Reprint **ISSN 1991-3036 (Web Version)**

International Journal of Sustainable Crop Production (IJSCP)

(*Int. J. Sustain. Crop Prod.*)

Volume: 10 **Issue: 1** **February 2015**

Int. J. Sustain. Crop Prod. 10(1): 11-18 (February 2015)

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ISSN-1991-3036 (Online)
Int. J. Sustain. Crop Prod. 10(1):11-18(February 2015)

MAPPING OF QUANTITATIVE TRAIT LOCI CONTROLLING RESISTANCE TO DARK LEAF SPOT (*Alternaria brassicicola*) DISEASE IN *Brassica oleracea* L.

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Accepted for publication on 10 January 2015

ABSTRACT

Doullah MAU, Okazaki K (2015) Mapping of quantitative trait loci controlling resistance to dark leaf spot (*Alternaria brassicicola*) disease in *Brassica oleracea* L. *Int. J. Sustain. Crop Prod.* 10(1), 11-18.

Dark leaf spot and black rot are important diseases in Brassicas worldwide. New sources of resistance to the diseases are urgently needed for sustainable management. Quantitative trait loci (QTL) controlling resistance to *Alternaria brassicicola* was mapped using cleaved amplified polymorphic sequences (CAPS) and sequence-related amplified polymorphism (SRAP) analysis with disease rating of F_3 families from a susceptible broccoli Green commet P09 × resistant cabbage Reiho P01. For dark leaf spot disease, 3rd/4th leaves of 30-day old plants were inoculated by detached leaf inoculation method. A total of 38 CAPS and 60 SRAP primer pairs were screened to assess parental polymorphism against dark leaf spot resistance. Ninety-two markers were distributed in 10 linkage groups (LGs) covering 320.5 cM, with average 3.56 cM interval between markers. The QTL effect was detected significantly in the interval between *IPI - FLC3* on LG 7 for dark leaf spot disease resistance explained 43.8 % of the phenotypic variation with LOD score of 4.27. This is the first QTL detected for resistance to *A. brassicicola*. One non-significant QTL on LG 2 in the interval between *CAM – GSA1* (LOD = 2.02) was also detected for resistance to the dark spot disease. The QTL, which was mapped to LG 7, could be useful for marker-assisted selection of dark leaf spot resistance breeding.

Key words: *genetic analysis, mapping, QTL, Alternaria leaf spot, resistance, Brassica oleracea* L.

INTRODUCTION

Brassica oleracea L. is one of the most important vegetable crops and have great economic importance worldwide. It includes many vegetables such as cabbage, broccoli, brussels sprouts, cauliflower and kale (Gomez-Campo, 1999). The crops suffer from several diseases of which Alternaria leaf spot is economically important worldwide (Camargo *et al*. 1995; Rotem 1998; Meah *et al*. 2002; Taylor *et al*. 2002; Chen *et al*. 2003).

Alternaria leaf spot of crucifers is potentially severe fungal disease that can affect virtually any cultivated crucifer (Doullah *et al*. 2009; King 1994). *Alternaria brassicicola* (Schw.) Wilts., a common seed-borne pathogen of *Brassica* crops, frequently attacks crucifer seed crops causing reduced seed yield and germination (Bassey and Gabrielson, 1983). The disease can appear in all cultivated crucifers at any stage of crop development (Doullah *et al*. 2006; Rotem 1998). Due to uncertainty of the level of disease and various sources of inoculum, the disease is difficult to control although several fungicides are prescribed to manage the diseases (Meah *et al*. 1992; King 1994; Taylor *et al*. 2002).

Resistance to *A. brassicicola* has been identified by screening genotypes of *B. napus* and *B. oleracea* (King 1994). We identified cultivar resistance to *A. brassicicola* in *B. oleracea* in our screening test (Doullah 2006) and assumed that this resistance may be useful for incorporating immunity to *Alternaria* diseases into the cultivated Brassicas, although gene(s) related to the resistance have not been identified yet.

Control of the disease is 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 (King 1994; Westman *et al*. 1999; Taylor *et al*. 2002; Vicente *et al*. 2002). Utilization of host resistance has been recognized as one of the most economic and effective control measures. New sources of resistance to dark leaf spot are urgently needed for sustainable agriculture. Despite the availability of resistant cultivars, little is known about the genetic control of resistance of Brassica crops against the disease. Recently molecular markers provide a useful tool for plant breeders to identify resistance genes and correlate their presence with disease severity symptoms following inoculation. Molecular markers can be used to study the genetic control of quantitative traits by establishing linkage associations between markers and quantitative trait loci (QTL) (Doullah *et al*. 2011; Tonguc *et al*. 2003). The main focus of our study were to map the resistance loci and to find markers linked to these loci that could be helpful in marker-assisted selection programs for breeding of dark leaf spot resistant varieties.

MATERIALS AND METHODS

Plant materials

Doubled hybrid (DH) broccoli line 'Green Commet (GC) P09' (*Brassica oleracea* subsp. *italica*) and cabbage DH line 'Reiho P01' (*B. oleracea* subsp. *capitata*) were selected. The 'GC P09' was susceptible to *Alternaria brassicicola* and 'Reiho' was tolerant. Seedlings were grown to 20-day old in greenhouse and were transferred to plastic pot (12 cm) (Doullah *et al*. 2006). F_1 progeny followed by F_2 and F_3 was developed from the cross between 'GC P09' and 'Reiho P01' where 'GC P09' served as mother parent according to Doullah *et al*. (2011).

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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.

Fungal isolate

Fungus *A. brassicicola* 'Akakura' isolate was used in this study (Doullah *et al*. 2006).

Plant inoculation

Third or fourth true leaf from 30-day old plant of parents and F_3 population as a representative of F_2 individual were inoculated by detached leaf inoculation test for dark leaf spot disease according to the method of Doullah (2006).

Isolation of genomic DNA

Healthy leaves harvested from the parents and 94 F_2 individual were used for genomic DNA extraction. Total genomic DNA was isolated according to Cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980) with minor modifications (Doullah 2006).

In this method, young leaves were taken from 4-5 week old plants and were freeze-dried. One gram of freezed dried leaf was macerated with liquid nitrogen using pestle and mortar. 1.5ml of 1.5 × CTAB (1.5% CTAB, 0.75M Tris-HCl (pH 8.0), 1.05M NaCl, 0.75% PVP) at 70°C was added to the macerated tissue and kept it warm at 55°C for 10 minutes. Equal volume of chloroform/isoamyl alcohol (24:1) was then added and mixed by shaking for 30 minutes at room temperature. The mixture was spin-down at 15000 rpm for 10 minutes at room temperature and resulting supernatant was decanted into another new tube. Volume of 150µl (1/10 volume of supernatant) of 10% CTAB (10% CTAB, 0.7M NaCl) at 50°C was added to the supernatant and kept it warm at 50°C for 5 minutes. To precipitate DNA, equal volume of buffer (1% CTAB, 5mM Tris-HCl (pH 8.0), 10mM EDTA) was added and mixed by shaking for 30 minutes at room temperature. The mixture was then centrifuged at 5000 rpm for 10 minutes. The supernatant was removed and 1.5ml of 1M NaCl-TE {1M NaCl, 10mM Tris-HCl (pH 8.0), 1mM EDTA} was mixed and warmed-up at 55°C for 2 hours to overnight to dissolve DNA. Equal volume of phenyl chloroform or chloroform (1:1) was added to the mixture. Supernatant was taken out after centrifugation at 3000 rpm for 10 minutes and was mixed with equal volume of isopropanol and kept for 2 minutes. The supernatant was removed after centrifugation at 12000 rpm for 5 minutes or 3000 rpm for 10 minutes. The pellet was then washed by 70% ethanol following centrifugation. After washing, the pellet was vacuum dried and dissolved in 50µl TE-RNase (1g/ml RNase, 1M NaCl, 10mM Tris-HCl (pH 8.0), 1mM EDTA) and kept at 55°C for 2 hours to overnight to complete dissolution and stored at 4°C.

Analysis of CAPS

Primer sequences used in CAPS (Cleaved Amplified Polymorphic Sequences) analysis are listed in Table 1. The primer sequences were designed based on the structural gene sequences published in the NCBI and TAIR databases, and in the reports of Kuittinen *et al*. (2002) and Inoue and Nishio (2004). 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 (Fig. 2). Polyacrylamide gel was prepared according to the method of (Kikuchi *et al*. 2004). The digestion was checked by 2% agarose gel before running polyacrylamide gel. An example of segregation pattern of F_2 progenies (GC P09×Reiho P01) in 13% polyacrylamide gel against CAPS primer is shown in Fig. 3.

Analysis of SRAP

Polymorphic detection by the sequence-related amplified polymorphism (SRAP) method was conducted according to the method of Li and Quiros (2001), with minor modifications (Doullah 2006). 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 Kikuchi *et al*. (2004) 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). An example of segregation pattern of F_2 progenies (GC P09 × Reiho P01) in 8% polyacrylamide gel against SRAP primer pairs is shown in Fig. 4.

Construction of Map

For making map, CAPS and SRAP markers were used. Linkage analyses were performed using JOIN MAP program, version 3.0 (Van Ooijen and Voorrips, 2001). The Kosambi mapping function was used to convert recombination frequencies into genetic (map) distances.

QTL analysis

The QTLs for *A. brassicicola* resistance were analyzed using a composite interval-mapping analysis (Zeng 1994) with Map QTL version 2.0 (Van Ooijien *et al*. 2000) and QTL Cartographer version 1.16 (Basten *et al*. 2002). 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.

RESULTS AND DISCUSSION

Disease resistance

The mean disease severity score against dark leaf spot was obtained by detached leaf inoculation method and are shown in Table 3. The two parents (susceptible and resistant) showed significant difference (t = 17.66; n = 27; *P* = 9.90) in symptom production against *Alternaria brassicicola*. F_1 hybrid showed susceptibility to the disease and F_3 plants gave a continuous distribution (Fig. 1), suggesting that dark leaf spot resistance is controlled quantitatively.

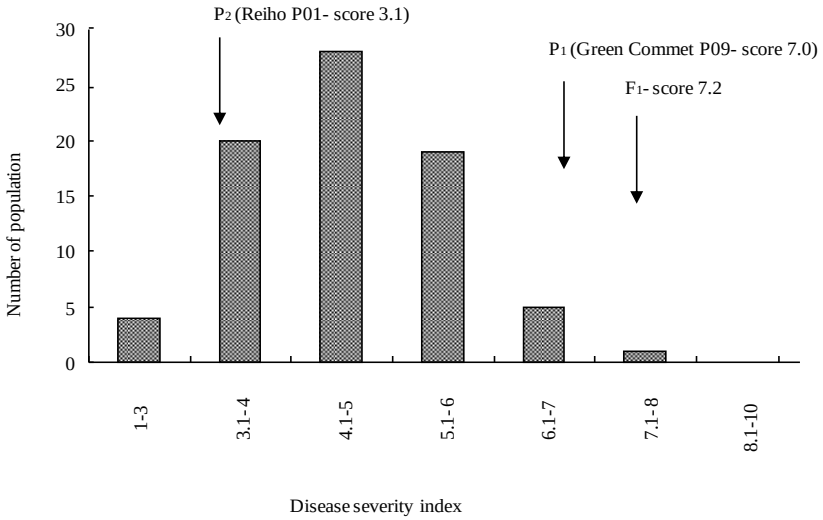

Fig.1. Frequency distribution of mean disease severity of dark leaf spot disease in F3 population (n = 29) Green commet

Linkage analysis

A total of 38 CAPS and 60 SRAP primer pairs were screened to assess parental polymorphism against dark leaf spot resistance. After restriction digestion of PCR products, polymorphic bands were detected in products generated by 35 CAPS primers pairs by electrophoresis (Table 1, Figs. 2 and 3). In the SRAP analysis, polymorphic markers were detected from 57 primer pairs (Fig. 4). Finally 92 markers were distributed in 10 linkage groups covering 320.5 cM and average interval between markers was 3.56 cM (Figure not shown) where 19 markers were in linkage groups 2 & 7 (Fig. 5).

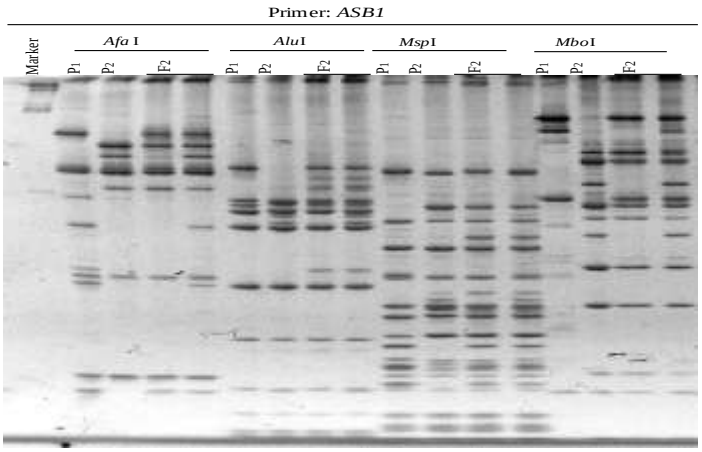

Fig. 2. Digestion of PCR amplicons by four restriction enzymes (*Afa*I, *Alu*I, *Msp*I and *Mob*I) using CAPS primer *ASB1* and separated on 13% polyacrylamide gel. Parents- Green commet P09 (P_1), Reiho P01 (P_2) and F_2 progenies derived from Green commet × Reiho are shown. Marker: λHind III

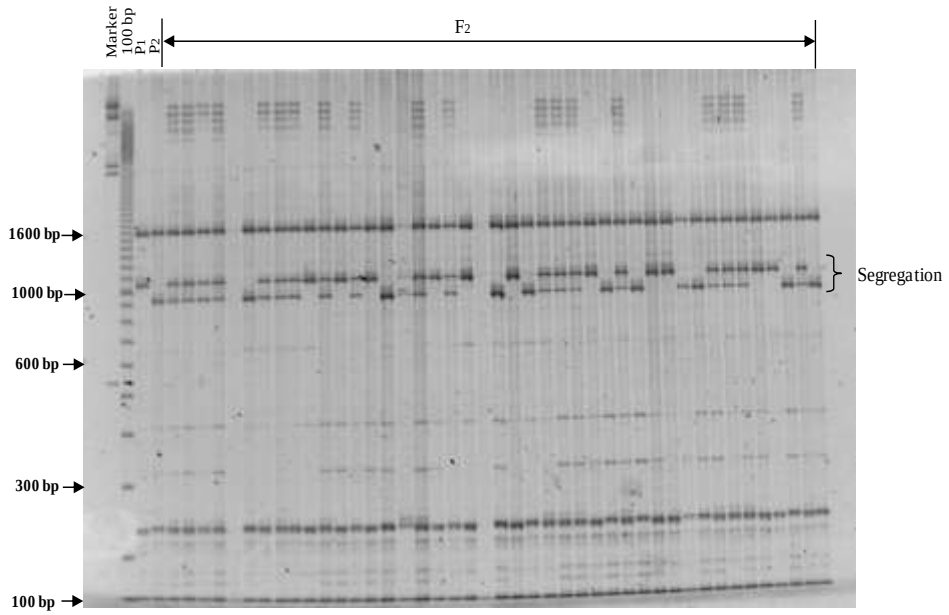

Fig. 3. CAPS- *PLC* marker and its segregation pattern of F_2 progenies (Green commet ×Reiho) in 13% polyacrylamide gel. Parents- Green commet P09 (P_1), Reiho P01 (P_2) and F_2 progenies derived from Green commet ×Reiho are shown. Marker: λHind III; 100bp: 100 base pair marker

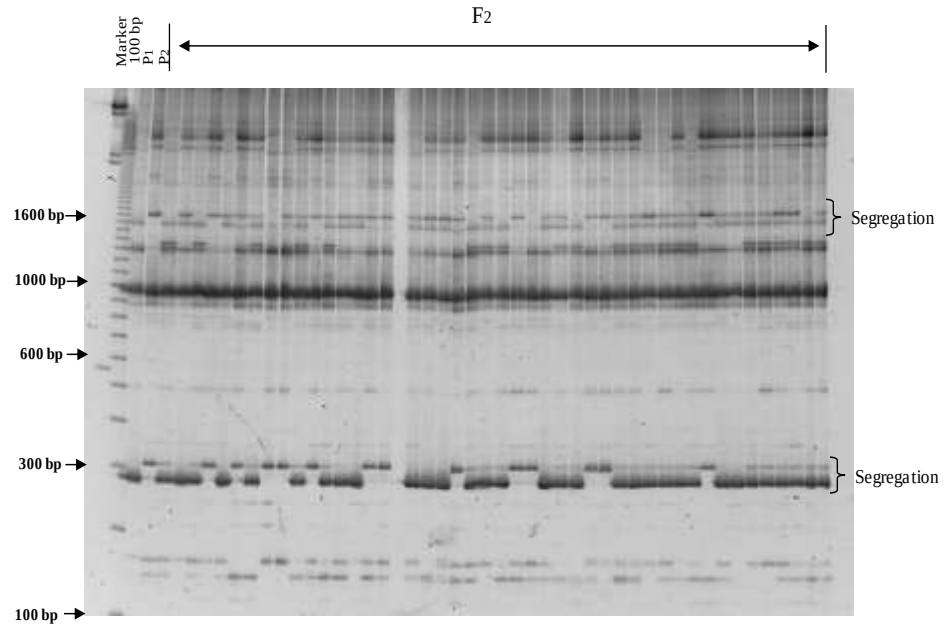

Fig. 4. SRAP- *F2-R1* marker pair and its segregation pattern of F_2 progenies (Green commet × Reiho) in 8% polyacrylamide gel. Parents- Green commet P09 (P_1), Reiho P01 (P_2) and F_2 progenies derived from Green commet × Reiho are shown. Marker: λHind III; 100bp: 100 base pair marker

Table 1. Primer sequences of CAPS markers for *Brassica oleracea* (Okazaki *et al*. 2006)

Name	Accession	Primer sequences (5' - 3')		PCR[d]	CAPS
		Forward	Reverse		
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. (2002) and [b]Inoume and Nishio (2004)
[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.

Table 2. Primer pairs used in SRAP (Okazaki *et al*. 2006)

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 reported by Li and Quiros (2001)

Table 3. Mean disease severity of Dark leaf spot (*Alternaria brassicicola*) of *Brassica oleracea* (Reiho P01, Green commet P09, F_1 and F_3 progenies derived from Green commet × Reiho)

	Dark leaf spot
Genotype	Mean (± SE[a])
Reiho P01	3.14 ± 0.36 (n[b] = 29)
Green Commet	7.00 ± 0.21 (n = 27)
F_1	7.21 ± 0.12 (n = 27)
F_3	4.66 ± 0.11 (n = 75)

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

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 dark leaf spot (*Alternaria brassicicola*) disease using F_3 populations derived from Green commet P09 × Reiho P01

Map interval	Linkage group[a]	LOD	Additive effect[b]	VE (%)[c]
A. brassicicola:				
CAM – GSA1	2	2.02	0.69	23.4
IPI – FLC3	7	4.27	-0.91	43.8

[a] Linkage groups are as in Figure 5
[b] Additive effect: ' – ' indicate resistance due to parent GC P09
[c] Percentage of variance explained by quantitative trait loci

Mapping QTLs for dark leaf spot

A total of 92 markers were used to associate markers and phenotypes in the mapping population (Table 1 & 2) discussed by Doullah (2006). The chromosomal locations of marker intervals, effect of each QTL and phenotypic variation are presented in Table 4.

For dark leaf spot disease resistance, the largest QTL effect was detected significantly in the interval between *IPI - FLC3* on LG 7, based on the LOD threshold of 4.27 in permutation test. This QTL explained up to 43.8% of the phenotypic variation. A small QTL effect explaining 23.4% of phenotypic variation was detected in the interval between *CAM1 – GSA1* on LG 2 (LOD = 2.02), which was not significant in the permutation test (Table 4 and Fig. 5).

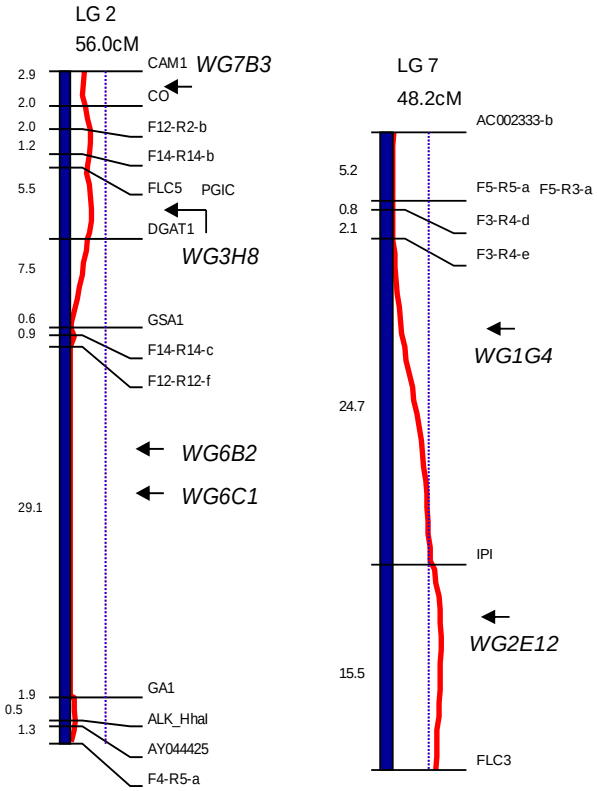

Fig. 5. QTLs of resistance to dark leaf spot disease on linkage group 2 & 7 constructed with CAPS, SRAP markers. Markers with romam letters have been incorporated from the linkage map of flowering time (Okazaki *et al*. 2006). LG: Linkage group; cM: Centi Morgan

Alternaria brassicicola (dark leaf spot) a common seed-borne pathogen of *Brassica* crops (Bassey and Gabrielson, 1983; King 1994; Rotem 1998). The inoculation was made by spraying inoculum directly on the detached leaf described by Doullah *et al*. (2006).

One QTL significantly associated with *A. brassicicola* resistance was found on LG 7 (LOD = 4.27) in the interval between *IPI – FLC3*. This is the first QTL identified for resistance to *A. brassicicola* to be mapped in *Brassica oleracea*. The additive effect of the QTL was – 0.91 indicating resistance from parent 'GC P09'. This may be due to transgressive segregation of some F_3 progenies where alleles from the susceptible parent were associated with resistance at one locus on LG 7. We identified one QTL on LG 2 interval between *CAM – GSA1*, which was not significant but revealed high LOD value of 2.02. The additive effect of the QTL was 0.69 indicating resistance has from parent Reiho P01. King (1994) determined heritability of resistance to *A. brassicicola* in *B. oleracea* but he did not map the QTL associated with the resistance.

Glucosinolates play an allelopathic role in plant resistance against fungi, bacteria, nematodes, herbivores and weeds (Giamoustaris and Mithen, 1997; Kushad *et al*. 2004). The level of Alternaria infection in leaves of *B. napus* was positively correlated with glucosinolate content but not for *Leptosphaeria maculans* (Giamoustaris and Mithen, 1997). Two QTLs associated with glucosinolate content were identified as GSL of LG 1 and ALK of LG 2 in our previous experiment using the same progenies (data not shown). We identified QTL for resistance to *A. brassicicola* on LG 7 indicating no relationship with glucosinolate content for disease development. This result is not in agreement with the result of Giamoustaris and Mithen (1997) who reported positive relationship between glucosinolate content and resistance of *A. brassicicola*. It may be explained that necrotrophic and hemibiotrophic pathogens like *Alternaria* spp. have ability to detoxify secondary metabolites within the host (Giamoustaris and Mithen, 1997).

In our studies, we identified QTLs resistance to *A. brassicicola* for the first time and we also reconfirmed the resistant locus using different cross combination of *B. oleracea* that would facilitate marker-assisted selection to improve dark leaf spot in *Brassica* crops.

CONCLUSION

Quantitative trait loci (QTL) controlling resistance to *A. brassicicola* was mapped using cleaved amplified plymorphic sequences (CAPS) and sequence-related amplified polymorphism (SRAP) analysis with disease rating of F_3 families from a susceptible broccoli 'GC P09' × resistant cabbage 'Reiho P01'. Detached leaf inoculation test was used for dark leaf spot disease. A total of 38 CAPS and 60 SRAP primer pairs were screened to assess parental polymorphism against the disease resistance where 92 markers were distributed in 10 linkage groups (LGs) covering 320.5 cM, with average 3.56 cM interval between the markers. One QTL resistance to *A. brassicicola* was detected significantly in the interval *IPI - FLC3* on LG 7 based on the LOD threshold of 4.27 explaining up to 43.8% of the phenotypic variation. This is the first QTL detected resistance to *A. brassicicola*.

ACKNOWLEDMENT

This work was supported by the Ministry of Education, Culture, Sports, Science and Technology, Government of Japan (Monbukagakusho-MEXT).

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