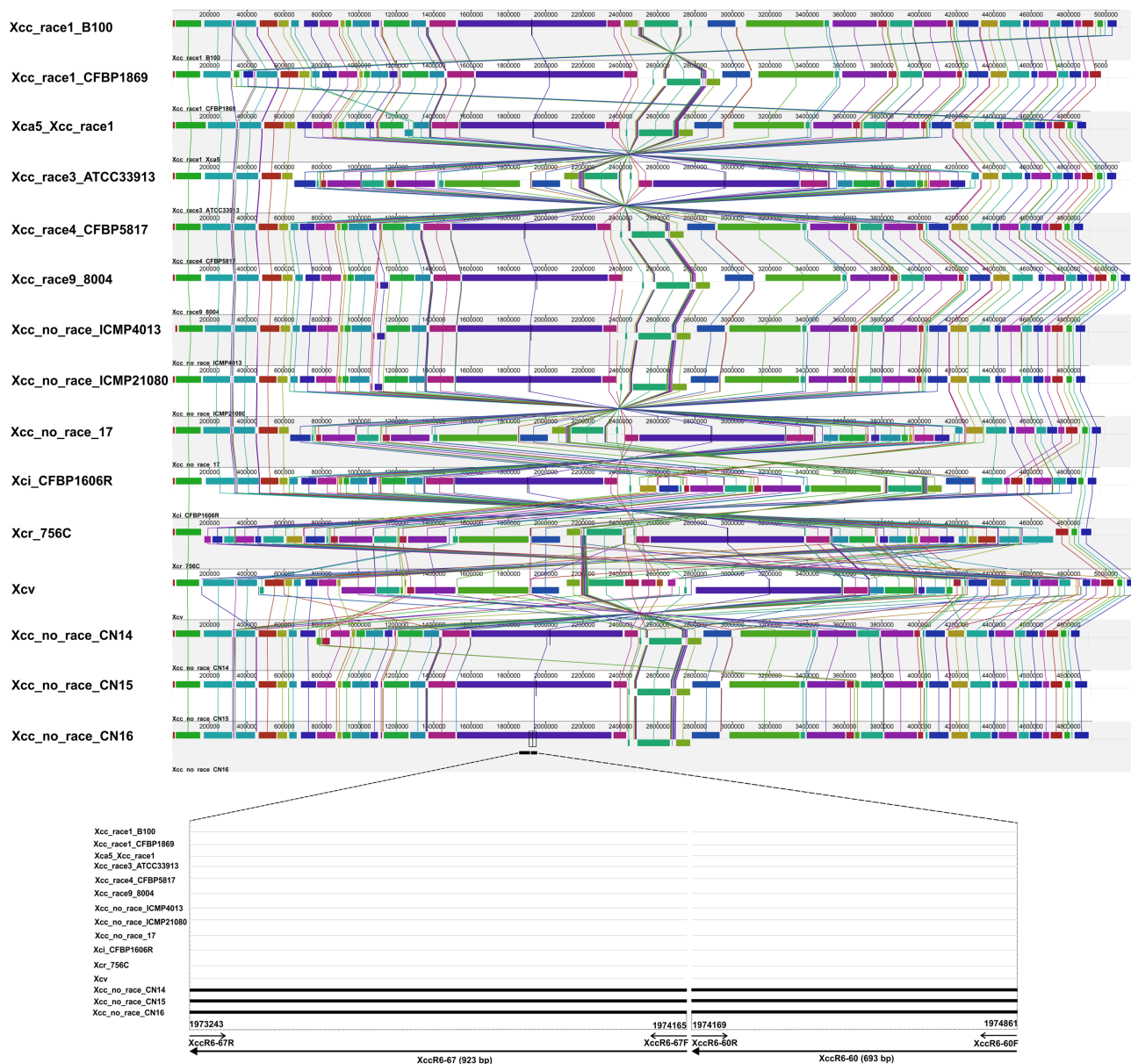
Fig. 1. Whole genome alignment of the publicly available genome sequences of *Xanthomonas campestris* pv. *campestris* (*Xcc*) races, strains and pathovars. The syntenic blocks are connected among the *Xcc* races/strains/*Xc* pathovars. The variable region among the *Xcc* races/strains/*Xc* subspecies are shown with the line diagram in the bottom of the alignment. Genomic blocks are shown as colored rectangles.

retrieved from the NCBI (https://www.ncbi.nlm.nih.gov/genome/) (Table 1). To develop molecular markers, we aligned the complete genome of available *Xcc* races (race 1, 3, 4, and 9), *Xcc* race-unknown strains, and *Xc* pathovars (Table 1) with 'progressiveMauve' tool (Darling et al., 2004). Further, this alignment was analyzed by the trial version of Geneious software program (http://www.geneious.com) and identified variable regions among the aligned genomes (Fig. 1).

PCR amplification. The variable regions among the genomes were considered for primer designing with the 'PCR Primer Stats' (http://www.bioinformatics.org/sms2/pcr_primer_stats.html) (Table 2). Further the designed primers were BLAST-searched (http://210.110.86.160/lab/home.html) to check the primer specificity. The PCR was performed with the bacterial gDNA. PCR reaction volume was total 20 μl consisting of 30 ng of gDNA as a template, 10 pmol of each primer, 0.25 mM dNTP mix, 5× GoTaq buf-

Table 2. List of *Xcc* race 6 specific primers used for in this study

Primer name	Primer sequence (5′ to 3′)	Length (bp)	Annealing conditions
XccR6-60F	TGTAGAATCTTTAGCTGGCGC	693	63°C, 40 s, 22 cycles
XccR6-60R	TTCAATGCATTCCCGACACG		
XccR6-67F	TTCCCATCTGAACACCCATT	923	57°C, 40 s, 25 cycles
XccR6-67R	GGCATCAGTTCAGCGATTAC		

Xcc, *Xanthomonas campestris* pv. *campestris*.

fer, 1 unit of GoTaq DNA Polymerase (Promega, Madison, WI, USA). The thermocycler conditions were as follows: 1× (94°C, 5 min); 22× (94°C, 30 s; 63°C, 40 s; 72°C, 45 s), 1× (72°C, 5 min). Then the PCR products were loaded on 1.5% agarose gel followed by gel electrophoresis and visualized with a gel documentation system. Furthermore, the sensitivity of the sequence-characterized amplified region (SCAR) primer sets was determined by using a dilution series (50 ng/μl, 5 ng/μl, 0.5 ng/μl, 0.05 ng/μl, and 0.005 ng/μl) of *Xcc* race 6 gDNA.

Cloning and sequencing. The genomic fragments (XccR6-60 and XccR6-67) specific to *Xcc* race 6 were amplified by PCR and purified with the Wizard SV gel and PCR cleanup system (Promega). Then, the purified PCR products were cloned with TOP cloner blunt kit (Enzynomics, Daejeon, Korea) following the manufacturer's instructions. The single colonies were checked by colony PCR and only positive clones were grown in a liquid LB media for overnight. Subsequently, the plasmid DNA was isolated and purified with a GeneAll kit (Seoul, Korea). The plasmid DNA samples were sequenced by ABI 3730XL DNA sequencer (Macrogen Inc., Seoul, Korea) using universal primers. The sequence alignment was carried out with the Clustal Omega online tool (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Bio-PCR analysis. Three-week-old cabbage plants were inoculated with eight reference races of *Xcc* (race 1-8) according to the method described by Afrin et al. (2018). Upon visible symptom of black rot, about 1 cm^2 infected area of inoculated leaf was collected and sliced into small pieces. Thereafter, sliced pieces of leaf were placed in 250 μl sterile water (40 min) and 10 μl of the soaked water was used as a template for the bio-PCR.

Results

Marker development. The availability of the complete genome sequences of *Xcc* races, *Xcc* strains, *Xc* pathovars and their re-alignment enabled us to develop *Xcc* race

specific molecular markers. In this study, we developed two SCAR markers XccR6-60 and XccR6-67, for rapid detection of race 6 from other *Xcc* races (Fig. 1). Since, the complete genome sequence of *Xcc* race 6 is not available yet, we analyzed the variable genomic regions among the aligned genomes. The result showed that the XccR6-60 and XccR6-67 fragments were specific to three race-unknown *Xcc* strains, including CN14, CN15, and CN16 (Fig. 1) and absent in the remaining genomes in the alignment. In the next step, we designed two sets of primers on XccR6-60 (693-bp) and XccR6-67 (923-bp) genomic fragments considering CN14, CN15, and CN16 as reference genome and amplified by the PCR.

The PCR analysis revealed that the XccR6-60 and XccR6-67 SCAR primer sets amplified about 692-bp and 917-bp fragments, respectively only from the gDNA of *Xcc* race 6 while no amplification was observed for the remaining tested samples (Fig. 2, Supplementary Fig. 1). Moreover, the sensitivity test of the SCAR primer sets revealed that they were sensitive enough to detect race 6 by PCR from total 0.05-0.5 ng template gDNA (Fig. 3).

Further, we cloned and sequenced the bands amplified by the SCAR primers. The sequence analysis showed that XccR6-60 primer set amplified fragment with 692-bp from *Xcc* race 6 (Supplementary Fig. 2A). Only 1-bp deletion at 515-bp position and two single nucleotide polymorphisms (SNPs) compared to the reference (CN14, CN15, and CN16). For XccR6-67, 6-bp deletions at 241-bp position 14 SNPs compared to the reference (CN14, CN15, and CN16) (Supplementary Fig. 2B).

The sequence obtained through the cloning of XccR6-60 and XccR6-67 fragments from *Xcc* race 6 was subjected to open reading frame (ORF) prediction with the ORF Finder tool of NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). The results indicated that the XccR6-60 and XccR6-67 fragments contained 381-bp (129-aa) and 720-bp (239-aa) ORF, respectively (Fig. 4, Supplementary Figs. 3 and 4). Thereafter, BLASTp search was performed using these proteins in the NCBI. The result showed that 129-aa protein sequence of XccR6-60 had a significant homology (99% identity) with DUF4189 domain-containing protein

Fig. 2. Gel electrophoresis (1.5% agarose) of the PCR products of *Xanthomonas campestris* pv. *campestris* (*Xcc*) race 6 specific se-quence-characterized amplified region primer sets (A, XccR6-60, 692-bp; B, XccR6-67, 917-bp) amplified from genomic DNA of *Xcc* races and pathovars. Lane M, 100 bp plus DNA ladder; lanes 1-8, *Xcc* race 1-8; lane *Xci*, *X. campestris* pv. *incane* (*Xci*); lane *Xcr*, *X. campestris* pv. *raphanin* (*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 *Pcc*, *Pectobacterium carotovora* subsp. *carotovora* (*Pcc*).
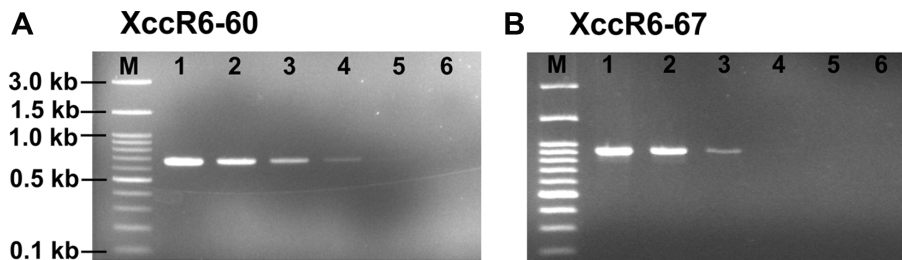

Fig. 3. Sensitivity of the *Xanthomonas campestris* pv. *campestris* (*Xcc*) race 6 specific SCAR primers (XccR6-60 and XccR6-67). (A) XccR6-60. (B) XccR6-67. Lane M, 100 bp plus DNA ladder; lane 1, 50 ng/μl gDNA; lane 2, 5 ng/μl; lane 3, 0.5 ng/μl; lane 4, 0.05 ng/μl; lane 5, 0.005 ng/μl; lane 6, negative control. For the PCR amplification, 1 μl DNA of *Xcc* race 5 from each dilutions was used as a template.

of *X. campestris* (WP_076054737.1). In case of XccR6-67, 239-aa protein sequence had a significant homology with a hypothetical protein of *X. campestris* (WP_076054735.1) (Supplementary Figs. 3 and 4). In addition, the conserved domain analysis using these proteins revealed that the 129-aa protein of XccR6-60 contained a conserved domain called 'Domain of unknown function (DUF4189)' while 239-aa protein of XccR6-67 contained a 'TrbL domain-containing protein' (WP_076054735.1) (Supplementary Figs. 3 and 4).

Bio-PCR detection from infected cabbage leaves. A bio-PCR was performed with artificially infected cabbage leaves with reference *Xcc* races (races 1-8) to detect the po-tentiality of race 6 specific primers (XccR6-60 and XccR6-67). The visible bands were only for those cabbage leaves inoculated with *Xcc* race 6 for both primer sets, while un-detected for rest of the samples inoculated with other races (Fig. 5).

Discussion

The present study was carried out to develop molecular marker for race-specific, rapid and precise detection of *Xcc* race 6 from other *Xcc* races/strains/pathovars. The new races of *Xcc* are emerging continuously in different bras-
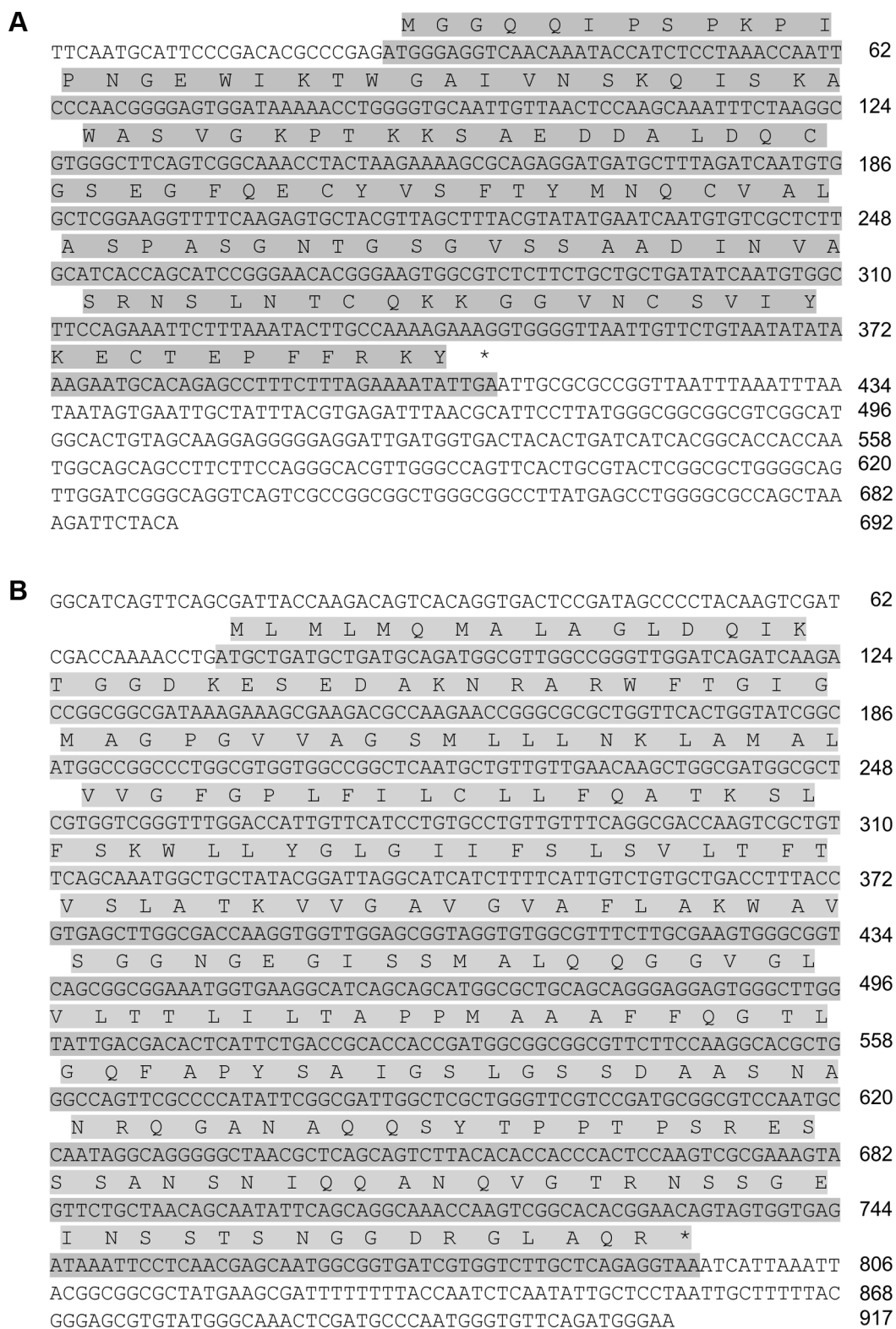
A

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                        M  G  G  Q  Q  I  P  S  P  K  P  I
TTCAATGCATTCCCGACACGCCCGAGATGGGAGGTCAACAAATACCATCTCCTAAACCAATT      62
   P  N  G  E  W  I  K  T  W  G  A  I  V  N  S  K  Q  I  S  K  A
CCCAACGGGGAGTGGATAAAAACCTGGGGTGCAATTGTTAACTCCAAGCAAATTTCTAAGGC     124
    W  A  S  V  G  K  P  T  K  K  S  A  E  D  D  A  L  D  Q  C
GTGGGCTTCAGTCGGCAAACCTACTAAGAAAAGCGCAGAGGATGATGCTTTAGATCAATGTG     186
 G  S  E  G  F  Q  E  C  Y  V  S  F  T  Y  M  N  Q  C  V  A  L
GCTCGGAAGGTTTTCAAGAGTGCTACGTTAGCTTTACGTATATGAATCAATGTGTCGCTCTT     248
 A  S  P  A  S  G  N  T  G  S  G  V  S  S  A  A  D  I  N  V  A
GCATCACCAGCATCCGGGAACACGGGAAGTGGCGTCTCTTCTGCTGCTGATATCAATGTGGC     310
    S  R  N  S  L  N  T  C  Q  K  K  G  G  V  N  C  S  V  I  Y
TTCCAGAAATTCTTTAAATACTTGCCAAAAGAAAGGTGGGGTTAATTGTTCTGTAATATATA     372
K  E  C  T  E  P  F  F  R  K  Y     *
AAGAATGCACAGAGCCTTTCTTTAGAAAATATTGAATTGCGCGCCGGTTAATTTAAATTTAA     434
TAATAGTGAATTGCTATTTACGTGAGATTTAACGCATTCCTTATGGGCGGCGGCGTCGGCAT     496
GGCACTGTAGCAAGGAGGGGGAGGATTGATGGTGACTACACTGATCATCACGGCACCACCAA     558
TGGCAGCAGCCTTCTTCCAGGGCACGTTGGGCCAGTTCACTGCGTACTCGGCGCTGGGGCAG     620
TTGGATCGGGCAGGTCAGTCGCCGGCGGCTGGGCGGCCTTATGAGCCTGGGGCGCCAGCTAA     682
AGATTCTACA                                                        692
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B

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GGCATCAGTTCAGCGATTACCAAGACAGTCACAGGTGACTCCGATAGCCCCTACAAGTCGAT      62
              M  L  M  L  M  Q  M  A  L  A  G  L  D  Q  I  K
CGACCAAAACCTGATGCTGATGCTGATGCAGATGGCGTTGGCCGGGTTGGATCAGATCAAGA     124
T  G  G  D  K  E  S  E  D  A  K  N  R  A  R  W  F  T  G  I  G
CCGGCGGCGATAAAGAAAGCGAAGACGCCAAGAACCGGGCGCGCTGGTTCACTGGTATCGGC     186
 M  A  G  P  G  V  V  A  G  S  M  L  L  L  N  K  L  A  M  A  L
ATGGCCGGCCCTGGCGTGGTGGCCGGCTCAATGCTGTTGTTGAACAAGCTGGCGATGGCGCT     248
  V  V  G  F  G  P  L  F  I  L  C  L  L  F  Q  A  T  K  S  L
CGTGGTCGGGTTTGGACCATTGTTCATCCTGTGCCTGTTGTTTCAGGCGACCAAGTCGCTGT     310
F  S  K  W  L  L  Y  G  L  G  I  I  F  S  L  S  V  L  T  F  T
TCAGCAAATGGCTGCTATACGGATTAGGCATCATCTTTTCATTGTCTGTGCTGACCTTTACC     372
 V  S  L  A  T  K  V  V  G  A  V  G  V  A  F  L  A  K  W  A  V
GTGAGCTTGGCGACCAAGGTGGTTGGAGCGGTAGGTGTGGCGTTTCTTGCGAAGTGGGCGGT     434
   S  G  G  N  G  E  G  I  S  S  M  A  L  Q  Q  G  G  V  G  L
CAGCGGCGGAAATGGTGAAGGCATCAGCAGCATGGCGCTGCAGCAGGGGAGGAGTGGGCTTGG     496
V  L  T  T  L  I  L  T  A  P  P  M  A  A  A  F  Q  G  T  L
TATTGACGACACTCATTCTGACCGCACCACCGATGGCGGCGGCGTTCTTCCAAGGCACGCTG     558
 G  Q  F  A  P  Y  S  A  I  G  S  L  G  S  S  D  A  A  S  N  A
GGCCAGTTCGCCCCATATTCGGCGATTGGCTCGCTGGGTTCGTCCGATGCGGCGTCCAATGC     620
  N  R  Q  G  A  N  A  Q  Q  S  Y  T  P  P  T  P  S  R  E  S
CAATAGGCAGGGGGCTAACGCTCAGCAGTCTTACACACCACCCACTCCAAGTCGCGAAAGTA     682
S  S  A  N  S  N  I  Q  Q  A  N  Q  V  G  T  R  N  S  S  G  E
GTTCTGCTAACAGCAATATTCAGCAGGCAAACCAAGTCGGCACACGGAACAGTAGTGGTGAG     744
 I  N  S  S  T  S  N  G  G  D  R  G  L  A  Q  R     *
ATAAATTCCTCAACGAGCAATGGCGGTGATCGTGGTCTTGCTCAGAGGTAAATCATTAAATT     806
ACGGCGGCGCTATGAAGCGATTTTTTTTTACCAATCTCAATATTGCTCCTAATTGCTTTTTAC     868
GGGAGCGTGTATGGGCAAACTCGATGCCCAATGGGTGTTCAGATGGGAA                 917
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Fig. 4. Nucleotide and amino acid sequences of the XccR6-60 (A) and XccR6-67 (B) genomic fragments cloned and sequenced from *Xanthomonas campestris* pv. *campestris* race 6. The open reading frames are presented in grey highlighted area: XccR6-60, 381-bp and XccR6-67, 720-bp. The deduced amino acid sequences are shown in the above of the nucleotide sequences: XccR6-60, 126-aa and XccR6-67, 239-aa. The nucleotide sequence is shown as reverse complement.
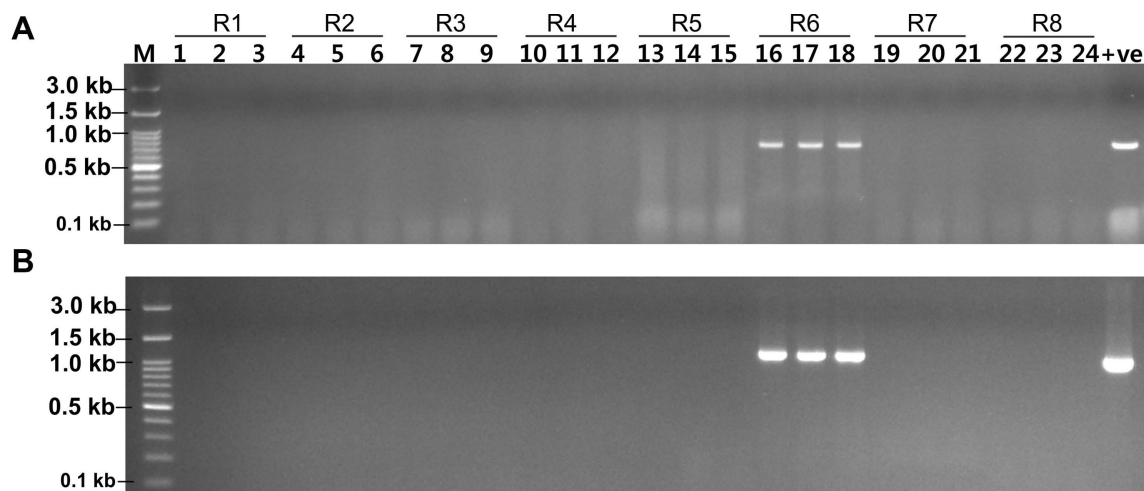
Fig. 5. Detection of *Xanthomonas campestris* pv. *campestris* (*Xcc*) race 6 by bio-PCR with artificially *Xcc* (races 1-8, R1-R8) inoculated cabbage leaves with XccR6-60 (A) and XccR6-67 (B) primer sets. Lanes 1-24, artificially inoculated leaf samples with *Xcc* races (lanes: 1-3, race 1; 4-6, race 2; 7-9, race 3; 10-12, race 4; 13-15, race 5; 16-18, race 6; 19-21, race 7; 22-24, race 8; +ve, gDNA of *Xcc* race 5 as a positive control; –ve, negative control; M, 100 bp plus DNA ladder).

sica vegetable growing regions of the world. For example, five physiological races of *Xcc* (0-4) have been reported by Kamoun et al. (1992) based on differential interactions with different *Brassica* spp. Thereafter, Vicente et al. (2001) noticed six races of *Xcc* (race 1-6). A total of nine *Xcc* races have been reported by Fargier and Manceau (2007). More recently, Cruz et al. (2017) identified two new races of *Xcc* (race 10 and race 11). These races were determined by traditional method with the differential cultivars and the pathogens. Korean *Xcc* isolates need to be determined their race identity. However, the cultivar-based race determination method is more expensive and time consuming. Therefore, PCR-based simple, reliable and race-specific detect is crucial for the race determination of Korean isolates. So far, our research group have developed molecular markers for race-specific detection of *Xcc* race 1, 3, 4, and 5 (Afrin et al., 2018, 2019; Rubel et al., 2017).

In this study, we developed PCR-based SCAR markers (XccR6-60 and XccR6-67) specific to *Xcc* race 6 through re-alignment of available complete genome sequences of fifteen *Xcc* races/strains/pathovars (Fig. 1). As the complete genome sequence of *Xcc* race 6 is not available yet, the variable genomic regions of known *Xcc* genome sequences were considered as targets to develop the race 6-specific molecular markers. A similar approach was recently reported by Afrin et al. (2019) to develop *Xcc* race 5-specific molecular marker whose complete genome is not yet sequenced. The SCAR markers developed in this study were able to detect and identify *Xcc* race 6 from other

Xcc races/strains/pathovars (Fig. 2, Supplementary Fig. 1). Further, these markers can detect *Xcc* race 6 from very low concentration of race 6 gDNA templates (total 0.05-0.5 ng) (Fig. 3). Moreover, the newly developed primer sets (XccR6-60 and XccR6-67) were highly sensitive as they were capable to detect race 6 directly from infected cabbage leaves within few hours by bio-PCR (Fig. 5). This suggests that newly developed SCAR markers provide a simple, specific, quick, low-cost and powerful method for identification of *Xcc* race 6. A similar bio-PCR technique was reported by Song et al. (2014) for *X. oryzae* pv. *oryzae* (*Xoo*) race K3a and by Afrin et al. (2018) for *Xcc* race 3. In the past, PCR-based molecular markers were successfully used for pathovar-specific detection of *Xanthomonas*. For example, *hrp* gene (hypersensitive reaction and pathogenicity) based PCR amplification technique has been used to distinguish *X. campestris* pathovars (Berg et al., 2005; Singh et al., 2014) and *X. axonopodis* pv. *vesicatoria* (Leite et al., 1994). Later, *hrcC* gene (encoding the outer membrane protein of the type III secretion system) based PCR assay for *Xcc* specific detection was also described (Zaccardelli et al., 2007). Moreover, Song et al. (2014) reported a SCAR marker for *Xoo* K3a race-specific detection. In addition, PCR-based molecular markers have also been reported for distinguishing fungal pathogens like *Fusarium oxysporum* f. sp. *melonis* race 2 and *F. oxysporum* f. sp. *cubense* race 4, which cause vascular wilt and panama disease in cucurbits and banana, respectively (Lin et al., 2009; Luongo et al., 2012). Wang et al. (2010) reported SCAR

Table 3. The results of NCBI BLAST search with the *Xcc* race 6 specific genomic fragments

Cloned fragments of *Xcc* race6	CN14		CN15		CN16	
	Identity (%)	E-value	Identity (%)	E-value	Identity (%)	E-value
XccR6-60	99.57	0.0	99.71	0.0	99.57	0.0
XccR6-67	97.72	0.0	97.72	0.0	97.72	0.0

Xcc, *Xanthomonas campestris* pv. *campestris*.

markers for race-specific detection of *Puccinia striiformis* f. sp. *tritici* Chinese races (CYR32 and CYR33), the causal agent of stripe rust of wheat.

The cloning and sequence analysis of XccR6-60 and XccR6-67 marker regions revealed that they have a considerably high homology with DUF4189 domain-containing protein (WP_076054737.1) and hypothetical protein (WP_076054735.1) of *X. campestris,* respectively. Further, domain analysis using protein sequence of XccR6-67 (239-aa) revealed that presence of TrbL domain (TrbL/VirB6 plasmid conjugal transfer protein, pfam04610) (Supplementary Fig. 4). VirB6 was reported as a core component of type IV secretion system (T4SS), which is involved in macromolecular translocation (Villamil Giraldo et al., 2012). Moreover, it has been reported that VirB6 domains direct the ordered export of a DNA substrate via the T4SS (Jakubowski et al., 2004).

The genomic fragments XccR6-60 and XccR6-67 were specific to race-unknown *Xcc* strain CN14, CN15, and CN16 but were not present in the other genomes analyzed (Fig. 1). Nevertheless, the primer sets designed to cover these regions were PCR amplified only from *Xcc* race 6 (Fig. 2). Besides, the NCBI BLAST search of the cloned sequence of XccR6-60 and XccR6-67 fragments from *Xcc* race 6 showed more than 99% and 97%, respectively identity with three race-unknown *Xcc* strains, CN14, CN15, and CN16 (Table 3). Therefore, it can be anticipated that these race-unknown *Xcc* strain (CN14, CN15, and CN16) might belong to race 6. However, more experimental evidences are required to support this hypothesis.

In conclusion, the newly developed SCAR markers (XccR6-60 and XccR6-67) could be useful for rapid, low-cost and reliable detection of race 6 from other *Xcc* races/strains/pathovars. Thus, these molecular markers would be valuable for early detection of this pathogen from infected Brassica crops worldwide.

Acknowledgments

Electronic Supplementary Material

Supplementary materials are available at Plant Pathology Journal website (http://www.ppjonline.org/).

References

Afrin, K. S., Rahim, M. A., Jung, H.-J., Park, J.-I., Kim, H.-T. and Nou, I.-S. 2019. Development of molecular marker through genome realignment for specific detection of *Xanthomonas campestris* pv. *campestris* race 5, the pathogen of black rot disease. *J. Microbiol. Biotechnol.* 29:785-793.

Afrin, K. S., Rahim, M. A., Rubel, M. H., Natarajan, S., Song, J.-Y., Kim, H.-T., Park, J.-I. and Nou, I.-S. 2018. Development of race-specific molecular marker for *Xanthomonas campestris* pv. *campestris* race 3, the causal agent of black rot of crucifers. *Can. J. Plant Sci.* 98:1119-1125.

An, S.-Q., Lu, G.-T., Su, H.-Z., Li, R.-F., He, Y.-Q., Jiang, B.-L., Tang, D.-J. and Tang, J.-L. 2011. Systematic mutagenesis of all predicted *gntR* genes in *Xanthomonas campestris* pv. *campestris* reveals a GntR family transcriptional regulator controlling hypersensitive response and virulence. *Mol. Plant.-Microbe. Interact.* 24:1027-1039.

Arias, R. S., Nelson, S. C. and Alvarez, A. M. 2000. Effect of soil-matric potential and phylloplanes of rotation-crops on the survival of a bioluminescent *Xanthomonas campestris* pv. *campestris*. *Eur. J. Plant Pathol.* 106:109-116.

Berg, T., Tesoriero, L. and Hailstones, D. L. 2005. PCR-based detection of *Xanthomonas campestris* pathovars in Brassica seed. *Plant Pathol.* 54:416-427.

Bogdanove, A. J., Koebnik, R., Lu, H., Furutani, A., Angiuoli, S. V, Patil, P. B., Van Sluys, M.-A., Ryan, R. P., Meyer, D. F., Han, S.-W., Aparna, G., Rajaram, M., Delcher, A. L., Phillippy, A. M., Puiu, D., Schatz, M. C., Shumway, M., Sommer, D. D., Trapnell, C., Benahmed, F., Dimitrov, G., Madupu, R., Radune, D., Sullivan, S., Jha, G., Ishihara, H., Lee, S.-W., Pandey, A., Sharma, V., Sriariyanun, M., Szurek, B., Vera-

Cruz, C. M., Dorman, K. S., Ronald, P. C., Verdier, V., Dow, J. M., Sonti, R. V., Tsuge, S., Brendel, V. P., Rabinowicz, P. D., Leach, J. E., White, F. F. and Salzberg, S. L. 2011. Two new complete genome sequences offer insight into host and tissue specificity of plant pathogenic *Xanthomonas* spp. *J. Bacteriol.* 193:5450-5464.

Bolot, S., Cerutti, A., Carrère, S., Arlat, M., Fischer-Le Saux, M., Portier, P., Poussier, S., Jacques, M.-A. and Noël, L. D. 2015. Genome sequences of the Race 1 and Race 4 *Xanthomonas campestris* pv. *campestris* strains CFBP 1869 and CFBP 5817. *Genome Announc.* 3:e01023-15.

Bolot, S., Guy, E., Carrere, S., Barbe, V., Arlat, M. and Noël, L. D. 2013a. Genome sequence of *Xanthomonas campestris* pv. *campestris* strain Xca5. *Genome Announc.* 1:e00032-12.

Bolot, S., Roux, B., Carrere, S., Jiang, B.-L., Tang, J.-L., Arlat, M. and Noël, L. D. 2013b. Genome sequences of three atypical *Xanthomonas campestris* pv. *campestris* strains, CN14, CN15, and CN16. *Genome Announc.* 1:e00465-13.

Chidamba, L. and Bezuidenhout, C. C. 2012. Characterisation of *Xanthomonas campestris* pv. *campestris* isolates from South Africa using genomic DNA fingerprinting and pathogenicity tests. *Eur. J. Plant Pathol.* 133:811-818.

Cook, A. A., Walker, J. C. and Larson, R. H. 1952. Studies on the disease cycle of black rot of crucifers. *Phytopathology* 42:162-167.

Cruz, J., Tenreiro, R. and Cruz, L. 2017. Assessment of diversity of *Xanthomonas campestris* pathovars affecting cruciferous plants in Portugal and disclosure of two novel *X. campestris* pv. *campestris* races. *J. Plant Pathol.* 99:403-414.

da Silva, A. C. R., Ferro, J. A., Reinach, F. C., Farah, C. S., Furlan, L. R., Quaggio, R. B., Monteiro-Vitorello, C. B., Van Sluys, M. A., Almeida, N. F., Alves, L. M. C., do Amaral, A. M., Bertolini, M. C., Camargo, L. E. A., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Ciapina, L. P., Cicarelli, R. M. B., Coutinho, L. L., Cursino-Santos, J. R., El-Dorry, H., Faria, J. B., Ferreira, A. J. S., Ferreira, R. C. C., Ferro, M. I. T., Formighieri, E. F., Franco, M. C., Greggio, C. C., Gruber, A., Katsuyama, A. M., Kishi, L. T., Leite, R. P., Lemos, E. G. M., Lemos, M. V. F., Locali, E. C., Machado, M. A., Madeira, A. M. B. N., Martinez-Rossi, N. M., Martins, E. C., Meidanis, J., Menck, C. F. M., Miyaki, C. Y., Moon, D. H., Moreira, L. M., Novo, M. T. M., Okura, V. K., Oliveira, M. C., Oliveira, V. R., Pereira, H. A., Rossi, A., Sena, J. A. D., Silva, C., de Souza, R. F., Spinola, L. A. F., Takita, M. A., Tamura, R. E., Teixeira, E. C., Tezza, R. I. D., Trindade dos Santos, M., Truffi, D., Tsai, S. M., White, F. F., Setubal, J. C. and Kitajima, J. P. 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* 417:459-463.

Darling, A. C. E., Mau, B., Blattner, F. R. and Perna, N. T. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. G*enome Res.* 14:1394-1403.

Desai, D., Li, J.-H., van Zijll de Jong, E., Braun, R., Pitman, A., Visnovsky, S., Hampton, J. and Christey, M. 2015. Draft genome sequences of two New Zealand *Xanthomonas camp-*

estris pv. *campestris* isolates, ICMP 4013 and ICMP 21080. *Genome Announc.* 3:e01247-15.

Fargier, E. and Manceau, C. 2007. Pathogenicity assays restrict the species *Xanthomonas campestris* into three pathovars and reveal nine races within *X. campestris* pv. *campestris*. *Plant Pathol.* 56:805-818.

Grimm, R. and Vogelsanger, J. 1989. Black rot disease on cabbage, irrigation and spreading. In: *Proceeding of the 7th International Conference on Plant Pathogenic Bacteria*, ed. by Z. Klement, pp. 225-229. Akademiai Kiado, Budapest, Hungary.

Jakubowski, S. J., Krishnamoorthy, V., Cascales, E. and Christie, P. J. 2004. *Agrobacterium tumefaciens* VirB6 domains direct the ordered export of a DNA substrate through a type IV secretion system. *J. Mol. Biol.* 341:961-977.

Jensen, B. D., Vicente, J. G., Manandhar, H. K. and Roberts, S. J. 2010. Occurrence and diversity of *Xanthomonas campestris* pv. *campestris* in vegetable *Brassica* fields in Nepal. *Plant Dis.* 94:298-305.

Kamoun, S., Kamdar, H. V., Tola, E. and Kado, C. I. 1992. Incompatible interactions between crucifers and *Xanthomonas campestris* involve a vascular hypersensitive response: role of the hrpK locus. *Mol. Plant-Microbe Interact.* 5:22-33.

Kim, B. S. 1986. Testing for detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds and seed disinfection. *Korean J. Plant Pathol.* 2:96-101.

King, E. O., Ward, M. K. and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *J. Lab. Clin. Med.* 44:301-307.

Leite, R. P. Jr., Minsavage, G. V., Bonas, U. and Stall, R. E. 1994. Detection and identification of phytopathogenic *Xanthomonas* strains by amplification of DNA sequences related to the hrp genes of *Xanthomonas campestris* pv. *vesicatoria*. *Appl. Environ. Microbiol.* 60:1068-1077.

Lema, M., Cartea, M. E., Sotelo, T., Velasco, P. and Soengas, P. 2012. Discrimination of *Xanthomonas campestris* pv. *campestris* races among strains from northwestern Spain by *Brassica* spp. genotypes and rep-PCR. *Eur. J. Plant Pathol.* 133:159-169.

Lin, Y.-H., Chang, J.-Y., Liu, E.-T., Chao, C.-P., Huang, J.-W. and Chang, P.-F. L. 2009. Development of a molecular marker for specific detection of *Fusarium oxysporum* f. sp. *cubense* race 4. *Eur. J. Plant Pathol.* 123:353-365.

Liu, Y.-C., Wang, S.-C., Yu, Y.-J., Fung, K.-M., Yang, M.-T., Tseng, Y.-H., Tsai, S.-F., Sun, H. S., Lyu, P.-C. and Chou, S.-H. 2015. Complete genome sequence of *Xanthomonas campestris* pv. *campestris* strain 17 from Taiwan. *Genome Announc.* 3:e01466-15.

Luongo, L., Vitale, S., Haegi, A. and Belisario, A. 2012. Development of SCAR markers and PCR assays for *Fusarium oxysporum* f. sp. *melonis* race 2-specific detection. *J. Plant Pathol.* 94:193-199.

Massomo, S. M. S., Nielsen, H., Mabagala, R. B., Mansfeld-Giese, K., Hockenhull, J. and Mortensen, C. N. 2003. Identification and characterisation of *Xanthomonas campestris*

pv. *campestris* strains from Tanzania by pathogenicity tests, Biolog, rep-PCR and fatty acid methyl ester analysis. *Eur. J. Plant Pathol.* 109:775-789.

Park, H. G. 2006. Genetical improvement of *Brassica* in Korea. *Acta Hortic.* 706:31-48.

Qian, W., Jia, Y., Ren, S.-X., He, Y.-Q., Feng, J.-X., Lu, L.-F., Sun, Q., Ying, G., Tang, D.-J., Tang, H., Wu, W., Hao, P., Wang, L., Jiang, B.-L., Zeng, S., Gu, W.-Y., Lu, G., Rong, L., Tian, Y., Yao, Z., Fu, G., Chen, B., Fang, R., Qiang, B., Chen, Z., Zhao, G.-P., Tang, J.-L. and He, C. 2005. Comparative and functional genomic analyses of the pathogenicity of phytopathogen *Xanthomonas campestris* pv. *campestris*. *Genome Res.* 15:757-767.

Roux, B., Bolot, S., Guy, E., Denancé, N., Lautier, M., Jardinaud, M.-F., Fischer-Le Saux, M., Portier, P., Jacques, M.-A., Gagnevin, L., Pruvost, O., Lauber, E., Arlat, M., Carrère, S., Koebnik, R. and Noël, L. D. 2015. Genomics and transcriptomics of *Xanthomonas campestris* species challenge the concept of core type III effectome. *BMC Genomics* 16:975.

Rubel, M. H., Robin, A. H. K., Natarajan, S., Vicente, J. G., Kim, H.-T., Park, J.-I. and Nou, I.-S. 2017. Whole-genome realignment facilitates development of specific molecular markers for races 1 and 4 of *Xanthomonas campestris* pv. *campestris*, the cause of black rot disease in *Brassica oleracea*. *Int. J. Mol. Sci.* 18:2523.

Saha, P., Kalia, P., Sharma, M. and Singh, D. 2016. New source of black rot disease resistance in *Brassica oleracea* and genetic analysis of resistance. *Euphytica* 207:35-48.

Schaad, N. W. and Alvarez, A. 1993. *Xanthomonas campestris* pv. *campestris*: cause of black rot of crucifers. In: *Xanthomonas*, eds. by J. G. Swings and E. L. Civerolo, pp. 51-55. Chapman and Hall, London, UK.

Singh, D., Raghavendra, B. T., Rathaur Singh, P., Singh, H., Raghuwanshi, R. and Singh, R. P. 2014. Detection of black rot disease causing pathogen *Xanthomonas campestris* pv. *campestris* by bio-PCR from seeds and plant parts of cole crops. *Seed Sci. Technol.* 42:36-46.

Soengas, P., Hand, P., Vicente, J. G., Pole, J. M. and Pink, D. A. C. 2007. Identification of quantitative trait loci for resistance to *Xanthomonas campestris* pv. *campestris* in *Brassica rapa*. *Theor. Appl. Genet.* 114:637-645.

Song, E.-S., Kim, S.-Y., Noh, T.-H., Cho, H., Chae, S.-C. and Lee, B.-M. 2014. PCR-based assay for rapid and specific detection of the new *Xanthomonas oryzae* pv. *oryzae* K3a race using an AFLP-derived marker. *J. Microbiol. Biotechnol.* 24:732-739.

Thieme, F., Koebnik, R., Bekel, T., Berger, C., Boch, J., Büttner, D., Caldana, C., Gaigalat, L., Goesmann, A., Kay, S., Kirchner, O., Lanz, C., Linke, B., McHardy, A. C., Meyer, F., Mittenhuber, G., Nies, D. H., Niesbach-Klösgen, U., Patschkowski, T., Rückert, C., Rupp, O., Schneiker, S., Schuster, S. C., Vorhölter, F.-J., Weber, E., Pühler, A., Bonas, U., Bartels, D. and Kaiser, O. 2005. Insights into genome plasticity and pathogenicity of the plant pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* revealed by the complete genome sequence. *J. Bacteriol.* 187:7254-7266.

Vicente, J. G., Conway, J., Roberts, S. J. and Taylor, J. D. 2001. Identification and origin of *Xanthomonas campestris* pv. *campestris* races and related pathovars. *Phytopathology* 91:492-499.

Vicente, J. G. and Holub, E. B. 2013. *Xanthomonas campestris* pv. *campestris* (cause of black rot of crucifers) in the genomic era is still a worldwide threat to brassica crops. *Mol. Plant Pathol.* 14:2-18.

Villamil Giraldo, A. M., Sivanesan, D., Carle, A., Paschos, A., Smith, M. A., Plesa, M., Coulton, J. and Baron, C. 2012. Type IV secretion system core component VirB8 from Brucella binds to the globular domain of VirB5 and to a periplasmic domain of VirB6. *Biochemistry* 51:3881-3890.

Vorhölter, F.-J., Schneiker, S., Goesmann, A., Krause, L., Bekel, T., Kaiser, O., Linke, B., Patschkowski, T., Rückert, C., Schmid, J., Sidhu, V. K., Sieber, V., Tauch, A., Watt, S. A., Weisshaar, B., Becker, A., Niehaus, K. and Pühler, A. 2008. The genome of *Xanthomonas campestris* pv. *campestris* B100 and its use for the reconstruction of metabolic pathways involved in xanthan biosynthsis. *J. Biotechnol.* 134:33-45.

Wang, B., Hu, X., Li, Q., Hao, B., Zhang, B., Li, G. and Kang, Z. 2010. Development of race-specific SCAR markers for detection of Chinese races CYR32 and CYR33 of *Puccinia striiformis* f . sp. *tritici*. *Plant Dis.* 94:221-228.

Williams, P. H. 1980. Black rot: a continuing threat to world crucifers. *Plant Dis.* 64:736-742.

Yuen, G. Y., Alvarez, A. M., Benedict, A. A. and Trotter, K. J. 1987. Use of monoclonal antibodies to monitor the dissemination of *Xanthomonas campestris* pv. *campestris*. *Phytopathology* 77:366-370.

Zaccardelli, M., Campanile, F., Spasiano, A. and Merighi, M. 2007. Detection and identification of the crucifer pathogen, *Xanthomonas campestris* pv. *campestris*, by PCR amplification of the conserved Hrp/type III secretion system gene *hrcC*. *Eur. J. Plant Pathol.* 118:299-306.