Screening of Cabbage (*Brassica oleracea* L.) Germplasm for Resistance to Black Rot

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ABSTRACT Black rot of *Brassica* crops is the most devastating disease which causes substantial yield reduction of cabbage throughout the world. The use of resistant cabbage cultivars could be inexpensive and effective measure to combat this destructive disease. We screened cabbage inbred lines for black rot disease resistance through bioassay and identified some novel lines that showed race-specific resistance to *Xanthomonas campestris* pv. *campestris* (*Xcc*) races. The pathogenicity test revealed that out of 27 cabbage lines, one (SCNU-C-4074), six (SCNU-C-3631, SCNU-C-3637, SCNU-C-3639, SCNU-C-4072, SCNU-C-4073 and SCNU-C-3273), two (SCNU-C-3273 and SCNU-C-4118), two (SCNU-C-3270 and SCNU-C-4118), two (SCNU-C-3470 and SCNU-C-41148) and four (SCNU-C-107, SCNU-C-3270, SCNU-C-3470 and SCNU-C-4059) were shown to be resistant to *Xcc* races 1, 2, 3, 5, 6 and 7, respectively while none of these showed resistance against race 4. Furthermore, these resistant and susceptible lines were evaluated by previously reported molecular markers for black rot resistance. The molecular screening results were also revealed the existence of race-specific resistance in these cabbage lines. This result will help *Brassica* breeder to develop race-specific black rot resistant cabbage cultivars.

Keywords Black rot resistance, *Xanthomonas campestris* pv. *campestris*, Cabbage, Bioassay, Molecular markers

INTRODUCTION

Cabbage (*Brassica oleracea* L.) is the most important vegetable among *Brassica* crops in the world due to its nutritional and health benefits (Lee *et al.* 2015). Black rot disease, caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Pammel) Dowson, is the most destructive diseases of the crops belonging to the Brassicaceae family (Williams 1980; Lema *et al.* 2012; Vicente and Holub 2013; Lee *et al.* 2015). The major host of *Xcc* is *B. oleracea* and its subspecies (Vicente *et al.* 2001). *Xcc* is mainly a seed born pathogen, but can alive in crop residues and cruciferous weeds and ornamentals (Cook *et al.* 1952; Roberts *et al.* 1999; Vicente *et al.* 2001; Lema *et al.* 2011).

This pathogen can enter into the leaf via insects, wounded tissues and hydathodes (leaf margin) and spreads through vascular tissues (Tonu *et al.* 2013). *Xcc* can spread via rain, wind, insects, agricultural equipments and irrigation water (Sharma *et al.* 2017). The characteristic disease symptoms are V-shaped chlorotic lesions at the leaf margins, necrotic and darkened veins which reduce the quality and production (Williams 1980; Kifuji *et al.* 2013; Tonu *et al.* 2013; Vicente and Holub 2013; Lee *et al.* 2015). In favorable condition, black rot disease can reduce crop yield more than 50% (Williams 1980). However, up to 100% yield loss has been reported in cabbage by farmers in Tanzania (Massomo *et al.* 2003). Black rot also decreases the market value of cabbage. Like other plant pathogenic

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bacteria, *Xcc* has been separated into different physiological races based on the response of differential cultivars with resistance genes and pathogens. Up to now, 11 physiological races of *Xcc* have been reported (Kamoun *et al.* 1992; Vicente *et al.* 2001; Fargier and Manceau 2007; Cruz *et al.* 2017). Initially, Kamoun *et al.* (1992) reported five *Xcc* races (0-4). Later, Vicente *et al.* (2001) reclassified *Xcc* into six races (1-6). Furthermore, Jensen *et al.* (2010) and Fargier and Manceau (2007) were added race 7 and races 8-9 to Vicente's classification, respectively. Newly, race 10 and race 11 have reported in Portugal (Cruz *et al.* 2017).

The sources of resistance for black rot in *B. oleracea* are very less (Ignatov *et al.* 1998). However, the existence of black rot resistance was reported in Japanese cabbage (Early Fuji, P143660) as well as in Penca kales (Bain 1952; Dickson and Hunter 1987; Ferreira *et al.* 1992; Ignatov *et al.* 1999). Ignatov *et al.* (1998) reported race-specific resistance to *Xcc* races 1 and 5 in Japanese cabbage lines and Penca kale landraces. The race-specific resistance to *Xcc* in Asian cabbages was said to be inherited from Penca kales (a Portuguese black rot resistant kale landrace) (Ignatov *et al.* 1998). There were many research reports describing *Xcc* resistant genes in *B. oleracea* and related species. Williams *et al.* (1972) have been stated that black rot is controlled by one major gene designated as *f* and the heterozygous condition is influenced by one dominant and one modifier gene. They screened 300 cultivars and inbred lines of cabbage for *Xcc* resistance and this *f* gene found only in Early Fuji, a Japanese black rot resistant cabbage cultivar. Dickson and Hunter (1987) reported that black rot resistance is governed by a single recessive gene in cabbage (PI436606, a cabbage line from China). There are some reported quantitative trait loci (QTLs) and associated markers for black rot resistance in different *Brassica* species. For instance, Camargo *et al.* (1995) described QTLs for black rot resistance based on restriction fragment length polymorphism (RFLP) loci in cabbage. They found that two QTLs on linkage group (LG) 1 and 9 showed resistance against black rot for both young and old plants while two additional QTLs on LG 2 only for young plant resistance. Kifuji *et al.* (2013) reported a QTL for black rot resistance using 161 EST-SNP markers and found that

QTL-1 located on LG C02 is the major QTL in cabbage. A major black rot resistance locus called *Xca1bo* has been mapped on Chromosome 03 in Indian cauliflower (Saha *et al.* 2014). Lee *et al.* (2015) reported a genetic linkage map where they identified four QTLs such as BRQTL-C1_1 and BRQTL-C1_2 on LG C01, BRQTL-C3 on LG C03 and BRQTL-C6 on LG 06. Among these QTLs, BRQTL-C1_2 designates as the most important QTL for black rot in cabbage while remaining three as minor QTLs. Sharma *et al.* (2016) reported black rot resistance locus *Xca1bc* on LG B-7 in Indian mustard (*Brassica carinata*). They also reported that black rot resistance in *B. carinata* was controlled by a single dominant gene. Now-a-days, molecular marker based genotyping are extensively used to screen disease resistance. However, the resistance sources in *B. oleracea* (C genome) are limited while major resistance sources have been described in A and B genomes (Taylor *et al.* 2002; Sharma *et al.* 2016).

Xcc can be retained in seeds, *Brassica* crop residues, and cruciferous weed and ornamentals which act as the sources of inoculum (Schaad and Dianese 1981). Thus, it is very difficult to control black rot disease through disease management practices including agrochemicals. For this reason, exploration of *Xcc* resistant sources is one of the best ways to abate crop loss from black rot disease. However, new pathogenic races can be evolved due to the intensive cultivation of resistant cultivars that is the main hinder of black rot resistance (Song *et al.* 2014). Therefore, in this study, we aimed to screen out novel cabbage lines which provide race-specific resistance against *Xcc* races.

MATERIALS AND METHODS

Plant materials

Twenty seven different cabbage (*Brassica oleracea* L.) inbred lines were tested in this study (Supplementary Table S1). Seeds were obtained from Department of Horticulture, Sunchon National University, Republic of Korea. Initially, seeds were sown in trays containing a nursery pot mixture at plant culture room (25±1°C temperature, 60% Relative humidity and 80-120 µmol/m²/second light intensity). Twenty two days after sowing, the seedlings were

transferred into the pot and kept at the glasshouse. For pathogenicity test, seedlings from each of the above mentioned inbred lines were used for screening against black rot disease.

Bacterial strains

A total of seven *Xanthomonas campestris* pv. *campestris* (*Xcc*) races (race 1, 2, 3, 4, 5, 6 and 7) were inoculated in the aforementioned cabbage line for pathogenicity test which are shown in Table 1. The *Xcc* races (race 1-7) were collected from the School of Life Sciences, University of Warwick, UK. All the *Xcc* races were grown on King's medium B (KB) for 48 hours at 30°C (King *et al.* 1954).

Inoculation and disease scoring

The cabbage inbred lines were inoculated at 35 days after sowing. The *Xcc* races were sub-cultured on KB at 30°C for 48 hours before inoculation. Thereafter, the bacteria were scraped from the culture plates and suspended in 10 mL of sterile water. Finally, three youngest leaves of each plants were inoculated by clipping

secondary veins with sterile forceps (maintained at least 10 inoculation points per leaf) followed by dipping into a bacterial suspension (10^8 - 10^9 CFU/mL) of *Xcc* races and maintained high humidity (Vicente *et al.* 2001). Three seedlings for each *Xcc* race were used for the inoculation. The disease symptoms of inoculated leaves were evaluated at 2 and 3 weeks after inoculation (WAI). The disease reaction was scored at 2 WAI for each inoculated leaf based on a 0-9 scale (Fig. 1) where 0 = no visible symptoms; 1 = small necrosis or chlorosis near the inoculation point; 3 = typical small V-shaped lesion with black veins; 5 = typical lesion half way to the middle vein; 7 = typical lesion succeeding to the middle vein; and 9 = lesion reaching the middle vein as previously described by Vicente *et al.* (2002). The highly resistant (HR), resistant (R), susceptible (S) and highly susceptible (HS) lines were characterized based on the scales 0, 2-3, 5-7, and 9, respectively (Fig. 1).

DNA extraction

The young leaves of each cabbage lines were collected and immediately frozen in liquid nitrogen. Then, the leaf

Table 1. *Xanthomonas campestris* pv. *campestris* (*Xcc*) races used for bioassay-based screening of cabbage lines.

Sl. No.	Bacterial race/strains	Source	Reference
1	<i>Xanthomonas campestris</i> pv. <i>campestris</i> Race 1 (B100)	UK	Vicente <i>et al.</i> 2001
2	<i>Xanthomonas campestris</i> pv. <i>campestris</i> Race 2 (3849A)	US	
3	<i>Xanthomonas campestris</i> pv. <i>campestris</i> Race 3 (5212)	UK	
4	<i>Xanthomonas campestris</i> pv. <i>campestris</i> Race 4 (CFBP 5817)	UK	
5	<i>Xanthomonas campestris</i> pv. <i>campestris</i> Race 5 (3880)	Australia	
6	<i>Xanthomonas campestris</i> pv. <i>campestris</i> Race 6 (6181)	Portugal	
7	<i>Xanthomonas campestris</i> pv. <i>campestris</i> Race 7 (8450A)	UK

Fig. 1. The disease reaction scoring criteria used in this study for black rot of cabbage. Scales: 0 = no visible symptoms; 1 = small necrosis or chlorosis near the inoculation point; 3 = typical small V-shaped lesion with black veins; 5 = typical lesion half way to the middle vein; 7 = typical lesion succeeding to the middle vein; and 9 = lesion reaching the middle vein (Vicente *et al.* 2002). 0, high resistant (HR); 1-3, resistant (R); 5-7, susceptible (S); 9, highly susceptible (HS).

samples were maintained at -80°C until use. The genomic DNA was isolated using a commercial kit (DNeasy Plant Mini Kit, QIAGEN, Germany) following the manufacturer's instructions. The integrity and purity of the DNA were assessed by gel electrophoresis (0.8% agarose) and Nanodrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA), respectively.

Polymerase chain reaction (PCR)

Previously reported markers were used to screen the cabbage inbred lines for black rot resistance (Table 2). The PCR amplification was performed in 20 μL reaction mixture, containing of 2X Prime Taq premix (Genet Bio, Republic of Korea), 10 pmoles of each forward and reverse primers (Macrogen Inc., Seoul, Republic of Korea) and 100 ng genomic DNA as template. The thermal cycle was set with an initial denaturation at 95°C for 5 minutes, followed by 30-35 cycles of denaturation at 95°C for 30 seconds, annealing at specific T_m to respective primer sets (Table 2) between 52 and 61°C for 30 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 5 minutes. Then, the PCR products were subjected to agarose

gel electrophoresis to visualize the bands. The concentration of agarose gel was varied based on amplicon size. Finally, results were compared with the bioassay results.

Cloning and sequencing

The polymorphic markers for black rot disease (BoGMS0971, BnGMS301, BoESSR291, BoESSR726 and OI10G06) were amplified by PCR using Phusion[®] High-Fidelity DNA Polymerase (New England Biolabs) from resistant (R) and susceptible (S) cabbage lines. The polymorphic bands were extracted from the gel and purified using the Wizard SV gel and PCR cleanup system (Promega, Madison, WI, USA) from both resistant and susceptible lines for each of the above mentioned markers. Then, cloning was done using TOP cloner blunt kit (Enzymomics, Daejeon, Republic of Korea) according to the manufacturer's instruction. Three independent PCR positive clones were sequenced with the universal primers (M13FpUC and M13RpUC) by ABI 3730XL DNA sequencer (Macrogen Inc., Seoul, Republic of Korea). Subsequently, the cloned sequences of R and S lines for each marker were aligned using the Clustal Omega online

Table 2. List of markers with their primer sequence used in the molecular screening for black rot resistance in cabbage.

Sl. No.	Markers	Chromosome	Primer sequence (5'.....3')	T_m ($^{\circ}\text{C}$)	Size (bp)	Marker type	Reference
1	BoESSR291	C03	F: AAGCTGGGGATGGAGAAGAT R: GCACCTAATCGAACCCCTTA	52	114	SSR	Izzah <i>et al.</i> 2014
2	BoESSR216	C01	F: GGTTTCCGCTATGTCCAGAA R: CGGAAGAAGACGTTGAGGAG	52	317	SSR	Izzah <i>et al.</i> 2014
3	BoESSR089	C01	F: ATGATCAGCGAAACCACTCC R: TGATACATCCCGTTTGCTCA	55	259	SSR	Izzah <i>et al.</i> 2014
4	BoESSR145	C01	F: GGGCGAGGATGGTTACTACA R: TCATACCCCAAGGCTATTTT	55	240	SSR	Izzah <i>et al.</i> 2014
5	BoESSR726	C01	F: CAATGGGTTACGCATGGTTT R: CGTTTGTGAAACAGCCATTG	55	228	SSR	Izzah <i>et al.</i> 2014
6	SSR739	C03	F: TAGGGTGAAAGGGAAGCTCA R: CGCTAATAATGGCGCTAAGG	52	182	SSR	Saha <i>et al.</i> 2014
7	BnGMS301	C01	F: AATATGCAGCATTCTAGACAAA R: ATCATTCTCGTGATGACACA	55	250	SSR	Cheng <i>et al.</i> 2009
8	BoGMS0971	C08	F: TAATCCGAACAACACGAA R: CACCCAATAAGCGATGAG	52	344	SSR	Li <i>et al.</i> 2011
9	OI10G06	C06	F: GACAAGTTCCCTTGTAATGGC R: TGTAATCATCACACATTTTGGG	52	109	SSR	Li <i>et al.</i> 2011
10	Iso0857_371bc	C09	F: AAGGAATTCCCCAGGATGTC R: TTGGCAACCCTAATGCTTTT	51	477	InDel	Lee <i>et al.</i> 2015

Multiple Sequence Alignment tool available at <https://www.ebi.ac.uk/Tools/msa/clustalo/>.

RESULTS

Screening of cabbage inbred lines for the black rot resistance

The disease reaction results of 27 cabbage inbred lines for black rot disease resistance are shown in Table 3 and Fig. 2. The tested cabbage lines provide race-specific resistance against different *Xanthomonas campestris* pv. *campestris* (*Xcc*) races. The result showed that cabbage line SCNU-C-4074 was resistant (Fig. 2) against *Xcc* race 1. A total of six cabbage lines were highly resistant to *Xcc* race 2 (SCNU-C-3273, SCNU-C-3631, SCNU-C-3637, SCNU-C-3639, SCNU-C-4072 and SCNU-C-4073). Similarly, two lines SCNU-C-3273 and SCNU-C-4118 were resistant to *Xcc* race 3. Likewise, lines SCNU-C-3270 (highly resistant) and SCNU-C-4118 (resistant) were resistant to *Xcc* race 5. Two cabbage lines (SCNU-C-3470 and SCNU-C-41148) were shown to be resistant against *Xcc* race 6. A total of four cabbage lines (SCNU-C-107, SCNU-C-3270, SCNU-C-3470 and SCNU-C-4059) were resistant to *Xcc* race 7 of which three lines (SCNU-C-3270, SCNU-C-3470 and SCNU-C-4059) were highly resistant. On the other hand, all of the tested lines were susceptible for *Xcc* race 4. However, some cabbage lines were resistant to more than one *Xcc* races. For example, line SCNU-C-3270 shown to be resistant against race 5 and 7 (highly resistant), line SCNU-C-3273 against race 2 (highly resistant) and 3, and SCNU-C-4118 against race 3, 5 and 6.

Molecular screening cabbage inbred lines for the black rot resistance

A total of 10 previously published markers (9 SSR and 1 InDel) for black rot resistance were selected for this study based on reported QTL information. Among those reported markers, only five showed polymorphic amplification and were able to distinguish resistant (R) and susceptible (S) cabbage lines against black rot disease (Table 3 and Supplementary Fig. S1). Thereafter, the bioassay and molecular screening results were compared and those

having more than 60% adaptability for each race were shown in Table 3. The outcome of the comparison showed that the OI10G06 marker separates R and S lines with 83.3% adaptability, respectively for *Xcc* race 1 followed by BnGMS301 (79.2%), BoESSR726 (79.2%) and BoESSR291 (66.7%). Similarly, BoESSR291 and OI10G06 can differentiate R and S lines against *Xcc* race 2 with 73.9% adaptability followed by BoESSR726 (65.2%) and BoGMS0971 (60.9%). In case of race 3, BnGMS301 can differentiate R and S lines with 81.5% adaptability followed by BoESSR291 (77.8%), BoESSR726 (74.1%) and OI10G06 (70.1%). For race 4, BnGMS301 showed 79.2% match with the bioassay results followed by BoESSR726 (75%), OI10G06 (70.8%) and BoESSR291 (66.7%). In case of race 5, BnGMS301 showed 91.3% match with the phenotypic screening results followed by BoESSR726 (78.3%), BoESSR291 (73.9%) and OI10G06 (69.6%). For race 6, BnGMS301 and BoESSR726 can separate R and S cabbage lines with 81.5% adaptability with the bioassay results followed by BoESSR291 (70.4%) and OI10G06 (66.7%). In case of race 7, BnGMS301, BoESSR291, OI10G06 and BoESSR726 showed 72.0%, 68.0%, 68.0% and 64.0% match with the bioassay results, respectively.

Sequence analysis of amplified DNA fragments using markers

The amplified polymorphic markers were further cloned and sequenced from R and S lines. Through sequencing, the genetic variations including insertion/deletions (InDels), single nucleotide polymorphisms (SNPs) between R and S lines for the polymorphic markers were detected (Supplementary Fig. S2). The BoGMS0971 marker amplified a 351-bp DNA fragment for R lines and 330-bp for S lines. The sequence alignment results showed that there was a total 21-bp deletions from S lines compared to R lines. This marker located on the intergenic region between two genes (Bo8g092870 and Bo8g092880) (Table 4). The BnGMS301 amplified 238-bp DNA fragment for S lines but 215-bp with total 27-bp deletion for R lines, which are located between two genes (Bo1g053300 and Bo1g053310). In case of the BoESSR291, the tested cabbage lines showed three different types of PCR bands. These bands were

Table 3. Comparison of bioassay and molecular screening results for black rot resistance in cabbage.

Sl. No.	Lines	Race 1		Race 2		BoESSR291		OI10G06		BoESSR726		BoGMS0971	
		P ²⁾		P ²⁾		G ^{y)}		G ^{y)}		G ^{y)}		G ^{y)}	
1	SCNU-C-106	HS ^{u)}	+	+	+	+	+	+	+	+	+	+	-
2	SCNU-C-107	n/a ^{b)}	-	+	+	-	+	-	+	+	+	+	+
3	SCNU-C-3122	S ^{v)}	+	-	+	+	+	+	+	+	+	+	+
4	SCNU-C-3270	S	+	-	+	+	+	+	+	+	+	+	+
5	SCNU-C-3273	S	+	+	+	+	+	+	+	+	+	+	-
6	SCNU-C-3305	HS	+	+	+	+	+	+	+	+	+	+	+
7	SCNU-C-3328	S	+	-	+	+	+	+	+	+	+	+	+
8	SCNU-C-3412	S	+	+	+	+	+	+	+	+	+	+	-
9	SCNU-C-3414	S	-	+	+	+	+	-	+	+	+	+	-
10	SCNU-C-3449	HS	+	+	+	+	+	+	+	+	+	+	+
11	SCNU-C-3470	n/a	+	+	+	+	+	+	+	+	+	+	+
12	SCNU-C-3631	S	-	+	+	+	+	-	+	+	+	+	-
13	SCNU-C-3637	S	+	+	+	+	+	+	+	+	+	+	-
14	SCNU-C-3639	S	+	+	+	+	+	+	+	+	+	+	+
15	SCNU-C-4059	S	+	+	+	+	+	+	+	+	+	+	-
16	SCNU-C-4066	S	+	+	+	+	+	+	+	+	+	+	-
17	SCNU-C-4071	S	-	+	+	+	+	-	+	+	+	+	-
18	SCNU-C-4072	n/a	-	+	+	+	+	-	+	+	+	+	-
19	SCNU-C-4073	S	+	+	+	+	+	+	+	+	+	+	-
20	SCNU-C-4074	R ^{w)}	-	-	+	+	+	-	+	+	+	+	-
21	SCNU-C-4085	S	+	+	+	+	+	+	+	+	+	+	-
22	SCNU-C-4105	S	+	+	+	+	+	+	+	+	+	+	+
23	SCNU-C-4118	S	+	-	+	+	+	+	+	+	+	+	+
24	SCNU-C-4161	HS	+	+	+	+	+	+	+	+	+	+	-
25	SCNU-C-4168	S	-	+	+	+	+	-	+	+	+	+	-
26	SCNU-C-4302	HS	+	+	+	+	+	+	+	+	+	+	+
27	SCNU-C-4320	HS	+	+	+	+	+	+	+	+	+	+	-
Matched			20.0	19.0	19.0	16.0	17.0	17.0	17.0	15.0	14.0		
Not matched			4.0	5.0	5.0	9.0	6.0	6.0	6.0	8.0	9.0		
% Adaptability			83.3	79.2	79.2	66.7	73.9	73.9	73.9	65.2	60.9		

Table 3. Continued 1.

Sl. No.	Lines	Race 3		Race 4		Race 5		Race 6		Race 7		Race 8		Race 9		Race 10		Race 11		Race 12		Race 13		Race 14		Race 15		Race 16		Race 17		Race 18		Race 19		Race 20		Race 21		Race 22		Race 23		Race 24		Race 25		Race 26		Race 27		Race 28		Race 29		Race 30		Race 31		Race 32		Race 33		Race 34		Race 35		Race 36		Race 37		Race 38		Race 39		Race 40		Race 41		Race 42		Race 43		Race 44		Race 45		Race 46		Race 47		Race 48		Race 49		Race 50		Race 51		Race 52		Race 53		Race 54		Race 55		Race 56		Race 57		Race 58		Race 59		Race 60		Race 61		Race 62		Race 63		Race 64		Race 65		Race 66		Race 67		Race 68		Race 69		Race 70		Race 71		Race 72		Race 73		Race 74		Race 75		Race 76		Race 77		Race 78		Race 79		Race 80		Race 81		Race 82		Race 83		Race 84		Race 85		Race 86		Race 87		Race 88		Race 89		Race 90		Race 91		Race 92		Race 93		Race 94		Race 95		Race 96		Race 97		Race 98		Race 99		Race 100		Race 101		Race 102		Race 103		Race 104		Race 105		Race 106		Race 107		Race 108		Race 109		Race 110		Race 111		Race 112		Race 113		Race 114		Race 115		Race 116		Race 117		Race 118		Race 119		Race 120		Race 121		Race 122		Race 123		Race 124		Race 125		Race 126		Race 127		Race 128		Race 129		Race 130		Race 131		Race 132		Race 133		Race 134		Race 135		Race 136		Race 137		Race 138		Race 139		Race 140		Race 141		Race 142		Race 143		Race 144		Race 145		Race 146		Race 147		Race 148		Race 149		Race 150		Race 151		Race 152		Race 153		Race 154		Race 155		Race 156		Race 157		Race 158		Race 159		Race 160		Race 161		Race 162		Race 163		Race 164		Race 165		Race 166		Race 167		Race 168		Race 169		Race 170		Race 171		Race 172		Race 173		Race 174		Race 175		Race 176		Race 177		Race 178		Race 179		Race 180		Race 181		Race 182		Race 183		Race 184		Race 185		Race 186		Race 187		Race 188		Race 189		Race 190		Race 191		Race 192		Race 193		Race 194		Race 195		Race 196		Race 197		Race 198		Race 199		Race 200		Race 201		Race 202		Race 203		Race 204		Race 205		Race 206		Race 207		Race 208		Race 209		Race 210		Race 211		Race 212		Race 213		Race 214		Race 215		Race 216		Race 217		Race 218		Race 219		Race 220		Race 221		Race 222		Race 223		Race 224		Race 225		Race 226		Race 227		Race 228		Race 229		Race 230		Race 231		Race 232		Race 233		Race 234		Race 235		Race 236		Race 237		Race 238		Race 239		Race 240		Race 241		Race 242		Race 243		Race 244		Race 245		Race 246		Race 247		Race 248		Race 249		Race 250		Race 251		Race 252		Race 253		Race 254		Race 255		Race 256		Race 257		Race 258		Race 259		Race 260		Race 261		Race 262		Race 263		Race 264		Race 265		Race 266		Race 267		Race 268		Race 269		Race 270		Race 271		Race 272		Race 273		Race 274		Race 275		Race 276		Race 277		Race 278		Race 279		Race 280		Race 281		Race 282		Race 283		Race 284		Race 285		Race 286		Race 287		Race 288		Race 289		Race 290		Race 291		Race 292		Race 293		Race 294		Race 295		Race 296		Race 297		Race 298		Race 299		Race 300		Race 301		Race 302		Race 303		Race 304		Race 305		Race 306		Race 307		Race 308		Race 309		Race 310		Race 311		Race 312		Race 313		Race 314		Race 315		Race 316		Race 317		Race 318		Race 319		Race 320		Race 321		Race 322		Race 323		Race 324		Race 325		Race 326		Race 327		Race 328		Race 329		Race 330		Race 331		Race 332		Race 333		Race 334		Race 335		Race 336		Race 337		Race 338		Race 339		Race 340		Race 341		Race 342		Race 343		Race 344		Race 345		Race 346		Race 347		Race 348		Race 349		Race 350		Race 351		Race 352		Race 353		Race 354		Race 355		Race 356		Race 357		Race 358		Race 359		Race 360		Race 361		Race 362		Race 363		Race 364		Race 365		Race 366		Race 367		Race 368		Race 369		Race 370		Race 371		Race 372		Race 373		Race 374		Race 375		Race 376		Race 377		Race 378		Race 379		Race 380		Race 381		Race 382		Race 383		Race 384		Race 385		Race 386		Race 387		Race 388		Race 389		Race 390		Race 391		Race 392		Race 393		Race 394		Race 395		Race 396		Race 397		Race 398		Race 399		Race 400		Race 401		Race 402		Race 403		Race 404		Race 405		Race 406		Race 407		Race 408		Race 409		Race 410		Race 411		Race 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Table 3. Continued 2.

Sl. No.	Lines	Race 5 P ²⁾	BnGMS301	BoESSR726	BoESSR291	OI10G06	Race 6 P ²⁾	BnGMS301	BoESSR726	BoESSR291	OI10G06
			G ^{y)}						G ^{y)}		
1	SCNU-C-106	HS	+	+	+	+	HS	+	+	+	+
2	SCNU-C-107	HS	+	+	-	-	HS	+	+	-	-
3	SCNU-C-3122	S	-	+	+	+	HS	-	+	+	+
4	SCNU-C-3270	R	-	+	+	+	S	-	+	+	+
5	SCNU-C-3273	n/a	+	+	-	+	HS	+	+	-	+
6	SCNU-C-3305	S	+	+	+	+	HS	+	+	+	+
7	SCNU-C-3328	HS	-	+	+	+	HS	-	+	+	+
8	SCNU-C-3412	S	+	+	+	+	S	+	+	+	+
9	SCNU-C-3414	n/a	+	+	-	-	HS	+	+	-	-
10	SCNU-C-3449	HS	+	+	+	+	S	+	+	+	+
11	SCNU-C-3470	n/a	+	+	+	+	R	+	+	+	+
12	SCNU-C-3631	S	+	+	-	-	HS	+	+	-	-
13	SCNU-C-3637	HS	+	+	-	+	HS	+	+	-	+
14	SCNU-C-3639	HS	+	+	+	+	S	+	+	+	+
15	SCNU-C-4059	S	+	+	+	+	S	+	+	+	+
16	SCNU-C-4066	HS	+	+	-	+	HS	+	+	-	+
17	SCNU-C-4071	HS	+	+	+	-	HS	+	+	+	-
18	SCNU-C-4072	HS	+	+	+	-	HS	+	+	+	-
19	SCNU-C-4073	HS	+	+	+	+	HS	+	+	+	+
20	SCNU-C-4074	n/a	-	-	+	-	S	-	+	+	-
21	SCNU-C-4085	S	+	+	+	+	HS	+	+	+	+
22	SCNU-C-4105	HS	+	+	+	+	S	+	+	+	+
23	SCNU-C-4118	R	-	-	-	+	R	-	-	+	+
24	SCNU-C-4161	S	+	+	+	+	HS	+	+	+	+
25	SCNU-C-4168	HS	+	+	-	-	HS	+	+	-	-
26	SCNU-C-4302	HS	+	-	+	+	S	+	-	+	+
27	SCNU-C-4320	HS	+	+	+	+	HS	+	+	+	+
Matched			21.0	18.0	17.0	16.0		22.0	22.0	19.0	18.0
Not matched			2.0	5.0	6.0	7.0		5.0	5.0	8.0	9.0
% Adaptability			91.3	78.3	73.9	69.6		81.5	81.5	70.4	66.7

Table 3. Continued 3.

Sl. No.	Lines	Race 7	BnGMS301	BoESSR291	OI10G06	BoESSR726
		P ^{z)}				
1	SCNU-C-106	HS	+	+	+	+
2	SCNU-C-107	R	+	–	–	+
3	SCNU-C-3122	HS	–	+	+	+
4	SCNU-C-3270	HR	–	+	+	+
5	SCNU-C-3273	n/a	+	–	+	+
6	SCNU-C-3305	S	+	+	+	+
7	SCNU-C-3328	HS	–	+	+	–
8	SCNU-C-3412	HS	+	+	+	+
9	SCNU-C-3414	n/a	+	–	–	+
10	SCNU-C-3449	S	+	+	+	+
11	SCNU-C-3470	HR	+	+	+	–
12	SCNU-C-3631	HS	+	–	–	+
13	SCNU-C-3637	HS	+	–	+	+
14	SCNU-C-3639	S	+	+	+	–
15	SCNU-C-4059	HR	+	+	+	+
16	SCNU-C-4066	S	+	–	+	+
17	SCNU-C-4071	HS	+	+	–	+
18	SCNU-C-4072	HS	+	+	–	+
19	SCNU-C-4073	S	+	+	+	+
20	SCNU-C-4074	HS	–	+	–	–
21	SCNU-C-4085	HS	+	+	+	+
22	SCNU-C-4105	HS	+	+	+	–
23	SCNU-C-4118	HS	–	–	+	–
24	SCNU-C-4161	HS	+	+	+	+
25	SCNU-C-4168	HS	+	–	–	+
26	SCNU-C-4302	S	+	+	+	–
27	SCNU-C-4320	HS	+	+	+	+
Matched			18.0	17.0	17.0	16.0
Not matched			7.0	8.0	8.0	9.0
% Adaptability			72.0	68.0	68.0	64.0

^{z)}P, phenotype based on disease reaction; ^{y)}G, genotype on molecular marker screening; ^{x)}HR, high resistant; ^{w)}R, resistant; ^{v)}S, susceptible; ^{u)}HS, high susceptible; ⁰⁾n/a, bioassay data not available.

114-bp DNA fragment for R lines and another two with InDels, where 21-bp deletions and 16-bp insertions from R line resulted in being susceptible to black rot disease. However, BoESSR291 showed high identity with a genomic fragment where putative promoter sequence of a gene encoding enzyme cystathionine gamma-synthase (Bo3g055310) was located. The BoESSR726 marker amplified a 238-bp DNA fragment for the R line while 228-bp for S line. There were 12-bp InDels for R line and 2-bp InDel for S line which are located in the intergenic region between genes with accession number Bo1g054960

and Bo1g054970. The OI10G06 marker amplified 127-bp DNA fragment for S lines and one with 18-bp deletions for R lines (109-bp), which are located in the intergenic region of two genes (Bo6g095520 and Bo6g095530) (Table 4).

DISCUSSION

Black rot is considered as one of the main threats of cabbage production in the world and it is very difficult to control. The race-specific resistance in *Brassica* crops is

Fig. 2. The resistant and susceptible cabbage lines against different races of *Xanthomonas campestris* pv. *campestris* (*Xcc*) at 2 weeks after inoculation. The letters a, b, c, d, e, f and g, represents *Xcc* race 1, 2, 3, 4, 5, 6 and 7, respectively.

Table 4. The tested molecular markers and their location on *Brassica oleracea* genome.

Maker name	Location ²⁾	Description
BoGMS0971	C8:31754531..31754753	Intergenic region (Bo8g092870 and Bo8g092880)
BnGMS301	C1:15386406..15386643	Intergenic region (Bo1g053300 and Bo1g053310)
BoESSR291	C3:21846613..21846644	Promoter region of a gene <i>cystathionine gamma-synthase</i> (Bo3g055310)
BoESSR726	C1:15967972..15968174	Intergenic region (Bo1g054960 and Bo1g054970)
OI10G06	C6:29898028..29898121	Intergenic region (Bo6g095520 and Bo6g095530)

²⁾The chromosomal locations are shown according to the ENSEMBL-based *Brassica oleracea* genome database.

linked to the hypersensitive response at inoculated area with incompatible race, nonetheless occasionally partial symptom appeared (Kamoun *et al.* 1992; Ignatov *et al.* 1997). However, recent reports described *Xcc* race 1, 4 and 6 as the major races while remaining as the minor races in *B. oleracea* (Ignatov *et al.* 1998; Vicente *et al.* 2001; Taylor *et al.* 2002; Chidamba and Bezuidenhout 2012; Lema *et al.* 2012; Rouhrazi and Khodakaramian 2014). In *B. oleracea*, Taylor *et al.* (2002) reported that 43% of the tested accessions were resistant to one or more of the minor *Xcc* races (2, 3, 5, and 6) and only one showed partial resistance to *Xcc* races 1, 3, 5, and 6. They also noted that resistance is more common to *Xcc* race 3 and 5 whereas very rare to race 1 in *B. oleracea*. Moreover, *Xcc* race 6 was predominant in *B. rapa* (Lema *et al.* 2015). But recently Cruz *et al.* (2017) reported the existence of races 4, 6 and 7 (encompassing 21 out of 33 isolates) in Portugal. In this study, we identified novel cabbage lines with race-specific hypersensitive response against different races of *Xcc*. The disease reaction results revealed that there were 1, 6, 2, 2, 2 and 4 cabbage inbred lines exhibiting resistance against *Xcc* race 1, 2, 3, 5, 6 and 7, respectively while none of tested lines were resistant to race 4 (Fig. 2 and Table 3). This result is consistent with previous report as described the race-specific resistance against *Xcc* in *Brassica* species by Ignatov *et al.* (1998). Besides, Tonu *et al.* (2013) also reported black rot resistance in *B. oleracea* in particular to *Xcc* race 3 and race 5. It should be noted that the phenotypic screening results revealed SCNU-C-4059 as resistant lines against race 7 but none of the tested markers correlated with the phenotype except BoGMS0971 which showed only 36% adaptability with bioassay results (Table 3).

The genetic inheritance of major black rot resistance genes has been elucidated in different *Brassica* species

including *B. rapa*, *B. carinata* and *B. napus* containing A, BC and AC genome, respectively (Guo *et al.* 1991; Ignatov *et al.* 2000; Vicente *et al.* 2002). However, the strong resistance source for *Xcc* race 4 was reported to be originated from A genome while resistance to both races 1 and 4 from B genome, and *B. juncea* (AB genome) was the most resistant species to races 1 and 4 (Taylor *et al.* 2002). To date, several QTLs for black rot resistance have been described in *B. oleracea* and other *B.* species (Camargo *et al.* 1995; Cheng *et al.* 2009; Kifuji *et al.* 2013; Izzah *et al.* 2014; Saha *et al.* 2014; Lee *et al.* 2015; Sharma *et al.* 2016). In this study, five reported SSR markers showed polymorphism in banding pattern for R and S lines of the tested cabbage lines. By using these markers, we were able to separate R and S lines which were consistent with bioassay based phenotypic screening results (Table 3 and Fig. 2), nevertheless, none of them perfectly match with phenotypic data. These published markers were located on four different chromosomes such as BnGMS301 and BoESSR726 on chromosome C01, BoESSR291 on chromosome C03, OI10G06 on chromosome C06, and BoGMS0971 on chromosome C08 (Fig. 3). Previous reports described several QTLs for black rot resistance on those chromosomes. For instance, Lee *et al.* (2015) described four QTLs on three different chromosomes where BRQTL-C1_2 on chromosome C01 was the main QTL for black rot resistance in cabbage. Interestingly, the tested polymorphic marker BnGMS301 located on chromosome C01. Tonu *et al.* (2013) found *Xcc*Bo(Reiho)2 on chromosome C08 as the major locus for black rot resistance in cabbage and the SSR marker BoGMS0971 was closely linked to it. The remaining two SSR markers namely BoESSR291 and OI10G06 were linked to the minor QTLs (BRQTL-C3 and BRQTL-C6, respectively)

Fig. 3. Chromosomes location of five reported polymorphic markers on *Brassica oleracea*.

Table 5. Genes around the tested polymorphic molecular markers in *Brassica oleracea* genome.

Makers	Gene ID	Description		
		NCBI ²⁾	TAIR ^{y)}	Bolbase ^{x)}
BoGMS0971	Bo8g092870	V-type proton ATPase subunit D-like	Vacuolar ATP synthase subunit D	V-type proton ATPase subunit D-like
	Bo8g092880	Citrate synthase 2, peroxisomal-like	Citrate synthase 1	Citrate synthase-like, large alpha subdomain
BnGMS301	Bo1g053300	(E)-beta-ocimene synthase, chloroplastic	Terpene synthase 02	Terpene synthase-like
	Bo1g053310	(E)-beta-ocimene synthase, chloroplastic	Terpene synthase 02	Uncharacterized protein
BoESSR291	Bo3g055310	Cystathionine gamma-synthase	Cystathionine gamma-synthase	Cystathionine gamma-synthase (CGS)
BoESSR726	Bo1g054960	Uncharacterized	ABC transporter family protein	ABC transporter D family member 2, chloroplastic
	Bo1g054970	Uncharacterized	Mitochondrial transcription termination factor family protein	Mitochondrial transcription termination factor-related protein
OI10G06	Bo6g095520	Uncharacterized	Unknown protein	Putative uncharacterized protein
	Bo6g095530	Protein argonaute 7-like	Argonaute family protein	Protein argonaute

²⁾The National Center for Biotechnology Information (NCBI); ^{y)}The Arabidopsis Information Resource (TAIR); ^{x)}The *Brassica oleracea* Genome Database (Bolbase).

(Lee *et al.* 2015). Plant disease resistance governed by an interaction between specific disease resistance gene (*R*) of the host plant and *avirulence gene* (*avr*) the pathogen and also known as “gene-for-gene” model for plant disease resistance (Flor 1971; Dangl and Jones 2001). The *R* genes codes for only five classes of proteins and those encoded

“nucleotide-binding site and leucine-rich-repeat” (NBS-LRR) proteins are the major *R* genes in “gene-for-gene” plant resistance (Van der Biezen and Jones 1998; Dangl and Jones 2001). The NBS-LRR type *R* genes were further subdivided into two classes based on N-terminal structures such as those having the intracellular signaling domains of

the *Drosophila* Toll and mammalian interleukin (IL)-1 receptors (TIR-NB-LRR), and others having coiled-coil domains (CC-NB-LRR) (Dangl and Jones 2001). None of the tested markers were located in neither the *R* genes nor any other annotated genes (Table 5). Therefore, it is crucial to identify *R* genes responsible for race-specific resistance in cabbage. Taking this into consideration, we performed crossing between R and S cabbage lines for each *Xcc* race (race 1, 2, 3, 5, 6 and 7) to develop mapping population. Thereafter, with molecular mapping, the specific *R* genes responsible for race-specific resistance could be elucidated.

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