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Construction of a Linkage Map and QTL analysis for Black Rot Resistance in *Brassica oleracea* L.

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ABSTRACT

For quantitative trait loci (QTL) controlling resistance to *Xanthomonas campestris* pv. *Campestris*, we constructed linkage map using cleaved amplified plymorphic sequences (CAPS) and sequence-related amplified polymorphism (SRAP) analysis with disease rating of F_3 families obtained from a susceptible broccoli and resistant cabbage [Green commet P09 × Reiho P01]. We established inoculation technique. In this technique, leaves from approximately 50-day old F_3 plants were inoculated by cutting 1.0 cm at mid vain near the margins. A total of 38 CAPS and 60 SRAP primer pairs were screened to assess parental polymorphism against black rot resistance. Ninety two markers were distributed in 10 linkage groups (LGs) covering 320.5 cM (centimorgan), with average 3.56 cM interval between markers. Two genomic regions on LG 2 and LG 9 were significantly associated with resistance to the disease. The analysis revealed QTLs in the map interval between *CAM1* – *GSA1* on LG 2 accounting for up to 10% of the phenotypic variation and one QTL in the map interval between *F12-R12e* – *BORED* on LG 9 explaining 16% phenotypic variation with LOD score of 3.09. Two additional non-significant QTLs on LG 3 in the interval between *CHI* – *ASB1* (LOD = 2.04) and on LG 7 in the interval between *IPI* – *FLC3* (LOD = 2.25) were also detected for resistance to the disease. The QTLs, which were mapped to LG 2 and LG 9 for the disease, could be useful for marker-assisted selection in resistance breeding.

Key words: Linkage map, QTL, Black rot, Resistance, *Brassica oleracea*

INTRODUCTION

Brassica oleracea L. is one of the most important crop species that includes many vegetables such as cabbage, cauliflower, broccoli, brussels sprouts and kale [1]. The crops suffer from several diseases of which black rot is economically important worldwide [2, 3, 4].

Black rot caused by *Xanthomonas campestris* pv. *campestris* is the most serious bacterial disease in *B. oleracea* [4]. The bacteria enter leaves through the hydathodes at leaf margins or wounds and cause systemic infection. The disease spreads through vascular tissue clogging vessels and producing V-shaped chlorotic lesions [5]. Infected veins turn black, and the interveinal tissue becomes chlorotic or necrotic. The bacteria are dispersed in rain, in splashed water and on plants and equipments and they survive for long periods on crop debris, seeds, and weeds [6]. Six races of *X. campestris* pv. *campestris* currently are recognized and a gene-for-gene model recently was proposed [7]. Race 1 and 4 are the most important races in *B. oleracea* crops especially in cabbage and cauliflower worldwide [8]. Control of the disease is very difficult and can only be achieved by the use of disease-free seeds and cultural practices, and the elimination of other potential inoculum sources (infected crop debris and cruciferous weeds).

Resistant cultivars could play an important role in reducing the losses due to the diseases [9, 10, 3, 8]. Utilization of host resistance has been recognized as one of the most economic and effective control measures. Recently molecular markers provided useful tool for plant breeders to identify resistance genes and correlate their presence with disease severity symptoms following inoculation. Molecular markers can also be used to study the genetic control of quantitative traits by establishing linkage associations between markers and quantitative trait loci (QTL) [11]. Resistance to black rot in *B. oleracea* has been reported as quantitative in nature, with varying numbers of genes involved [2, 8].

In the present study, we focused on genetic analysis of black rot resistance by using CAPS and SRAP markers. Our results indicate that the resistant locus in the different cross combination of *B. oleracea* which could facilitate marker assisted selection for improvement of black rot disease resistant variety.

MATERIALS AND METHODS

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Plants materials

Susceptible broccoli doubled hybrid (DH) line 'Green Commet (GC) P09' (*Brassica oleracea* subsp. *italica*) was crossed as the female parent to the resistant DH line 'Reiho P01' (*B. oleracea* subsp. *capitata*). The 'GC P09' was susceptible to *Alternaria brassicicola* and *Xanthomonas campestris* pv. *campestris* diseases where the Reiho was tolerant. Seedlings were grown to 20-day old in greenhouse and were transferred to plastic pot (12 cm). F_1 progeny was developed from the cross GC P09 × Reiho P01. Progenies of F_2 and F_3 were generated from selfing of F_1 populations. Young leaves from each parent and 94 individual of F_2 plants were collected for DNA extraction and the plants were allowed to grow for bud self-pollination to generate F_3 seeds for evaluation of resistance to the disease.

Bacterial isolate

X. campestris pv. *campestris* (isolate no. *Xcc-03-01967*) used in this study was obtained from Gene Bank of National Institute of Agrobiological Resources, Japan and was maintained on YDC (Yeast Dextrose Calcium Carbonate) agar slant at 4°C. For inoculation, a 48h new culture grown on YDC medium at 28°C was used. Bacteria were grown on YDC medium at 28°C for 48h before inoculation. Bacterial cells were scraped from the plates and suspended in saline solution (0.85% NaCl) and adjusted to 10^8 CFU/ml (0.2OD A_{600} nm on a Jenway 6030 colorimeter).

Plant inoculation

Approximately 50-day old plants were inoculated with *X. campestris* pv. *campestris*. Leaves were inoculated by cutting 1.0 cm with mid vein near the margins using nail cutter [12]. A total of 1,128 F_3 plants (obtained from 94 F_2 plants) were used for inoculation to know the disease reaction. In this inoculation technique, three youngest leaves of each F_3 plant and twelve plants of each F_3 strain were inoculated. For every inoculation, nail cutter was dipped into the bacterial solution. V-shaped symptom area (cm^2) was measured after 14 days of inoculation (Figure 1) following the equation of {(width × length) ÷ 2}. Plants with a mean disease area <0.50 cm^2 were classified as resistant, those having 0.51-0.90 cm^2 were border line resistant and those having of >0.90 cm^2 were classified as susceptible to the disease.

Detection of DNA polymorphism

Healthy young leaves taken from 4-5 weeks old plants were harvested from the parents and 94 F_2 individuals and were used for genomic DNA extraction. Total genomic DNA was isolated according to Cetyltrimethylammonium bromide (CTAB) method [13] with minor modifications.

The primer sequences used in CAPS (Cleaved Amplified Polymorphic Sequences) analysis were designed based on the structural gene sequences published in the NCBI and TAIR databases, and in the reports of [14, 15].

Table 1. CAPS markers used in this study [23]

Name	Accession	Primer sequences (5' - 3') Forward	Reverse	PCR[d]	CAPS
ACS2	AB086353	AGCTACATGCAACAGCCATG	AGTCGTTGTCTTCTTCCTCG	C	P(*Afa*I)
ACT1[b]	AF044573	TGGTTGGGATGAACCAGAAG	CATCAATTCGATCACTCAGG	C	P(*Alu*I)
ALK	AY044425	TCAACTGCTACGCCCTGATT	ACTCGTTTCCCATCTCCTGA	C	P(*Mbo*I)
ASB1[b]	AF195511	CGCAACCCAAGAATGCAATC	ACCAGAACATTCCATCCACT	C	P(*Mbo*I)
BOHM13[b]	Z97060	TATGCACTTCCGGTCAGACC	CAGCTTATCTCTCAACTCTG	C	P(*Mbo*I)
BORED	X64464	GTGGCCAGGCTATCACNTTYGG	AGTCGCTGTGTAGTTTGCYTGNCC	C	P(*Mbo*I)
BTPT	U13632	AGATCTCCCACGATGCAGAG	AGTAAGTCAGCAAAGAGAAC	C	P(*Msp*I)
CAM1	AJ427337	GTTCAAGGAAGCCTTTAGCC	AGAGATAGCTTAGCCGAAGC	C	P(*Alu*I)
CAM2	AJ427338	TGACCGATGACCAGATCTCA	GTCGCAACCGAATCAAGTTC	C	P(*Mbo*I)
CHI[a]	M86358	GTGGAAGGGAAAAACTACGGAGGAG	CCGGTTTCAGGGATACTATCATCTT	C	P(*Alu*I)
CO	AF016010	ATGTTCAAACAAGAGAGTAAC	CTTTATTTTTGGCCATAGAAT	C	P (*Alu*I)
DGAT1	AF164434	GGCGATTTTGGATTCTGGAG	CGGTTCATCAGGTCATGGTA	C	P(*Mbo*I)
FLC1	AY115674	GAGGAATCAAATGTCGATAA	CTAATAAAGCAGTGGGAGAG	C	P(*Mbo*I)
FLC2	AF116527	CCATGAGCTACTAGAACTTG	CTAATAAAGCAGTGGGAGAG	C×2	P(*Alu*I)
FLC3	AY115673	GTGGAATCAAATGTCGGTGG	CTAATAAAGCAGTGGGAGAG	C	P(*Alu*I)
FLC5[c]	-	AGTGTGGAAGGGATGTGAAAG	TGAGGTTACAGACGTCTAAC	C	P(*Xsp*I)
GA1[a]	U11034	CAAGGATACCAAAAGAGATAATGC	CGTTTTCTCCACCATATTGATC	C	P(*Afa*I)
GAPB	M64118	GGCTAGAAGTCGCTGAATTC	TGGTAGAGACATCAGAGCAC	C	P(*Mbo*I)
GSA1	U03773	ACCAGCTTCTAACCGATGCT	GTATATCCTCGGGAGTGTGA	C	P(*Alu*I)
GSL	AF399834	TGGCATCGTCACTTCTGACA	CTAATGCTACTCGCGACCAT	C×2	P(*Mbo*I)
GTR	AC002333	GACATCATCCARAARCAYCARAC	CTCTCCTTCCATCACTTCCTTA	C	P(*Mbo*I)
IPI[b]	AF236092	ATGCTGTTCAAAGACGCCTC	TACAGCTTCACCGAGAGTTC	C	P(*Mbo*I)
MYR	Z21978	CATAAAGCTTCTTCATGGAC	TCATGCATCAGCGAGCTTCT	C×2	P(*Alu*I)
MSI1	AF016846	GAGATCGAGGAGCGACTAAT	TGTTGTCCTCAGCAACACTG	C	P(*Afa*I)
NDPK3	AB072239	GGTCTTCTCTCAGTTTCACT	AGTTATCGCCATAGATCCAC	C	P(*Msp*I)
NITe	AF380304	ACATCTCTGGAACGTTGCAT	CCTTGAGTAATGTCCGACC	C×2	P(*Map*I)
PGIC[a]	X69195	TCGAACCCGGGAGAGGTAGACCA	TGCTGTCAGCACTAATCTTGCG	C	P(*Alu*I)
PLD2	AF090444	GGAGTATCCAAGACGCTTAC	CAGCAGCAATGTAGAGACAG	C	P(*Mbo*I)
SFR2[b]	X98520	TCGGCTACAGAATCTCTCAC	TCTAGATCAGCAGCTGCTAG	C	P(*Alu*I)
TFL1[a]	D87519	GGTTTCACGAGTGGCTTATTCC	CCGTCGTCATCCTCACCTTC	C	P(*Afa*I)
TMT1[b]	AF387791	TCCTGCCTGAAACTGTTGAG	ACCTCCCAAGCTTCTCTTTG	C	P(*Alu*I)

Primer sequences obtained from [a]Kuittinen et al. and [b]Inoume and Nishio [14, 15]

[c]The *FLC5*-specific primer pair was designed based on the first intron of *FLC5*. [d]C, common bands; ×2, multiple bands; P, polymorphic bands.

The CAPS markers used in this study are shown in Table 1. Annealing temperature and extension time for PCR were set according to the primer sequence and gene size. The amplicons were digested with one of four restriction enzymes (*Afa*I, *Alu*I, *Msp*I or *Mob*I) and were separated on 8-15% polyacrylamide gel according to gene size. Restriction enzyme was chosen according to their digestion of PCR amplicons against respective primer. Polyacrylamide gel was prepared according to the method of [16]. The digestion was checked by 2% agarose gel before running polyacrylamide gel.

Polymorphic detection by the sequence-related amplified polymorphism (SRAP) method was conducted according to the method of [17] with minor modifications. For amplification, a standard PCR cocktail with the primer pairs listed in Table 2 was

used. The first five cycles of PCR were performed at 95°C for 30 seconds for denaturing, 35°C for 30 seconds for annealing and 72°C for 2 minutes for extension. The annealing temperature was raised to 50°C for another 35 cycles.

The success of the amplification was checked by electrophoresis of the PCR products in a 1% agarose gel. 10 × loading buffer (Takara Biomedicals, Japan) was added to the PCR products and mixed well prior to loading (2μl) in the agarose gel. Amplified DNA fragments were loaded onto a native 8% polyacrylamide gel that was made according to method of [16] and separated at a power of 100 V for 1.5 hours and 250 V for 3 hours. The gel was subsequently stained with a Gelstar solution (0.1μl/10ml) (Takara Biomedicals, Japan).

Construction of Map and QTL analysis

Linkage analysis of the markers was performed using JOIN MAP programe, version 3.0 [18]. The Kosambi mapping function was used to convert recombination frequencies into genetic (map) distances. The QTL for *X. campestris* pv. *campestris* resistance were analyzed using a composite interval-mapping analysis [19] with Map QTL version 2.0 [20] and QTL Cartographer version 1.16 [21]. A forward-backward stepwise regression was performed to choose co-factors before performing QTL detection. A 1,000-permutation test was performed with QTL Cartographer to estimate the appropriate significance threshold for analysis. A minimum logarithm of the odds ratio (LOD) threshold of 2.4, corresponding to a genome-wise significance level of 0.10, was chosen.

Table 2. SRAP markers used in this study [23]

Primer sequences (5' – 3')			
Forward		Reverse	
F1a	TGAGTCCAAACCGGATA	R1	GACTGCGTACGAATTAAT
F2	TGAGTCCAAACCGGAGC	R2	GACTGCGTACGAATTTGC
F3	TGAGTCCAAACCGGAAT	R3	GACTGCGTACGAATTGAC
F4	TGAGTCCAAACCGGACC	R4	GACTGCGTACGAATTTGA
F5	TGAGTCCAAACCGGAAG	R5	GACTGCGTACGAATTAAC
F12	TGGATTGGTCCCGGATC	R12	TCGGTCATAGAATTGCT
F13	GGAACCAATCCCGGATG	R13	GGTACTAAGGAATTCGA
F14	AAACTCCGTCCCGGACT	R14	TACCTAAGCGAATTCAG
F18	CGTAAACTCCCCGGCAA	R18	ACTGAGATCCAATTCCG
F19	TACTGTTGCCCCGGCAT	R19	CCCGTTTTTGAATTCTC

[a]F1-F5 and R1-R5 correspond to the me1-me5 and em1-em5 primer pairs [17].

RESULTS

Disease resistance

Inoculation of plants by leaf cut method was highly effective in establishing the disease judged by symptom expression on the susceptible parent and controls. Symptom began appearing 5 days after inoculation as a water-soaked lesion around the cut site. On the susceptible plants, lesions enlarged as they progressed towards the midrib resulting in typical chlorotic, V-shaped lesions. On resistant plants, lesions were restricted in size and were often associated with a small necrotic area surrounding the cut portion after 15 days of inoculation (Figure 1).

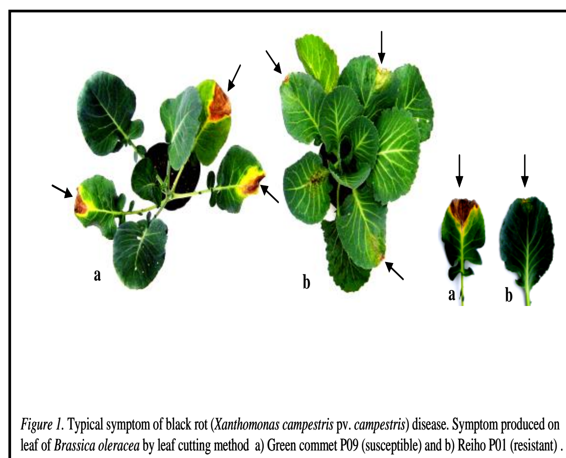

Figure 1. Typical symptom of black rot (*Xanthomonas campestris* pv. *campestris*) disease. Symptom produced on leaf of *Brassica olerac*ea by leaf cutting method a) Green commet P09 (susceptible) and b) Reiho P01 (resistant) .

The mean diseased leaf area (DLA) is shown in Table 1. The susceptible parent GC P09 was scored with a mean DLA of 2.22 cm^2 while the mean DLA of resistant parent Reiho P09 was 0.49 cm^2. The over all mean DLA of the two parents differed significantly (t = 8.87; n = 40; *P* = 2.89), but no significant difference was found between susceptible parent and F_1 hybrid (t = 0.87; n = 7; *P* = 0.42) for black rot disease development. The DLA of F_3 population indicated that black rot resistance is a quantitative trait with normal distribution (Figure 2).

Table 3. Mean diseased leaf area (cm^2) of black rot (*Xanthomonas campestris pv. campestris*) diseases of *Brassica oleracea* plants (Reiho P01, Green commet P09, F_1 and F_3 progenies derived from Green commet × Reiho)

	Black rot
	Mean (± SE[a])
Reiho P01	0.49 ± 0.07 (n = 44)
Green Commet	2.22 ± 0.20 (n = 40)
F_1	2.92 ± 0.41 (n = 7)
F_3	0.88 ± 0.05 (n = 81)

[a]SE: Standard error
[b]n: Number of plants included in the estimate

Linkage analysis

A total of 38 CAPS and 60 SRAP primer pairs were screened to assess parental polymorphism against black rot resistance. After restriction digestion of PCR products, polymorphic bands were detected in products generated by 35 CAPS primers pairs by electrophoresis. In the SRAP analysis, polymorphic markers were detected from 57 primer pairs. Finally 92 markers were distributed in 10 linkage groups covering 320.5 cM and average interval between markers was 3.56 cM (Figure 3).

Mapping QTLs for black rot diseases

A total of 92 markers were used to associate markers and phenotypes in the mapping population. The chromosomal locations of marker intervals, effect of Each QTL and Phenotypic variation are presented in

Table 4 and Figure 3.

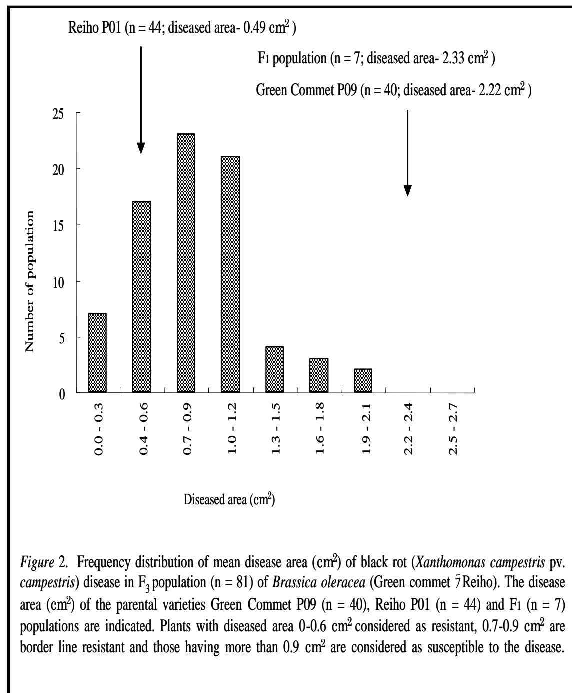

Figure 2. Frequency distribution of mean disease area (cm^2) of black rot (*Xanthomonas campestris* pv. *campestris*) disease in F_3 population (n = 81) of *Brassica oleracea* (Green commet × Reiho). The disease area (cm^2) of the parental varieties Green Commet P09 (n = 40), Reiho P01 (n = 44) and F1 (n = 7) populations are indicated. Plants with diseased area 0-0.6 cm^2 considered as resistant, 0.7-0.9 cm^2 are border line resistant and those having more than 0.9 cm^2 are considered as susceptible to the disease.

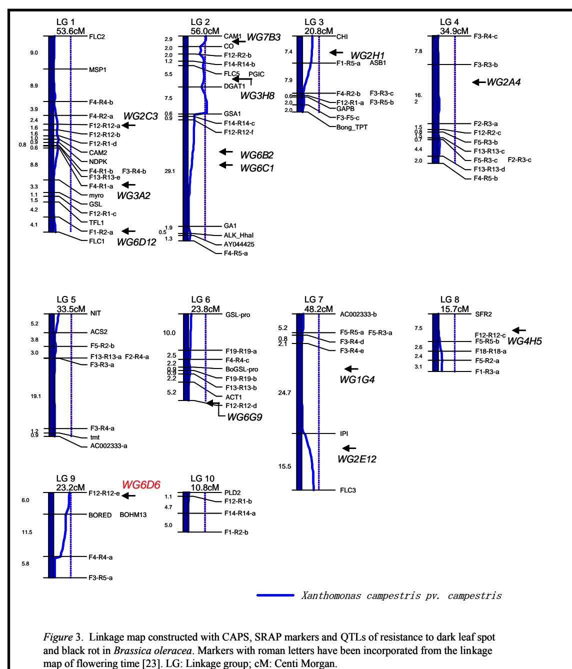

Figure 3. Linkage map constructed with CAPS, SRAP markers and QTLs of resistance to dark leaf spot and black rot in *Brassica oleracea*. Markers with roman letters have been incorporated from the linkage map of flowering time [23]. LG: Linkage group; cM: Centi Morgan.

Interval-mapping analysis for black rot disease using DLA revealed one region containing QTL located on LG 2 with LOD threshold of 3.94 and one QTL on LG 9 with LOD threshold of 3.09 in permutation test. Two markers on LG 2 and 2 markers on LG 9 were significantly associated with DLA and explained smaller fractions of the phenotypic variation (Table 4). The QTL on LG 2 explained 10.1% of the phenotypic variation, where 16.1% for the QTL on LG 9. Other two non-significant QTLs, one was detected on LG 3 in the interval between *CHI – ASB1* (LOD = 2.04) explaining 6% phenotypic variation and one was on LG 7 (LOD = 2.25) in the interval between *IPI – FLC3* explaining 14.7% phenotypic variation (Table 4 and Figure 3).

Table 4. The map intervals, linkage group, logarithm of odds (LOD), the percentage of explained phenotypic variance (VE) and additive effects of quantitative trait loci (QTL) detected for black rot (*Xanthomonas campestris* pv. *campestris*) disease using F_3 populations derived from Green commet P09 × Reiho P01

Map interval	Linkage group[a]	LOD	Additive effect[b]	VE (%)[c]
CAM1 – GSA1	2	3.94	-0.20	10.1
CHI – ASB1	3	2.04	0.18	6.0
IPI – FLC3	7	2.25	-0.24	14.7
F12-R12-e – BORED	9	3.09	0.25	16.1

[a] Linkage groups are as in Figure 3.
[b] Additive effect: ' – ' indicate resistance due to parent GC P09.
[c] Percentage of variance explained by quantitative trait loci.

DISCUSSION

*Black rot (*X. campestris *pv.* campestris*) is the most important bacterial disease of crucifers [2, 6, 7]. Several infection methods like spraying method [2], pinning method [22] and clipping method [2, 24, 7] have been reported. In the present study, the inoculation of leaves by cutting 1.0 cm with mid vein near the margins was highly effective in establishing the disease.*

Although the most useful sources of resistance are present in the A and B genomes (*B. rapa* and *B. nigra*) and absent in C genome (*B. oleracea*) [6, 7, 8], some authors have reported resistant genes to black rot in *B. oleracea*. Existence of a major gene *'f'* controlling resistance to black rot has been reported [25] based on segregation data from the crosses involving the highly resistant Japanese cabbage cultivar 'Early Fuji' and the susceptible Badger Inbreds-3, -5, -8, and –13. In *B. oleracea* resistance to race 1 is controlled by a dominant gene *R1* and recessive gene *r5* is responsible for resistance to race 5, but he did not identified QTL for the resistance [24]. A dominant resistance gene (*Xca3*) to race 3 in DH BOH 85c and PI436606 of *B. oleracea* are reported [8] whereas recessive resistance gene to race 1 and 3 in *B. oleracea* line Badger Indred-16 acts quantitatively. Resistance is inherited either as a dominant or recessive trait, which depends on genetic background of cultivars as shown by resistant gene to race 3 which is dominant in DH BOH 85c background and recessive in Badger inbred-16 background [2].

We identified one QTL on LG 2 (LOD = 3.94) in the map interval between *CAM1 – GSA1* and one QTL on LG 9 (LOD = 3.09) in the map interval between *F12-R12e - BORED* resistance to the *X. campestris* pv. *campestris*. We also found two more QTLs on LG 3 and LG 7 that were not significant in the permutation test. Black-rot-resistance QTLs has been found in the map interval between *wg6g5-wg2g11* on LG 1 and *wg8a9b-wg4d7* on LG 9 using RFLP

markers in *B. oleracea* [2]. In order to compare our QTLs with the QTLs identified [2], we incorporated RFLP markers into our map from linkage map of flowering time constructed by [25] which was also included in [2] because the same cross combinations were used to construct the map in our study and [25]. We identified position of the RFLP markers [2] in our map (shown by large italic capital letters eg. *WG2C3*). We found that QTL on LG 9 (LOD = 3.09) corresponds to the QTL in the map interval between *wg6g5-wg2g11* on LG 1 identified by [2] because the RFLP marker *WG6D6* was identified at the locus which was closely located at position of *Xanthomonas*-resistant QTL in both our map and Camargo's map. The additive effect of QTL on LG 2 was –0.20, which indicated that resistance has come from parent GC P09 for cryptic gene effect of transgressive segregation of F_3 progenies for DLA. This study will facilitate marker-assisted selection to improve black rot disease resistant in *Brassica* crops.

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