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ORIGINAL PAPER

Identification of QTLs that control clubroot resistance in *Brassica oleracea* and comparative analysis of clubroot resistance genes between *B. rapa* and *B. oleracea*

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Abstract To perform comparative studies of *CR* (clubroot resistance) loci in *Brassica oleracea* and *Brassica rapa* and to develop marker-assisted selection in *B. oleracea*, we constructed a *B. oleracea* map, including specific markers linked to *CR* genes of *B. rapa*. We also analyzed *CR*-QTLs using the mean phenotypes of F_3 progenies from the cross of a resistant double-haploid line (Anju) with a susceptible double-haploid line (GC). In the nine linkage groups obtained (O1-O9), the major QTL, *pb-Bo(Anju)1*, was derived from Anju with a maximum LOD score (13.7) in O2. The QTL (LOD 5.1) located in O5, *pb-Bo(GC)1*, was derived from the susceptible GC. Other QTLs with smaller effects were found in O2, O3, and O7. Based on common markers, it was possible to compare our finding *CR*-QTLs with the *B. oleracea CR* loci reported by previous authors; *pb-Bo(GC)1* may be identical to the *CR*-QTL reported previously or a different member contained in the same *CR* gene cluster. In total, the markers linked to seven *B. rapa CR* genes were mapped on the *B. oleracea* map. Based on the mapping position and markers of the *CR* genes, informative comparative studies of *CR* loci between *B. oleracea* and *B. rapa* were performed. Our map discloses specific primer sequences linked to *CR* genes and includes public SSR markers that will promote pyramiding *CR* genes in intra- and inter-specific crosses in Brassica crops. Five genes involved in glucosinolates biosynthesis were also mapped, and *GSL-BoELONG* and *GSL-BoPro* were found to be linked to the *pb-Bo(Anju)1* and *Bo(GC)1* loci, respectively. The linkage drag associated with the *CR*-QTLs is briefly discussed.

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Introduction

Clubroot disease is caused by a soil-borne obligate biotroph, *Plasmodiophora brassicae*, and is one of the most devastating diseases in Brassica crops worldwide (Crute et al. 1980; Hirai 2006). The pathogen causes gall formation on roots in the shape of a club or spindle. Severely infected roots cannot take up sufficient amounts of nutrients and water, and such infected plants are stunted and wilt in direct sunlight, causing a reduction in yield of both vegetable and oleiferous Brassicas (Voorrips 1995). Agricultural practices such as crop rotation, application of calcium to raise the pH, good drainage and sanitation, as well as use of agrochemicals are insufficient to control clubroot disease (Dobson et al. 1983; Voorrips 1995; Niwa

et al. 2008). In addition, it is difficult to control the disease because the pathogen survives in soil as resting spores for long periods (Wallenhammar 1996). Therefore, development of genetically resistant cultivars to minimize crop losses caused by clubroot disease is highly desired.

Various genetic loci encoding clubroot resistance (*CR*) were extensively screened in *Brassica oleracea* (Dixon and Robinson 1986; Crips et al. 1989; Dias et al. 1993; Manzanares-Dauleux et al. 2000). However, only a few resistant populations such as German cabbage landrace 'Bindsachsener' and 'Böhmerwaldkohl' are available for resistance breeding with *B. oleracea* (Crute et al. 1983; Crips et al. 1989; Voorrips 1995; Diederichsen et al. 2009). Rutabaga cv. 'Wilhelmsburgar' is a known source of resistance to the clubroot pathogen in *Brassica napus*. Chiang and Crete (1983) successfully transferred *CR* genes from *B. napus* to *B. oleracea*. Although cultivars resistant to clubroot disease are bred using such strategies, production of durable resistant *B. oleracea* cultivars has not been successful due to poor knowledge of the inheritance of resistance and complexity of the plant–pathogen interaction.

Studies have disagreed as to whether clubroot resistance in *B. oleracea* is a qualitative or a quantitative trait. In reports describing clubroot resistance as qualitative, clubroot resistance can be either dominant (Chiang and Crete 1983) or recessive (Chiang and Crete 1970; Yoshikawa 1983). When employing QTL (quantitative trait loci) analysis for genetic behavior of resistance to clubroot, on the other hand, authors usually conclude that clubroot resistance in *B. oleracea* progenies is quantitative and under polygenic control, illustrating the existence of one or two major QTLs and some QTLs with minor effects (Landry et al. 1992; Figdore et al. 1993; Grandclement and Thomas 1996; Voorrips et al. 1997; Moriguchi et al. 1999; Rocherieux et al. 2004; Nomura et al. 2005). Because none of these studies has disclosed specific primer sequences or sequences of restriction fragment length polymorphism (RFLP) markers linked to the *CR* genes, additional studies that disclose these data are necessary to develop marker-assisted selection for clubroot resistance in *B. oleracea* (Hirai 2006). In *Brassica rapa*, many studies have demonstrated oligogenic control of clubroot resistance (reviewed by Hirai 2006; Piao et al. 2009). In total, eight *CR* loci were mapped and allocated to five different *B. rapa* chromosomes. The authors reported the specific primer sequences of the DNA markers linked to the *B. rapa CR* genes so that marker-assisted selection is now available for clubroot resistance. It is also now possible to compare whether the published *CR* genes are mutually homologous. The genes *Crr2*, *CRc*, *Crr4*, and *Crr1* map to R1, R2, R6, and R8 of *B. rapa* linkage groups, respectively. The genes *CRa, CRb, CRk*, and *Crr3* map to R3. *CRk* and *Crr3* map closely together in R3 but are not at the same locus because these two loci were derived from different *CR* origins and had been inoculated with different isolates and conditions (Sakamoto et al. 2008). Therefore, it is important to clarify whether the *CR* genes identified so far are homologous; intra- and inter-specific comparison of *B. oleracea* and *B. rapa* is a means to address this issue.

The aims of this study were (1) to identify PCR-based markers linked to *CR* genes in *B. oleracea*, (2) to report the specific marker sequences of the *CR* genes to develop marker-assisted selection to clubroot resistance in *B. oleracea*, and (3) to perform a comparative study of *CR* genes in *B. rapa* and *B. oleracea* by simultaneously mapping the DNA markers linked to the *CR* genes identified in this study and in previous reports.

Materials and methods

Plant materials

Double-haploid (DH) lines were obtained from the clubroot-resistant cabbage cultivar 'Anju' (Nippon Norin Seed Co., Japan) and the susceptible broccoli cultivar 'Green Comet' (GC) (Takii & Co., Ltd.) using microspore culture. A preliminary inoculation test using a *Plasmodiophora brassicae* isolate revealed that the Anju DH line (P01) and GC DH line (P04) were resistant and susceptible, respectively. The GC DH line (P04) was crossed as the female parent to the Anju DH line (P01). A single F_1 plant was self-pollinated to produce F_2 seeds. F_2 plants were self-pollinated to produce F_3 seeds.

Pathogen isolation and inocula preparation

The isolate of *P. brassicae* that was determined as race 4 using the inoculation method of Williams (1966) was provided by A. Kiso (Musashino Seed Co., Ltd.). The pathogen was propagated on turnips, and the clubs in infected roots were stored at −20°C until required. Inocula were prepared from slowly thawed clubs. The clubs were ground in distilled water using a mortar and pestle, and the homogenized tissue was squeezed through four-layered gauze. The squeezed fluid was gathered and centrifuged at $1000 \times g$ for 10 min at room temperature. The pellet, which contained resting spores, was suspended in sterile distilled water. The final resting spore concentration was adjusted to 10^7 spores/ml using a hemocytometer.

Test for clubroot resistance

Seeds were germinated in 8-cm-diameter plastic pots containing soil in a greenhouse. Twelve plants of each

Table 1 DNA markers used in this study

Marker symbols	Types of Markers used	Species of Marker origin	Notes
BRAS	SSR	*B. napus*	Cited from Piquemal et al. (2005), Radoev et al. (2008)
BnGMS	SSR	*B. napus*	Cited from Cheng et al. (2009)
BRMS	SSR	*B. rapa*	Cited from Suwabe et al. (2006)
BrSTS	STS	*B. rapa*	Cited from Saito et al. (2006)
CB	SSR	*B. napus*	Cited from Piquemal et al. (2005), Radoev et al. (2008)
FITO	SSR	*B. oleracea*	Cited from Iniguez-Luy et al. (2008)
F_R_	SRAP	*B. oleracea*	Cited from Okazaki et al. (2007)
HC352R	SCAR	*B. rapa*	Hayashida et al. (2008). Primers were designed from sequences collected from the NCBI nucleotide database.
IGF	SNP	*B. napus*	Qiu et al. (2006). Primers were designed from sequences collected from the IMSORB nucleotide database.
KBr	SSR	*B. rapa*	Designed from terminal sequences of BAC clones released from the *B. rapa* genome project.
KBr_N1	SSR	*B. rapa*	Designed from terminal sequences of BAC clones released from the *B. rapa* genome project.
MD	SSR	*B. napus*	Cited from Radoev et al. (2008)
ME_OD_ ME_GA_	SRAP	*B. oleracea*	Cited from Li et al. (2003)
MR	SSR	*B. napus*	Cited from Radoev et al. (2008)
m6R	STS	*B. rapa*	Cited from Sakamoto et al. (2008)
Na	SSR	*B. napus*	Cited from Piquemal et al. (2005), Radoev et al. (2008)
Ni	SSR	*B. nigra*	Cited from Piquemal et al. (2005), Radoev et al. (2008)
Ol	SSR	*B. oleracea*	Cited from Piquemal et al. (2005), Radoev et al. (2008)
pW pX	CAPS	*B. napus*	Udall et al. (2005). Primers were designed from sequences collected from the NCBI nucleotide database.
TCR05	SCAR	*B. rapa*	Cited from Piao et al. (2004)

parent and 12 F_1 plants were used for the inoculation test. For the F_3 test, a subset of 94 F_3 progeny obtained from randomly selected F_2 plants was sown, and 12 plants per F_3 progeny were used for phenotypic evaluation. One-week-old seedlings were inoculated by applying 1 ml of spore suspension at the bottom of the stem base of each seedling (pipette method). The inoculated plants were grown during spring months in a greenhouse at a maximum temperature of 25°C. The soil was kept moist throughout the test. Four plants in each F_3 strain were grouped to make one replication, and each strain was tested in randomized complete block design with three replications. The plants were evaluated for clubroot infection 6 weeks after inoculation. The roots were thoroughly washed. The status of each root system was rated on a scale of 0–5, where 0 = no clubs, 1 = a few small clubs usually confined to lateral roots, 2 = moderate clubbing on lateral roots, 3 = larger clubs on lateral roots and slight swelling of main roots, 4 = larger clubs in main roots, and 5 = severe clubbing (no roots left, only one big gall). The disease severity index (DI) was calculated from the results as the mean value for the 12 F_3 seedlings. The phenotype evaluation was carried out twice, once in 2006 and once in 2008, using the same F_3 seed obtained from each F_2 plant, and the mean grades of the two F_3 progeny tests were calculated.

Detection of DNA polymorphism

Healthy leaves harvested from the parents and 94 F_2 individuals were used for genomic DNA extraction. Total genomic DNA was isolated using the cetyltrimethyl-ammonium bromide method (Murray and Thompson 1980). The DNA markers used in this study are shown in Table 1. Polymorphic detection using the sequence-related amplified polymorphism (SRAP) method was conducted according to the method of Li and Quiros (2001), with minor modifications. Simple sequence repeat (SSR) markers were obtained from Piquemal et al. (2005), Suwabe et al. (2006), Iniguez-Luy et al. (2008), Radoev et al. (2008), and Cheng et al. (2009). The *B. rapa* bacterial artificial chromosome (BAC) end sequences were obtained from the *B. rapa* genome project (http://www.brassica-rapa.org/BrGP/geneticMap.jsp) and the DDBJ search engine ARSA (http://arsa.ddbj.nig.ac.jp/top-j.html). SSR primers were then designed using the read2Marker program (Fukuoka et al. 2005) or FastPCR software (Kalendar

et al. 2009) and are denoted by KBr and KBr_N1, respectively. Structural genes were amplified using the primer sequences reported by Kuittinen et al. (2002), Okazaki et al. (2007), and Gao et al. (2007). Sequences of the RFLP probes (pW, pX, IGF) reported by Udall et al. (2005) and Qiu et al. (2006) were obtained from the NCBI nucleotide database, and the specific primers were designed for amplification of sequences of the RFLP probes. The obtained PCR products were used for cleaved amplified polymorphic sequences (CAPS) analysis. Similarly, the sequences of the RFLP markers, WG1G5 and WG6H1, which were linked to *B. oleracea CR*-QTLs (Nomura et al. 2005), were obtained from the NCBI database, and specific primers were designed to amplify those markers. The WG markers had been originally developed by Dr. Thomas Osborn (University of Wisconsin), and thereafter were renamed as pW markers; the RFLP markers, WG1G5 and WG6H1 were renamed pW216 and pW237, respectively. The primer sequences are shown in electronic Supplementary Table 2.

Annealing temperatures and extension times for PCR were determined according to the primer sequence and gene size. The PCR products were digested with one of four restriction enzymes (*Afa*I, *Alu*I, *Msp*I, or *Mbo*I). To identify the positions of the *B. rapa CR* genes in the *B. oleracea* map, we used the PCR-based markers reported by Suwabe et al. (2003, 2006), Piao et al. (2004), Saito et al. (2006), and Sakamoto et al. (2008). In addition, to identify the position of another *CR* gene, *CRa*, in *B. rapa*, a primer pair, HC352F2 and HC352R2, was designed as 5′-gctacaccaaaagattcgag-3′ and 5′- tgtccttcatagacaatgac-3′ based on the closest marker (HC352; Accession No. AB302983) to *CRa*. The amplified product was digested with *Alu*I before polyacrylamide gel electrophoresis.

Electrophoresis was conducted using an 8–13% polyacrylamide gel (Kikuchi et al. 2003). The gel was stained with a Gelstar solution (0.1 μl/10 ml; Takara Biomedicals, Japan).

Construction of the map and QTL analysis

Linkage analysis of the markers was performed using the program Antmap 1.2 (Iwata and Ninomiya 2006). QTLs for clubroot resistance were analyzed using a composite interval mapping (CIM) analysis (Zeng 1994) with QTL Cartographer version 2.5 (Basten et al. 2002). A 1,000-permutation test was performed with QTL Cartographer to estimate the appropriate significance threshold of a logarithm of odds (LOD) score for analysis.

Synteny analysis of the QTL regions

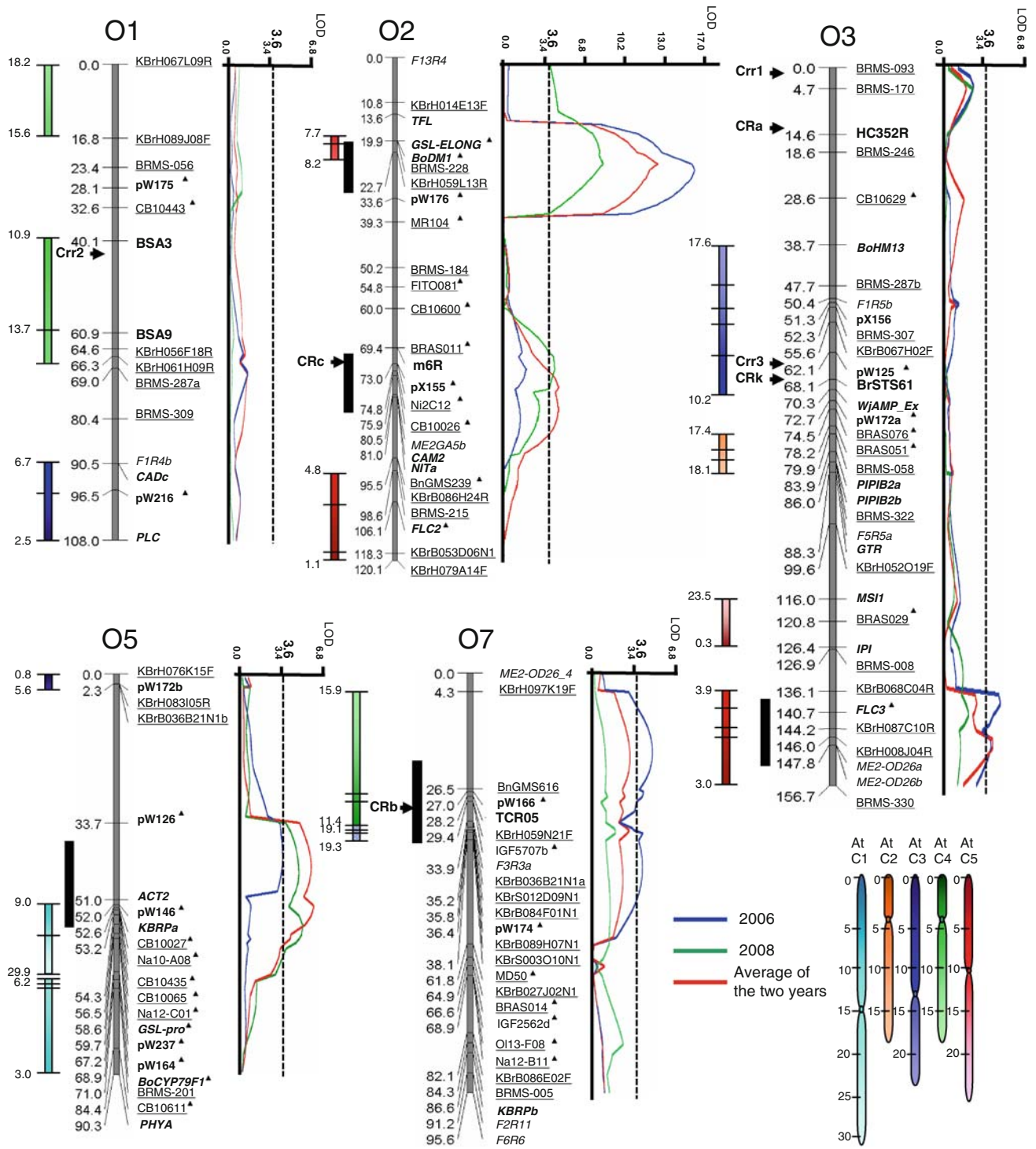
To identify syntenic regions between the *B. rapa* and *B. oleracea* genomes, KBr markers designed with the BAC sequences released from *BrGP* were aligned with the JWF3p map published on the *BrGP* web site (http://www.brassica-rapa.org/BRGP/index.jsp). In addition, we compared our map with the *B. rapa* maps published by Suwabe et al. (2006) and Sakamoto et al. (2008). To identify homologous regions between the *Arabidopsis thaliana* genome and our map, the sequences harboring the markers were aligned with the *A. thaliana* genome sequence using BLASTn in DDBJ. Based on a threshold value of $E < 10^{-10}$, we identified regions that were relatively conserved between the *A. thaliana* genome and our map. Groups of two or more markers showing homology and collinearity with *A. thaliana* were regarded as syntenic regions.

Fig. 1 Linkage map developed in a segregating F_2 population of broccoli 'GC' (P04) × cabbage 'Anju' (P01), and LOD profiles for clubroot resistance. LOD score profiles of the first test, second test, and the average of the two tests are shown as *blue*, *green*, and *red lines*, respectively. The threshold value (3.6) for the average of the two tests is shown as a *dashed line*. Linkage groups (O1, O2, O3, O5, O7) that internationally agree with *B. oleracea* reference linkage group nomenclature are indicated at the top of each linkage group. Locus names are indicated on the *right side* of linkage groups, and map distances in centimorgans are on the *left*. The markers used to assign linkage groups are marked with *black triangles*. The regions syntenic with *A. thaliana* are shown to the *left* of the linkage groups as *colored vertical bars*, which represent different chromosomes of *A. thaliana*. The positions of the homologous markers of *A. thaliana* are shown with the *horizontal lines* attached to the *colored vertical bars*, and the megabase distances in both ends of each syntenic region are given to the *left*. The details of the homologous markers of *A. thaliana* are shown in the supplementary data. Confidence intervals for QTL positions above 10:1 are indicated with *vertical black boxes*. *Arrows* indicate locations of the markers linked to *B. rapa CR* genes. Markers are denoted as follows. SRAP markers: *italic*; CAPS markers: *bold italic*; SSR markers: *underlined*; STS markers: *bold roman*

Results

Construction of linkage maps

The linkage map of the F_2 progeny derived from GC × Anju was constructed using SSR, CAPS, SRAP, insertion/deletion, and sequence-tagged site (STS) markers (Table 1; Fig. 1). For the SRAP analysis, 20 polymorphic loci were detected using combinations of 9 forward primers and 8 reverse primers, as reported by Li and Quiros (2001) and Okazaki et al. (2007). Of the 46 KBr_N markers designed from the BAC sequences that were derived from the *B. rapa* genome project, 9 polymorphic SSR loci were detected. The other 530 SSR markers, including 288 KBr, 96 BRMS, 58 CB, 20 FITO, 19 BnGMS, 23 Na/Ol, 12 MD/MR, 13 BRAS, and 1 Ni markers, were used so that 121 polymorphic SSR markers were detected. By amplifying structural genes and RFLP sequences, 71 polymorphic bands were obtained in insertion/deletion and CAPS



markers. The genetic linkage map we constructed spans nine linkage groups with a total distance of 1,048.6 cM. The length of each linkage group and the number of markers included in each linkage group are given in the electronic supplementary Table 1. To align our map to the internationally accepted Brassica map, we used public SSR, CB, IGF, pW, and pX markers reported by Parkin et al. (2005), Piquemal et al. (2005), Udall et al. (2005), Qiu et al. (2006), and Okazaki et al. (2007).

QTL analysis for clubroot resistance

For the inoculation test, the resistant parent 'Anju'(P01) had a DI of 0, whereas the susceptible parent 'GC'(P01)

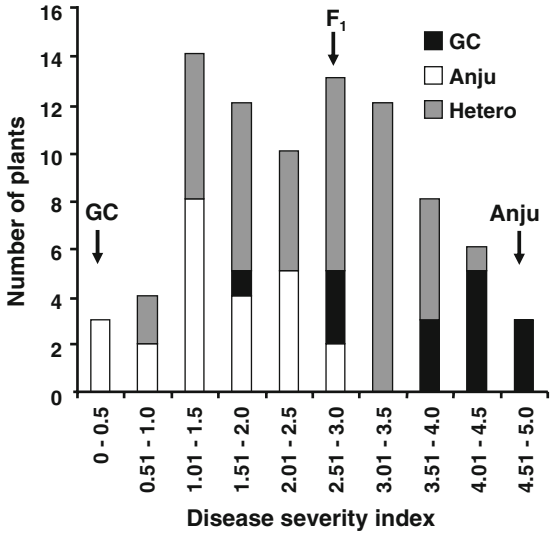


Fig. 2 Frequency distribution of disease severity index in the F_3 progeny. Arrows indicate positions of the parental and F_1 plants in the distribution. The homozygotes of 'Anju' (P01), homozygotes of 'GC' (P04), and the heterozygotes at the KBrH059L13R locus in the F_2 population are shown by *open bars*, *black bars*, and *gray bars*, respectively

had a DI of 5 (Fig. 2). The F_1 population had a DI of 2.7. The mean values for disease severity of F_3 progenies showed a continuous distribution pattern. QTL analysis was performed using the appropriate significance threshold calculated in the permutation test and detected several significant QTLs (Table 2; Fig. 1). These results indicated that Anju resistance was controlled by a polygenic system.

QTL analysis was carried out separately for each of the 2006 and 2008 tests and the average of the 2 years. Five QTLs for clubroot resistance were detected in O2 (two regions), O3, O5, and O7. Among them, the largest QTL located in O2 was detected in both years and in the combined data of the 2 years. The scores for the minor effect QTLs varied between the 2 years; the QTLs located in O3 and O7 exceeded the significance threshold score in 2006, and the minor effect QTLs in O2 and O5 were significant in 2008. These minor QTLs were significant for the combined data of the 2 years, except for the QTL located in O7 that had a score of 3.1, comparable to the threshold score (3.5). The largest QTL effect (LOD of 13.7) for clubroot resistance was detected between the loci *TFL* and *pW176* on O2 and was closely linked to marker KBrH059L13R (Fig. 1; Table 2). This QTL explained 47% of the total phenotypic variation. This *CR* locus was named *pb-Bo(Anju)1*. The QTL located in O5 came from the susceptible broccoli parent (Table 2), and therefore this *CR* locus was named *pb-Bo(GC)1*. Despite high susceptibility of the susceptible parent GC to *P. brassicae*, this *CR* locus accounted for only 9% of the variation, suggesting that there may be epistatic genes that interact with *Bo(GC)1* in other regions of the genome. Other small-effect QTLs found in O2, O3, and O7, which came from the resistant parent, accounted for 16% of the variation. These *CR* loci on O2, O3, and O7 were

Table 2 Summary of QTLs detected for clubroot resistance against *P. brassicae*

Name	Linkage Group		Closest marker	Marker position (cM)	LOD	R^{2a}	Additive effect[b]	Dominance effect
PbBo(Anju)1	O2	Average	KBrH059L13R	22.5	13.7	0.47	1.31	0.03
		1st Test	KBrH059L13R	22.6	17.3	0.62	1.44	0.08
		2nd Test	KBrH059L13R	22.2	8.8	0.26	0.86	0.26
PbBo(Anju)2	O2	Average	CAM2	80.1	4.9	0.04	0.16	0.62
		1st Test	ME2GA5b	76.3	2.1 ns	0.02	0.22	0.53
		2nd Test	pX155	72.5	4.6	0.05	0.01	0.51
PbBo(Anju)3	O3	Average	KBrH008J04R	146.5	4.1	0.09	0.48	0.5
		1st Test	KBrB068C04R	138.3	4.3	0.11	0.41	−0.1
		2nd Test	FLC3	140.8	1.9 ns	0.03	0.22	−0.02
PbBo(Anju)4	O7	Average	KBrB084F01N1	35.8	3.1 ns	0.03	0.37	0.07
		1st Test	KBrB089H07N1	36.5	4.3	0.03	0.42	0.18
		2nd Test	KBrB084F01N1	35.7	1.9 ns	0.01	0.23	0.12
PbBo(GC)1	O5	Average	CB10027	53.2	5.1	0.09	−0.54	−0.19
		1st Test	ACTb	44.2	3.1 ns	0.06	−0.53	−0.04
		2nd Test	CB10435	57.8	4.7	0.16	−0.72	0.17

The inoculation tests carried out in 2006 and 2008. The threshold values for the average of the two tests, first test, and second test, were 3.6, 3.8, and 3.6, respectively

ns Non-significant at 0.05 probability with 1,000 permutation tests

[a] R^2 The proportion of the phenotypic variance explained by each QTL

[b] Additive effects of the GC allele

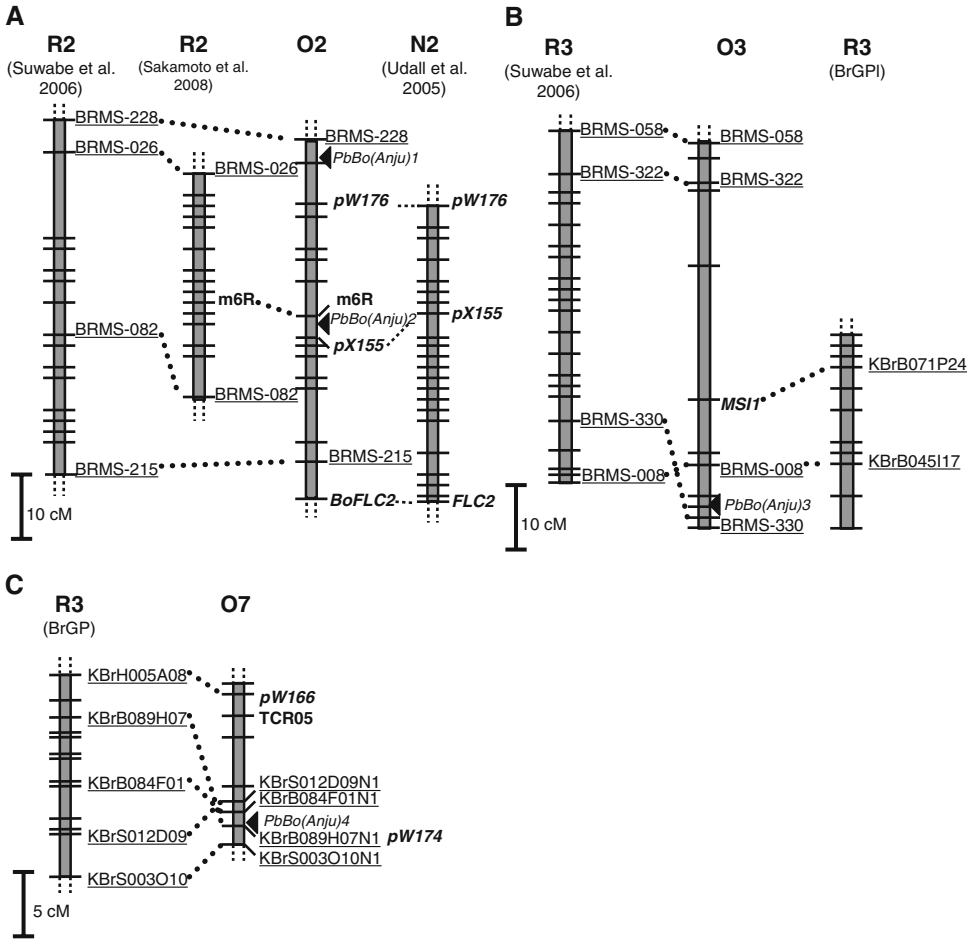


Fig. 3 Identification of the homologous region of *B. oleracea* linkage groups containing *CR*-QTLs with that of *B. rapa* (Suwabe et al. 2006; Sakamoto et al. 2008, BrGP) and *B. napus* (Udall et al. 2005) linkage groups. Positions of molecular markers are shown as *horizontal lines*, and only the names of markers commonly mapped to each linkage group are shown here. The positions of *pbBo(Anju)1* and *pbBo(Anju)2* (**a**), *pbBo(Anju)3* (**b**), and *pbBo(Anju)4* (**c**) are shown

named *pb-Bo(Anju)2*, *pb-Bo(Anju)3*, and *pb-Bo(Anju)4*, respectively.

Phenotypic DI at the KBrH059L13R marker that was closely linked to the major QTL indicated that higher resistance was associated with the homozygous Anju genotype versus the homozygous GC genotype, with the heterozygotes having varying resistance levels (Fig. 2).

Mapping of molecular markers closely linked to *B. rapa CR* genes

The synteny map data with the common BRMS and other molecular markers revealed that the region of O2 harboring *pb-Bo(Anju)1* and *pb-Bo(Anju)2* exhibits conserved synteny to the corresponding region of R2 of *B. rapa* where *CRc* was detected (Fig. 3a). The distal end of O3 harboring *pb-Bo(Anju)3* corresponded to that of R3 (Fig. 3b). The marker TCR05 that was closely linked to *CRb* was mapped to the middle of O7 where the *pb-Bo(Anju)4* QTL (closest marker, KBrS012D09N1) was located (Fig. 3c). Around this region, several markers that originated from the BAC sequences of R3 of *B. rapa* were also mapped. In addition, the pW166 marker that was closely linked to TCR05 was similar to the sequence of the BAC clone (KBrH005A08) that originated from R3. These results indicated that the region in O7 harboring *pb-Bo(Anju)4* is homologous to a part of the *B. rapa* R3 chromosome where *CRb* was mapped. Similarly, the published markers that were closely linked to *B. rapa CR* genes were mapped to our map; BSA3 (closely linked to *Crr2*) mapped to O1. BrSTS61 (*Crr3*), BRMS-093 (*Crr1*), and HC352b (*CRa*) mapped to O3 (Figs. 1, 4).

Discussion

Quantitative resistance to *P. brassicae*

In the phenotypic evaluation, the F_1 plants obtained from the cross of susceptible GC and resistant Anju were partially resistant to *P. brassicae*. The F_3 progenies revealed typical continuous distributions for clubroot resistance. This type of continuous trait is controlled not only by multiple genes but also by a few individual genes that reveal continuous distribution in their progeny due to environmental effects

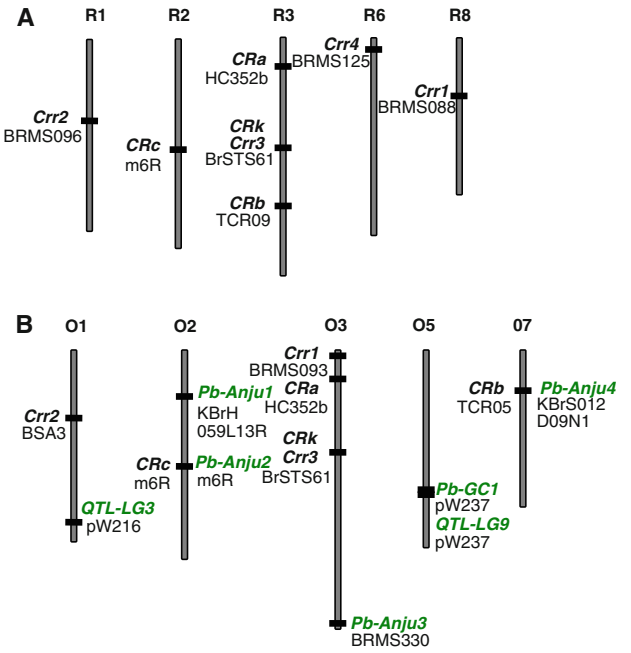


Fig. 4 Schematic of chromosomal locations of *CR* loci and the linked markers in *B. rapa* and *B. oleracea*. The numbers at the top indicate internationally agreed upon *Brassica* reference linkage groups. **a** Eight *B. rapa CR* genes and the linked markers: *Crr1*, *Crr2*, *Crr4* (Suwabe et al. 2006); *CRc*, *CRk* (Sakamoto et al. 2008); *CRa* (Hayashida et al. 2008); *CRb* (Piao et al. 2004); *Crr3* (Saito et al. 2006). **b** The *B. rapa CR* genes and the markers used to map them to the *B. oleracea* linkage groups are indicated on the *left side* of the linkage groups, and the *B. oleracea CR* genes and the markers used are on the *right side* of the linkage groups: *QTL-LG3*, *QTL-LG9* (Nomura et al. 2005); *pb-Anju 1-4*, *pb-GC1* (this study)

and experimental error when measuring the phenotype. In our study, the QTL analysis that was performed using the appropriate significance threshold successfully detected several significant QTLs, indicating that Anju resistance was controlled by a polygenic system. The fact that no plants exhibited transgressive segregation beyond the range between the two parents suggests that a large number of QTLs did not contribute to clubroot resistance in the progeny, and every QTL for clubroot resistance/susceptibility converged into either of the parental genotypes, a resistant homozygote (Anju type), or a susceptible homozygote (GC type). Alternatively, the DI used in this study, which was not a continuous variable, may be not suitable for detecting transgressive segregation in the progeny.

In the QTL analysis, we detected one major locus (*pb-Bo(Anju)1* on O2 that accounted for 47% of the variation) and some QTLs with minor effects on O2, O3, O5, and O7. The QTL with the largest effect exhibited good stability in the 2006 and 2008 tests. The other QTLs showed annual variation, which may be due to an environmental effect or inoculation conditions. The minor effect QTLs were significant for at least one of the two inoculation tests, and three of the four minor QTLs were significant using the combined data of the two tests. A candidate QTL (LOD of 3.1), detected in the distal end of O7, was comparable to the threshold score (3.5). Therefore, although the minor QTL effects are thought to be located on O2, O3, O5, and O7, it will be essential to isolate the individual QTLs through recombination and selection in the progeny to reassess the effect of individual QTLs.

Genotyping at the KBrH059L13R marker that was closely linked to *pb-Bo(Anju)1* showed that the homozygous Anju genotypes were highly resistant, whereas the homozygous GC genotypes were susceptible, and the heterozygotes tended to express partial resistance. This result suggests that *pb-Bo(Anju)1* acts as a partially dominant gene in heterozygous plants or F_1 plants and has notably established stable expression in homozygous plants. It is well known that major QTL effects that account for more than 50% of the phenotypic variation contribute to *B. oleracea* clubroot resistance (Landry et al. 1992; Voorrips et al. 1997; Rocherieux et al. 2004). The intermediate value of DI to clubroot resistance in F_1 plants is in agreement with the report of Figdore et al. (1993), who reported that the DI of the F_1 plant derived from the cross of susceptible cauliflower and resistance broccoli was intermediate, and the F_2 population studied revealed the polygenic control of clubroot resistance that involves one major QTL effect and some minor QTL effects. The stable expression of resistance in homozygotes at the *pb-Bo(Anju)1* locus indicates that the *CR* genes cumulatively act in a dose-dependent manner, which is consistent with the results reported by Suwabe et al. (2003) and Nomura et al. (2005). Previous studies identified *B. oleracea* clubroot resistance genes in either a dominant (Hansen 1989; Landry et al. 1992; Figdore et al. 1993; Laurens and Thomas 1993) or recessive manner (Yoshikawa 1983; Voorrips and Visser 1991). The expression levels of the *CR* genes that are relevant to the dominant-recessive relationship are thought to be determined by the specific *CR* gene itself, differential races of *P. brassicae*, diverse genetic backgrounds of plants, environmental conditions, etc. Which factor contributes the most to the control of *CR* gene expression remains to be determined. Furthermore, studies are needed to understand the interaction of resistance genes with pathogenicity genes.

Linkage drag between the *CR* genes and glucosinolate pathway genes

The largest QTL, *pb-Bo(Anju)1*, was located on the distal end of O2. The QTL *Bo(GC)1* was located on the central region of O5. In those regions, *GSL-BoELONG* and *GSL-BoPro*, which control the chain elongation of aliphatic glucosinolates, were found to be linked to the *pb-Bo(Anju)1* and *Bo(GC)1* loci, respectively. This observation indicates

that this linkage drag can lead to a correlation between specific glucosinolates and resistance to *P. brassicae*. In fact, Chong et al. (1985) found that the level of goitrin, a sulfur-containing metabolite that modulates thyroid hormone production, was higher in selected clubroot-resistant cabbages than in commercial cultivars. Similarly, Chiang et al. (1989) found a correlation between a low level of thiocyanate and clubroot disease in broccoli. In our study, using simultaneous mapping of the *CR* genes and the glucosinolate pathway genes, we clarified the reason why these groups found correlations between specific glucosinolates and resistance to *P. brassicae*. In general, glucosinolates and their breakdown products are thought to play a role in disease resistance against insects and fungal pathogens (Glen et al. 1990; Menard et al. 1999). However, no evidence has been reported showing that glucosinolates and their hydrolysis products are protective against *P. brassicae* (Chong et al. 1985; Chiang et al. 1989). Likewise, when we mapped the glucosinolate pathway genes to the *B. oleracea* map, no QTLs were detected around the positions of *GSL-BoALK*, *GSL-BoOH*, or *GSL-BoCYP79F1*, and the positions of *GSL-BoELONG* and *GSL-BoPro* were outside the confidence intervals of the QTLs detected around the two genes. Thus our data indicate that glucosinolate biosynthesis genes are not *CR* genes.

QTLs of clubroot resistance in *B. oleracea*

Recent studies on QTL analysis of *CR* genes have revealed that clubroot resistance in *B. oleracea* is characterized by oligogenic inheritance (Landry et al. 1992; Figdore et al. 1993; Grandclement and Thomas 1996; Voorrips et al. 1997; Moriguchi et al. 1999; Rocherieux et al. 2004; Nomura et al. 2005). Landry et al. (1992) identified two QTLs, *CR2a,* and *CR2b*, in the progeny derived from the cross between resistant Rutabaga and susceptible CrGC No85. Voorrips et al. (1997) identified two *CR* loci, *pb-3* and *pb-4*, and a few minor *CR* QTLs in a population of DH lines of F_1 plants obtained between cabbage landrace 'Bindsachsener' and broccoli 'Greenia'. Nomura et al. (2005) identified three QTLs for clubroot resistance in a population from a cross between cabbage and the Kale line 'K269'. Rocherieux et al. (2004) reported differential QTLs from different isolates of *P. brassicae* and found that one QTL (*pb-Bo1*) acts as a major resistance gene against the three isolates. In our study, we identified five QTLs, *pb-Bo(Anju)1*, *pb-Bo(Anju)2*, *pb-pb-Bo(Anju)3*, *pb-Bo(Anju)4*, and *pb-Bo(GC)1*, from the genetic and phenotypic analysis of a cross of resistant cabbage and susceptible broccoli.

It is difficult to compare the map positions of *CR* loci identified so far, due to the lack of common DNA markers in the published *B. oleracea* linkage groups. Based on the few common markers linked to *CR* genes, however, Voorrips et al. (1997) revealed that *CR2a* (Landry et al. 1992) and *pb-4* (Voorrips et al. 1997) are linked to the common marker 2NA8. However, those two *CR* genes are not likely to be identical because of their different origins; *CR2a* is derived from *CR* Rutabaga, and *pb-4* is from *CR* cabbage landrace. Similarly, we attempted to compare the *CR* genes published in *B. oleracea* as follows: Landry et al. (1992), Rocherieux et al. (2004), and Moriguchi et al. (1999) identified *CR2b*, *pb-Bo1*, and *QTL-LG1*, respectively, which were mapped to the distal end of the largest linkage groups in their *B. oleracea* maps. Voorrips et al. (1997) also detected *pb-3* at the distal end of the large linkage group. These data suggest that the largest linkage groups identified in these studies probably correspond to the largest linkage group (O3) in our map; thus, collectively the five *B. oleracea CR* genes, *CR2b*, *pb-3*, *pb-Bo1*, *QTL-LG1*, and *Pb-Bo(Anju)3*, map to either end of O3. In fact, based on the marker 4NE11, which was mapped as a common RFLP marker to the distal ends of the largest linkage groups in Landry et al. (1992) and Voorrips et al. (1997), Voorrips et al. (1997) detected *pb-3* at the marker 4NE11, whereas Landry et al. (1992) detected *CR2b* on the opposite sides of the marker 4NE11 in the largest linkage group. This mapping is consistent with our data that map the *CR* genes at both ends of the largest linkage group (O3) (Fig. 4).

Nomura et al. (2005) identified the major effect QTL (*QTL-LG3*) that is linked to the marker WG1G5 (equal to pW216) in linkage group 3. Using a specific primer to amplify the marker pW216 sequence that was collected from the NCBI database, we mapped this marker to O1 in our map. The QTLs found in our study were not detected in this region, indicating that the major *CR* locus reported by Nomura et al. (2005) is different from all the *CR* loci found in our study and may be lacking in our plant materials. Alternatively, this result suggests that some of the *CR* genes in *B. oleracea* are differentially expressed against the various *P. brassicae* races. The differential response of *CR* genes to the isolates was reported in *B. rapa* (Suwabe et al. 2006; Sakamoto et al. 2008) and in *B. oleracea* (Rocherieux et al. 2004). Nomura et al. (2005) also identified a minor QTL (*QTL-LG9*) that is linked to the marker WG6H1 (equal to pW237) in linkage group 9. The marker pW237 was mapped to the central region of O5 in our map where the *CR* locus *pb-Bo(GC)1* was detected, suggesting that *QTL-LG9* and *pb-Bo(GC)1* may be the same locus. Alternatively, the two *CR* loci could be different members located in the same *CR* gene cluster.

Comparative analysis of *CR* genes between *B. rapa* and *B. oleracea*

Extensive QTL analyses of clubroot resistance in *B. rapa* were recently conducted using public SSR markers and *CR* genes and linked markers (Suwabe et al. 2003, 2006; Hirai et al. 2004; Piao et al. 2004; Matsumoto et al. 2005; Saito

et al. 2006; Hayashida et al. 2008; Sakamoto et al. 2008). As a result, *CR* genes were found in *B. rapa* as follows: *CRa* (Matsumoto et al. 1998), *CRb* (Piao et al. 2004), *Crr3* (Hirai et al. 2004), *Crr1*, *Crr2*, *Crr4* (Suwabe et al. 2003, 2006), and *CRc* and *CRk* (Sakamoto et al. 2008). In total, eight CR loci were mapped and allocated to five different chromosomes (Fig. 4a). In our study, we mapped the *B. rapa CR* gene markers to the *B. oleracea* map so that the marker m6R that is closely linked to *CRc* was mapped to the central region of O2 where we detected a minor QTL, *pb-Bo(Anju)2*. In addition, the marker TCR05 that is linked to *CRb* was mapped to O7, where *pb-Bo(Anju)4* was detected. Moreover, using several anchor markers available in both the *B. oleracea* and *B. rapa* genomes, we showed that the *B. rapa* chromosomal regions harboring *B. rapa CR* gene-specific markers are homologous to the corresponding region of *B. oleracea* (Fig. 3), indicating that the linkage of the *CR* genes versus the specific markers established in the *B. rapa* genome is maintained in *B. oleracea* to some extent. Therefore, these results raise the possibility that the *pb-Bo(Anju)2* and the *pb-Bo(Anju)4* loci in *B. oleracea* are homologous to *CRc* and *CRb* in *B. rapa*, respectively. However, it is difficult to conclude whether the QTLs that are linked to the same molecular markers involve just one gene or family members of clustered *CR* genes. Microsynteny analysis in those regions in *B. rapa* and *B. oleracea* is needed to identify the relationship between these *CR* loci.

The marker BSA3, which is closely linked to *Crr2*, and the marker BrSTS61, which is closely linked to *Crr3*, were mapped to O1 and O3, respectively. In the flanking regions of these markers, we did not detect any counterpart QTLs. On the other hand, we detected the major-effect *pb-Bo(Anju)1*, moderate-effect *pb-Bo(GC)1*, and minor-effect *pb-Bo(Anju)3* on O2, O5, and O3, respectively, where none of the published *CR* loci derived from the *B. rapa* genome were included. These results raise the following possibilities: First, *Crr2* and *Crr3* are the original *CR* genes in the *B. rapa* genome, whereas *pb-Bo(Anju)1*, *pb-Bo(GC)1*, and *pb-Bo(Anju)3* originally existed in *B. oleracea*. If so, interchange and pyramiding of *CR* genes between *B. rapa* and *B. oleracea* through interspecific hybridization will be useful for clubroot resistance breeding; success in this regard would support this hypothesis. Second, the *CR* genes responded differently to different isolates used in this study and in previous studies (Suwabe et al. 2003; Hirai et al. 2004). Third, the linkage of the *CR* genes versus the specific markers established in the *B. rapa* genome is not maintained in the corresponding region of the *B. oleracea* genome. To address these questions, cloning of *CR* genes and QTL analyses using various races are required to characterize the relationship between *CR* genes and races of *P. brassicae*.

Genetic origin and diversification of *CR* genes

R-genes (NBS-LRR) are clustered mainly in *A. thaliana* chromosomes 1, 4, and 5 (Jones 2001). This cluster of disease resistance genes is termed the major recognition complex. Studies have reported that the regions harboring the *B. rapa CR* genes overlap in major recognition complexes of the *A. thaliana* genome (Suwabe et al. 2006; Piao et al. 2009). In our study, we showed that the regions containing *pb-Bo(Anju)1* and *pb-Bo(Anju)3* correspond to the top of *A. thaliana* chromosome 5 (Fig. 1, Supplementary Table S3). *pb-Bo(Anju)2* and *pb-Bo(Anju)4* correspond to the middle of chromosome 4, and *pb-Bo(Anju)4* corresponds to the distal end of chromosome 1. This observation demonstrates that the regions harboring the *B. oleracea CR* genes correspond to the major recognition complexes of the *A. thaliana* genome, as in the case of the *CR* loci in *B. rapa*.

Fuchs and Sacristain (1996) identified a *CR* locus (*RPB1*) in *A. thaliana* chromosome 1. Jubault et al. (2008) mapped two *CR* loci, *pb-At1*, *pb-At4*, in chromosomes 1 and 4, respectively, and the other *CR* loci, *pb-At5.1* and *pb-At5.2*, in chromosomes 5. In *B. rapa*, *Crr1*, *Crr2*, and *CRb* are syntenic with the central region of *A. thaliana* chromosome 4 (Suwabe et al. 2006; Piao et al. 2009). However, the region harboring *Crr3* corresponds to *A. thaliana* chromosome 3 (Saito et al. 2006). These results indicate that the *CR* loci of crucifer crops, including *B. oleracea*, may express lineage commitment-related *CR* genes that originated from the common ancestor of crucifer plants. At present, however, as a result of the long-term host–parasite co-evolution, *Brassica CR* genes have diversified against the various *P. brassicae* races (Suwabe et al. 2006; Sakamoto et al. 2008; Rocherieux et al. 2004). This study will promote a more comprehensive description of different *CR* genes of *B. rapa*, *B. oleracea*, as well as *B. napus*.

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