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ORIGINAL PAPER

Fine mapping of the clubroot resistance gene, *Crr3*, in *Brassica rapa*

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Abstract A linkage map of Chinese cabbage (*Brassica rapa*) was constructed to localize the clubroot resistance (CR) gene, *Crr3*. Quantitative trait loci analysis using an F_3 population revealed a sharp peak in the logarithm of odds score around the sequence-tagged site (STS) marker, OPC11-2S. Therefore, this region contained *Crr3*. Nucleotide sequences of OPC11-2S and its proximal markers showed homology to sequences in the top arm of *Arabidopsis* chromosome 3, suggesting a synteny between the two species. For fine mapping of *Crr3*, a number of STS markers were developed based on genomic information from *Arabidopsis*. We obtained polymorphisms in 23 *Arabidopsis*-derived STS markers, 11 of which were closely linked to *Crr3*. The precise position of *Crr3* was determined using a population of 888 F_2 plants. Eighty plants showing recombination around *Crr3* locus were selected and used for the mapping. A fine map of 4.74 cM was obtained, in which two markers (BrSTS-41 and BrSTS-44) and three markers (OPC11-2S, BrSTS-54 and BrSTS-61) were cosegregated. Marker genotypes of the 21 selected F_2 families and CR tests of their progenies strongly suggested that the *Crr3* gene is located in a 0.35 cM segment between the two markers, BrSTS-33 and BrSTS-78.

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Introduction

Clubroot disease caused by *Plasmodiophora brassicae* is one of the most serious diseases in Chinese cabbage (*Brassica rapa*) and other *Brassica* crops. *P. brassicae* is a soil-borne, obligate pathogen. The pathogen infects the roots of plants, which start abnormal growth and finally result in massive galls, called clubs. This abnormal growth prevents the roots from taking up water and nutrients, resulting in the slow growth of host plants. Consequently, the disease reduces crop quality and the commercial value of the products. Because the pathogen survives as resting spores for long time periods in the soil, it is hard to control the disease by cultural practice or agrochemicals. Thus, the breeding of resistant cultivars is one of the most effective

approaches to minimize crop loss from infection with this pathogen.

Although no high-resistance resources have been found among varieties of Chinese cabbage, resistance resources to the pathogen have been found in some European turnips (Wit 1964; Williams 1966; Yoshikawa 1993). Clubroot resistance (CR) cultivars of Chinese cabbage have been bred by introducing the resistance gene from CR European turnips into Chinese cabbage (Yoshikawa 1981). Yoshikawa (1993) reported that resistance in European turnips seems to be controlled by a single dominant gene and some genes with minor effects. In addition, European turnips have at least three independent dominant genes that each confers resistance to different *P. brassicae* pathotypes (Wit 1964; Toxopeus and Janssen 1975; Crute et al. 1980).

Recent molecular marker technology has revealed four independent CR genes in *B. rapa*. *Crr1* and *Crr2*, derived from the European turnip Siloga, were reported by Suwabe et al. (2003). Piao et al. (2004) found *CRb* derived from the CR Chinese cabbage cultivar, 'CR Shinki'. Another CR gene, *Crr3* derived from the European turnip Milan White has also been reported (Hirai et al. 2004). DNA markers linking these four CR genes have also been developed. Simple sequence repeat (SSR) markers, BRMS088 and BRMS096, link to *Crr1* and *Crr2*, respectively (Suwabe et al. 2003). Several molecular markers linked to *CRb* have been developed by converting amplified fragment length polymorphism (AFLP) markers into sequence-characterized amplified region (SCAR) markers (Piao et al. 2004). OPC11-2S linked to *Crr3* has been developed from a random amplified polymorphic DNA (RAPD) fragment (Hirai et al. 2004). These markers can be used for marker-assisted selection (MAS) in the breeding of Chinese cabbage cultivars. However, molecular markers are sometimes monomorphic in a breeding population. Therefore, a single linkage marker is not enough for breeders and a set of linkage markers should be developed for MAS.

From sequence analysis of the linkage markers to *Crr1* and *Crr2*, we have recently found that these two regions have homology to the central part of chromosome 4 of *Arabidopsis thaliana* (Suwabe et al. 2006). These findings suggest that these two loci originate from the same region of the ancestral genome. In contrast, it is unknown whether *Crr3* and *CRb* evolved from a common origin to that of *Crr1* and *Crr2*. Therefore, increasing the number of linkage markers of *Crr3* and *CRb* may be useful to understand their evolutionary origin, as well as to breed CR cultivars with MAS.

In this article, we describe the fine mapping and quantitative trait loci (QTL) analysis of *Crr3*. A number of DNA markers linked to *Crr3* were developed using the *Arabidopsis* DNA database. Synteny analysis of the genome region around *Crr3* suggested that this locus originated from a chromosomal segment different from *Crr1, Crr2* and *CRb*. The usefulness of these new molecular markers and the evolutionary origin of CR loci are discussed.

Materials and methods

Plant materials

A CR-turnip inbred line, N-WMR-3, was used as the pollen parent. This is a parental line of the F_1 hybrid CR-turnip cultivar, 'CR Shinano', which has a CR trait derived from the European fodder turnip, Milan White (Otani et al. 1982). The Chinese cabbage doubled haploid (DH) line, A9709, which is susceptible to the clubroot pathogen, was used as the seed parent. A population of 81 F_3 families was obtained by bud pollination of each F_2 line derived from crossing A9709 and N-WMR-3. The F_3 population was used for genetic and linkage analyses. The F_4 plants, obtained by selfing the F_3 plants, were used for scoring the disease index (ID) (Suwabe et al. 2003) for the CR trait. These scores were used as the phenotype of each F_3 line, as described previously (Hirai et al. 2004).

For fine mapping, a population of 888 F_2 plants was obtained by selfing the F_1 plants derived from crossing of the above parentage. This population was screened with two DNA markers linked to *Crr3*, BrSTS-20 and BrSTS-26, to identify recombinants in this region. The selected 80 plants were used for fine mapping around *Crr3* locus. Parts of the selected plants were selfed to obtain F_3 seeds. The F_3 plants were used for scoring the ID, which was used as the phenotype of each F_2 family.

Pathogen and CR test

The Ano-01 isolate of the clubroot pathogen (*P. brassicae*), which was maintained by infection of the Chinese cabbage cultivar 'Muso' (Takii & Company Ltd, Kyoto, Japan), was used for the test. The infection profile of this isolate has been reported by Kuginuki et al. (1999). The host range of the isolate was narrower than that of Wakayama-01, the isolate used in a previous study (Suwabe et al. 2003).

The CR test was performed three times from September 2005 to December 2005, as previously described (Hirai et al. 2004), with minor modifications of the culturing conditions, shown below. The infected plants were grown in a greenhouse maintained at a minimum temperature of 25°C under natural daylight

conditions. Tungsten lights (2 μmol/m^2/s) were turned on from sunset to 9:00 p.m. to maintain long-day conditions. The mean ID of the triplicated tests was used for subsequent genetic analysis.

Development of genetic markers linked to *Crr3*

PCR primers were designed based on the *Arabidopsis* genome database at the National Center for Biotechnology Information (http://www.ncbi.nih.gov/). Based on the exon sequences, primers were designed using Primer3 software (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Rozen and Skaletsky 2000) to amplify ~1 kb fragments containing introns to obtain maximal polymorphisms. The primers were used to amplify genomic fragments of *B. rapa*. The amplified fragments of the expected sizes were cut from the gel, extracted using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA), and ligated to a pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA, USA) or a pGEM-T vector (Promega Corporation, Madison, WI, USA). The resultant plasmids were sequenced with an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the sequence was used to design new primer pairs for detection of polymorphisms. In some cases, primers based on the *Arabidopsis* genome were directly used for detection of polymorphisms in *B. rapa*. TCR05, a linkage marker for *CRb* (Piao et al. 2004), was also examined in this study. However, because amplification of the TCR05 fragment was rather faint, the amplified fragment was sequenced and the flanking sequence was obtained by thermal asymmetric interlaced (TAIL)-PCR (Liu and Whittier 1995). A pair of new primers was designed and used for the linkage marker of *CRb* (Table 1). Two RFLP probes of *B. napus*, BN142 and BN308 (Harada et al. 1988), were sequenced to develop PCR-based markers for *B. rapa* (Table 1). The nucleotide sequences reported here have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB265743–AB265780.

Marker analysis

Genomic DNA was isolated from fresh or freeze-dried leaves using the CTAB method (Murray and Thompson 1980). The redesigned marker was amplified by PCR in 10 μl of reaction mixture containing 10 ng of genomic DNA, 1 × PCR buffer (supplied by TaKaRa Bio, Otsu, Japan), 0.25 U of *Taq* DNA polymerase (TaKaRa Bio), 2 nmol of each dNTP, and 5 pmol of each primer. The reaction was performed using a GeneAmp PCR System 9700 (Applied Biosystems) with the following parameters: 1 cycle of 94°C for 30 s; 35 cycles of 94°C for 30 s, optimized annealing temperature for 30 s (Table 1), and 72°C for 1 min; and a final extension at 72°C for 3 min. SSR markers of *B. rapa* were amplified as previously reported (Suwabe et al. 2002, 2006). RAPD analysis was conducted with Operon primers (Operon Biotechnologies, Huntsville, AL, USA) as described previously (Hirai et al. 2004). Amplified products were separated on a 1.5% or 3% agarose gel.

Linkage analysis and map construction

Segregation of each marker in the F_3 population ($n = 67$) (Hirai et al. 2004) and the screened F_2 population were scored. Based on the obtained data, a linkage map at the region for *Crr3* was constructed using JoinMap ver. 3.0 (Van Ooijen and Voorrips 2001). A minimum logarithm of odds (LOD)-likelihood score of 4.0 was used for map construction. The Kosambi map function (Kosambi 1944) was used to calculate the genetic distance between markers. A QTL analysis of CR was carried out using MapQTL ver. 4.0 (Van Ooijen et al. 2000). An interval mapping analysis (Lander and Botstein 1989; Van Ooijen 1992) was conducted for detection of QTL.

Results

Development of DNA markers based on genomic information of *Arabidopsis*

Preliminary genome mapping and QTL analysis of the F_3 population revealed that the SSR markers, BRMS058 and BRMS206, are linked to the CR gene, *Crr3*. The mapping data indicated that the CR locus, *Crr3*, was located between BRMS058 and BRMS206. These linkage markers and the previously identified STS marker, OPC11-2S, showed sequence homology to the top arm of the *Arabidopsis* chromosome 3 (Table 1) (Hirai et al. 2004). Therefore, it is likely that this region of the *B. rapa* genome has conserved synteny with the top arm of the *Arabidopsis* chromosome 3. We then tried to add more linkage markers around the *Crr3* locus using genomic information of *Arabidopsis*.

We designed 82 primer pairs based on open reading frames (ORFs) in chromosome 3 of the *Arabidopsis* genome. These primers were used for the amplification of genome sequences from the parent *B. rapa* plants. Of the 82 primer pairs tested, 48 pairs (59%) amplified fragments of the expected sizes. Two of the primers, BrSTS-20 and BrSTS-28, amplified clear polymorphic fragments. However, the remaining 46 primer pairs amplified monomorphic bands or unclear polymorphic

Table 1 Linkage markers for *Crr3* developed in this study and sequences of the primers used for detection of polymorphism

Maker name	Maker type[a]	Forward primer (5′-3′)	Origin[b]	Fragment size (bp)[c]	Tm (°C)	Locus tag	Linkage group	Accession no[d]
–	–	Reverse primer (5′-3′)	–			–		
BrSTS-08a	CAPS (*Pst*I)	CGGCTGCTTCAATAGATTAAGTTT	Br	300	55	At3g11964	LG 3	AB265743
		CAGAGAACTAATGAATCCGACTTG	Br					AB265744
BrSTS-08b	STS	CTAGTGATGATTCAAGTCGTAGTCA	Br	130	54	At3g11964	LG 5	AB265745
		CACTGACTAAGAAACTTAGCTACTG	Br					AB265746
BrSTS-10	CAPS (*Sau*3AI)	TCCCGAGCTTCACCATCAAGACACC	Br	150	55	At3g08560	LG 1	AB265747
		AAGTGTTCCTCCCTCCACCTCCTGA	Br					AB265748
BrSTS-16	CAPS (*Hin*fI)	TTTGACTACGAGCTCTACAACAGG	At	1000	55	At3g08900	LG 5	
		AGTTGCAAGTGTGATGAAGTAAGG	At					
BrSTS-20	STS	CTTCAGAACATCAGAAAGGGTCTT	At	1000	55	At3g09200	LG 2 (R3)	
		TTGTTAATCTTGGTTGGGATGTTA	At					
BrSTS-26	STS	GATCCTGGACTGTTGATTTATACT	Br	1200	53	At3g04120	LG 2 (R3)	AB265749
		GAATAATGTTGAATGAAGCAGCTC	Br					AB265750
BrSTS-26b	STS	GTTGCTAGAGTTGTTCTCCAGAGG	Br	1300	55	At3g04120	LG 5	
		GAATAATGTTGAATGAAGCAGCTC	Br					
BrSTS-28	STS	GGAAAGACACTTGTTTCCAGAACT	At	1000	52	At3g04840	LG 5	
		AAACATTCTGAAGAGGGTAGATGC	At					
BrSTS-30	CAPS (*Afa*I)	CTCAAGGTTCCCCACCCTATAACT	Br	300	53	At3g05530	LG 3	AB265751
		CCTTGTAAGAATCGCATTCAAGAT	Br					AB265752
BrSTS-33	CAPS (*Hin*fI)	AGGCTCTTCTAAATCAAAGCAAGA	At	500	52	At3g06654	LG 2 (R3)	
		ATTCCCTTGAAGTGATCTTTTTGA	At					
BrSTS-35	STS	AGGTCGTTAAGGATCGTATCAATG	At	250	54	At3g07170	LG 2 (R3)	AB265753
		TCTCCACATCTAATTAGTCCTCCT	Br					AB265754
BrSTS-41	CAPS (*Afa*I)	AGCAGATCTATCCGACGCAGGTTA	Br	100	55	At3g06700	LG 2 (R3)	AB265755
		CTTGGCCATCTCTGTACAAACTGT	Br					AB265756
BrSTS-44	STS	GAGAAGACGATTTGAAGTCGGAGT	Br	1200	55	At3g06760	LG 2 (R3)	AB265757
		GCTGTATCCTCCTTTTCTCAACCT	Br					AB265758
BrSTS-48	CAPS (*Alu*I)	TGTTCTGTCTTTCTCTCGATGCAT	Br	300	55	At3g06580	LG 3	AB265759
		GCTGTGCAAGTTCTTTCTAAAAGC	Br					AB265760
BrSTS-53	CAPS (*Xba*I)	TTCGATCTCTCTAGTTTCATCTCA	Br	200	52	At3g06060	LG 5	AB265761
		AGCATATCCATAAACACCCATCTA	Br					AB265762
BrSTS-54	STS	CGTATAGACATAGAAGACATGGAAGC	Br	400	55	At3g06190	LG 2 (R3)	AB265763
		GTGTTTATGCTGATTCCTTCACAG	Br					AB265764
BrSTS-55	CAPS (*Eco*T22I)	ACATAGTATTGACGCCAAATATGC	Br	200	52	At3g06610	LG 5	AB265765
		AACATCAGCGGCATTGATCTTAA	Br					AB265766
BrSTS-59	CAPS (*Dra*I)	GATTCTCCAAGTACAGCTCAAATT	Br	300	52	At3g05910	LG 8	AB265767
		TTCCTAGTAAACACATCACAACAT	Br					AB265768
BrSTS-61	CAPS (*Hin*fI)	CCATCCGCAGGAGTTAAAGTTGTA	Br	100	55	At3g05970	LG 2 (R3)	AB265769
		AGAAAACGGCTGAGGGTTACTAC	Br					AB265770

Table 1 continued

Maker name	Maker type[a]	Forward primer (5′-3′)	Origin[b]	Fragment size (bp)[c]	Tm (°C)	Locus tag	Linkage group	Accession no[d]
–	–	Reverse primer (5′-3′)	–			–		
BrSTS-67	CAPS (*Hin*fI)	ATTAACGCTAATCAGTGCAATTA	Br	300	50	At3g05500	LG 3	AB265771
		GAGAAACTTTAATTCTTTAATCTGAA	Br					AB265772
BrSTS-69	STS	TAAGATGCTACCTAAGATGAAATG	Br	500	50	At3g05010	LG 2 (R3)	AB265773
		GAAGCAAATCAAGACCTATAACAA	Br					AB265774
BrSTS-72	CAPS (*Hin*fI)	AGAGGTAACAAACACTCAACACCA	Br	200	55	At3g05560	LG 2 (R3)	AB265775
		ACTCTAAGCCAATCCCTCACATT	Br					AB265776
BrSTS-78	SSR	CTCTCCTCTAACCTGTTCCAAGAA	Br	200	55	At3g05840	LG 2 (R3)	AB265777
		GGTGTATCCACACACTCATCAAGT	Br					AB265778
OPC11-2S	STS	GTAACTTGGTACAGAACAGCATAG	Br	1200	53	Hirai et al. (2004)	LG 2 (R3)	
		ACTTGTCTAATGAATGATGATGG	Br			(At3g06270)		
TCR05-R[e]	STS	CGTTGGAGTACATCTGGGTGCCGAG	Br	200	60	Piao et al. (2004)	LG 2 (R3)	AB265779
		GTGGGGCCAACGTACTGCAGCTAA	Br			(At4g20150)		AB265780
BN142CAPS	CAPS (*Hin*fI)	CCGTGCAGACACATTTGTTCATCT	Bn	300	54	Harada et al. (1988)	LG 2 (R3)	
		GATCAAGTTTAGGTTAACTGATGG	Bn			*B. napus* cDNA		
BN308CAPS	CAPS (*Pst*I)	ACGCTTCACTATACTATTCTGGAG	Br	800	54	Harada et al. (1988)	LG 2 (R3)	
		ATCTTGGTGCCATCAACACGGTAT	Bn			*B. napus* cDNA		

[a] Restriction enzymes used to cut the amplified fragments is shown in the parenthesis

[b] Origin of the primer sequence based on. Br *Brassica rapa*, At *Arabidopsis thaliana*, Bn *Brassica napus*

[c] The size of the fragment amplified was estimated by agarose gel electrophoresis

[d] Accession numbers for A9709 (upper) and N-WMR-3 (lower)

[e] Re-designed primer sequences. See text

bands. Two of the 46 primer pairs (BrSTS-16 and BrSTS-33) showed polymorphic bands after the amplified products were digested with restriction enzymes. The primers based on the *Arabidopsis* genome sequence were used for directly scoring the genotype of these four loci (Table 1). The remaining monomorphic and unclear polymorphic fragments amplified from both parent plants were cloned and sequenced. New primer pairs were designed when nucleotide polymorphisms were found between the sequences of the parental lines. Based on the nucleotide sequences, eleven, seven and one of the 44 pairs were successfully converted to CAPS, indel and SSR markers, respectively. Consequently, 23 polymorphic markers were developed (Table 1), all of which were codominant. Two PCR-based markers, BN142CAPS and BN308CAPS, were also developed from RFLP probes of *B. napus*.

Mapping and QTL analysis

The above-mentioned 23 markers were used for construction of a linkage map based on the segregating F_3 population (Hirai et al. 2004). These markers were mapped in three linkage groups (LGs), LG 2, LG 3 and LG 5. Eleven markers were mapped in LG 2, where the previously identified CR locus, *Crr3*, was mapped (Fig. 1). LG 2 also included five RAPDs, 11 SSRs and two previously developed STSs (OPC11-1S and OPC11-2S; Hirai et al. 2004). The presence of SSR markers (Suwabe et al. 2006) and RFLP probe-derived markers (BN142CAPS and BN308CAPS) indicated that LG 2 corresponds to R3 of the internationally agreed *Brassica* reference linkage groups (see http://www.brassica.info/information/lg_assigments.htm), whereas the correspondence of the other two LGs with *Brassica* reference linkage groups was uncertain. The order of the marker loci mapped in the three LGs in *B. rapa* was the same as that in *Arabidopsis* chromosome 3 (Fig. 2), except for one change in LG 5. Four markers in LG 2, OPC11-2S, BrSTS-33, BrSTS-54 and BrSTS-61, were cosegregated in this population.

QTL analysis was conducted using the constructed map. A sharp peak in the LOD score was observed at the above cosegregated loci (Fig. 1, bold types). The peak LOD score was 27.2. This position explained 85.9% of the phenotypic variation. Therefore, *Crr3* is most likely to be located in a region around these four markers. TCR05-R, a linkage marker for *CRb* originally developed by Piao et al. (2004), was used for mapping in this population. This marker was mapped in the same linkage group, LG 2 (R3), at a distance of 37.9 cM from the peak LOD score of the present CR (Fig. 1). Moreover, the

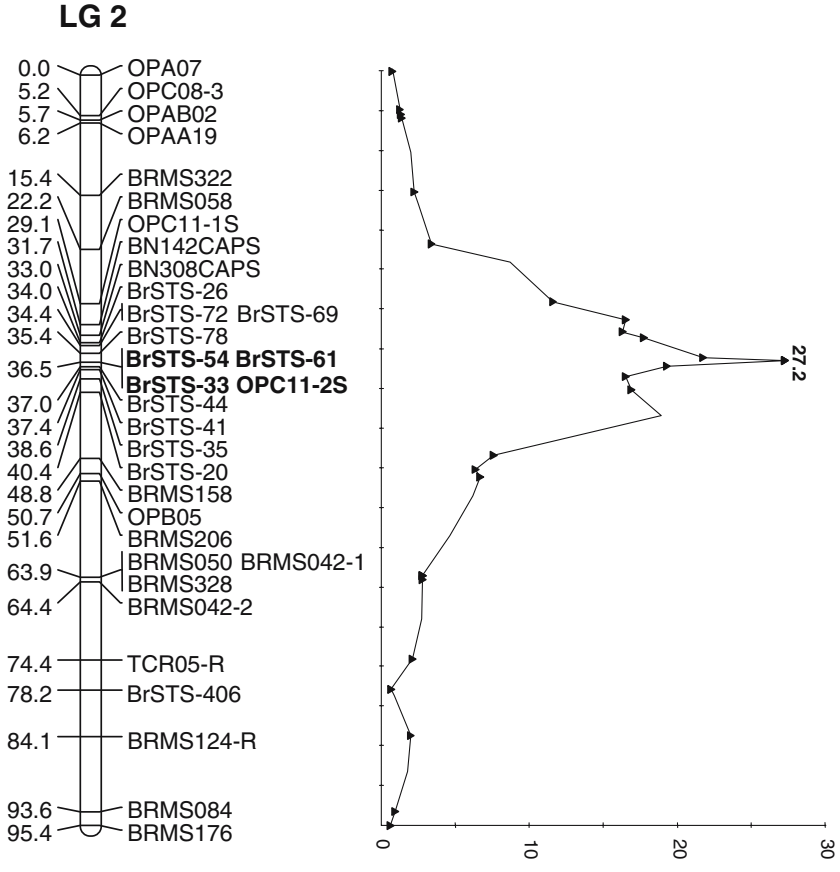

Fig. 1 A partial linkage map of the region containing *Crr3* and QTL analysis for clubroot resistance. Left: A linkage map containing the *Crr3* locus. Marker names and their genetic distances (cM) are indicated at the right and left sides of the linkage map, respectively. Markers prefixed with BRMS are SSR markers (Suwabe et al. 2002, 2006). Those prefixed with OP are RAPD and RAPD-derived STS markers (Hirai et al. 2004). Other markers were developed in this study (Table 1). Markers at the peak of the LOD score are shown in *bold* type. Right: The QTL likelihood-profile for the CR trait. LOD score and map distance are indicated on the *x* and *y* axes, respectively. The positions of markers are indicated with *filled triangles* on the LOD profile. The peak value of the LOD score is also shown

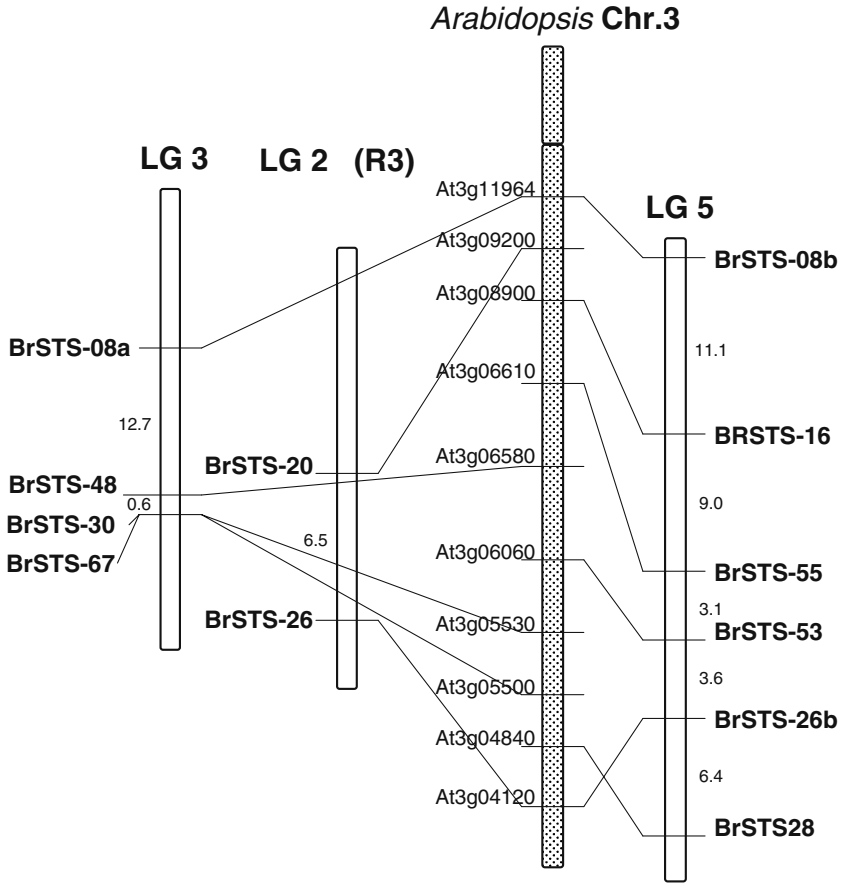

Fig. 2 Three linkage groups (LGs) of *B. rapa* showing synteny to the top arm of *Arabidopsis* chromosome 3. LGs of *B. rapa* and *Arabidopsis* chromosome 3 are represented by *open* and *dotted boxes*, respectively. The *Arabidopsis* chromosome is shown upside down. Markers on LG 2 (R3) are only partly shown here (see Fig. 1). Figures are not to scale

LOD score at TCR05-R was 2.04 and did not show any peak. This observation was confirmed by another STS marker linked to *CRb*, BrSTS-406, which was developed in this study. Therefore, *CRb* and *Crr3* are thought to be different loci that are independent of each other. These results clearly show the monogenic nature of CR in this population, as suggested previously (Hirai et al. 2004).

Fine mapping of *Crr3*

A total of 888 F_2 plants were used to obtain more detailed information of the map position of *Crr3*. The seedlings of the population were first selected for recombination between two DNA markers, BrSTS-20 and BrSTS-26, which are located ca. 4 cM upstream and 3 cM downstream from the peak position of the LOD score in the segregating F_3 population, respectively (Fig. 1). The 80 selected F_2 individuals were then used to construct a detailed linkage map around *Crr3* (Fig. 3). A partial map of 4.74 cM was obtained. We already reported two linkage markers, OPC11-1S and OPC11-2S for *Crr3*. Among them, only OPC11-2S was mapped at this distance. Two markers (BrSTS-41 and BrSTS-44) and three markers (OPC11-2S, BrSTS-54 and BrSTS-61) were cosegregated, even in this population. F_3 seeds were then obtained by selfing of the selected F_2 plants. The number of F_3 seeds obtained was enough for 21 F_2 families. These seeds were then used for the CR test. The results are illustrated in Fig. 4. CR scores of the families were clearly segregated into three ID scores: (1) less than 0.1, (2) more than 2.7, and (3) intermediate (from 0.6 to 1.8). Because QTL analysis suggested that CR in this population was monogenic in nature, these segregants could be classified into three corresponding categories: (1) homozygotes for the resistant allele, (2) homozygotes for the susceptible allele, and (3) heterozygotes, respectively (Fig. 4). The CR scores and genotypes of the F_2 families were compared. The genotypes between BrSTS-33 and BrSTS-78 (0.35 cM) did not show discrepancy with the ID scores obtained. Therefore, the CR locus, *Crr3*, is likely to be located within this region.

Discussion

Use of genomic information of *Arabidopsis* to obtain genetic markers in *B. rapa*

The model plant *A. thaliana* is a close relative of *Brassica* species that are extensively cultivated as oil and

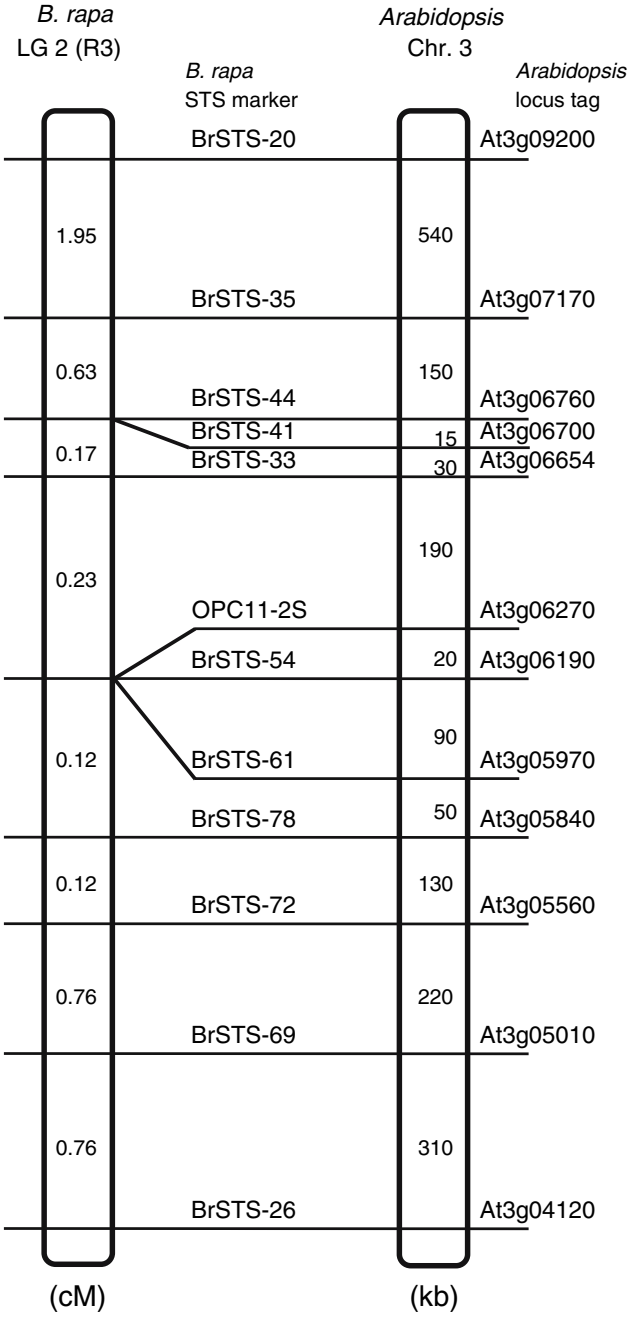

Fig. 3 A partial linkage map of *B. rapa* in the region of *Crr3* (LG 2 (R3)) based on an F_2 population ($n = 888$) and a corresponding physical map of *Arabidopsis*. Marker names of the *B. rapa* LG and gene loci of *Arabidopsis* are shown at the left and right sides of *Arabidopsis* chromosome 3, respectively. Genetic distances between markers in the *B. rapa* linkage map are given in cM. Approximate nucleotide distances between the loci of *Arabidopsis* are shown in kb. Two markers (BrSTS-41 and BrSTS-44) and three markers (OPC11-2S, BrSTS-54 and BrSTS-61) were cosegregated, respectively, in this mapping population

vegetable crops worldwide. The whole genome of *A. thaliana* has been sequenced (Arabidopsis Genome Initiative 2000), and *Arabidopsis* genome information has been effectively used in comparative genome analysis of *Brassica* species. In addition, some parts of the genome structure are conserved, even in distantly related species, such as rice (Mayer et al. 2001), tomato (Ku et al. 2000) and legumes (Kevei et al. 2005).

Brassica species and *Arabidopsis* are classified in the same family, Brassicaceae, and are thought to have evolved from a common ancestor ca. 14.5–20.4 million years ago (Yang et al. 1999). The genome sizes of diploid *Brassica* species are estimated to be three- to four-fold larger than those of *Arabidopsis* (Johnston et al. 2005). Many studies have compared *Brassica* and *Arabidopsis* genomes based on linkage and/or physical maps (Lagercrantz 1998; Axelsson et al. 2001; Rana et al. 2004; Suwabe et al. 2006). These studies have revealed that small genomic segments of *Arabidopsis* are triplicated and dispersed throughout the *Brassica* genome. In these small segments, the order of genes is conserved, although some inversions and large-scale deletions have been found (Cavell et al. 1998; O'Neill and Bancroft 2000; Ryder et al. 2001). Therefore, the genomic information of *Arabidopsis* can be used for the fine mapping of *Brassica* genomes.

We effectively used the *Arabidopsis* genome information for the detailed mapping of the targeted regions in the *B. rapa* genome around *Crr3*. Because exon sequences are generally conserved in evolution, we designed primers based on the exon sequences of *Arabidopsis*. However, 41% of the primer pairs did not amplify DNA fragments in *B. rapa*. Several hypotheses could account for this failure of amplification. Because all gene loci in the *Arabidopsis* genome have not been experimentally characterized, some of the ORFs that we chose to use for primer design may be less conserved, nonfunctional genes. Thus, divergence of exon sequence during evolution could have caused the failure of amplification. Alternatively, some of the coding regions of the *Arabidopsis* genome may have been lost in the *Brassica* genome during the course of evolution, as shown by Rana et al. (2004). We included introns for the targeted region of the amplification to obtain polymorphisms. Insertion of long introns into the *Brassica* DNA may also have caused failure of the amplification. After sequencing the amplified fragments, we obtained 23 polymorphic markers, half of which were mapped in the target region of LG 2. The overall percentage of the obtained linkage markers (11/82 = 13.4%) may be useful for future studies on *Brassica* species, since we do not have any information on the divergence of triplicated sequences in this region.

The rest of the polymorphic STS markers were mapped in different LGs (LG 3 and LG 5), even though their primers were designed based on the same

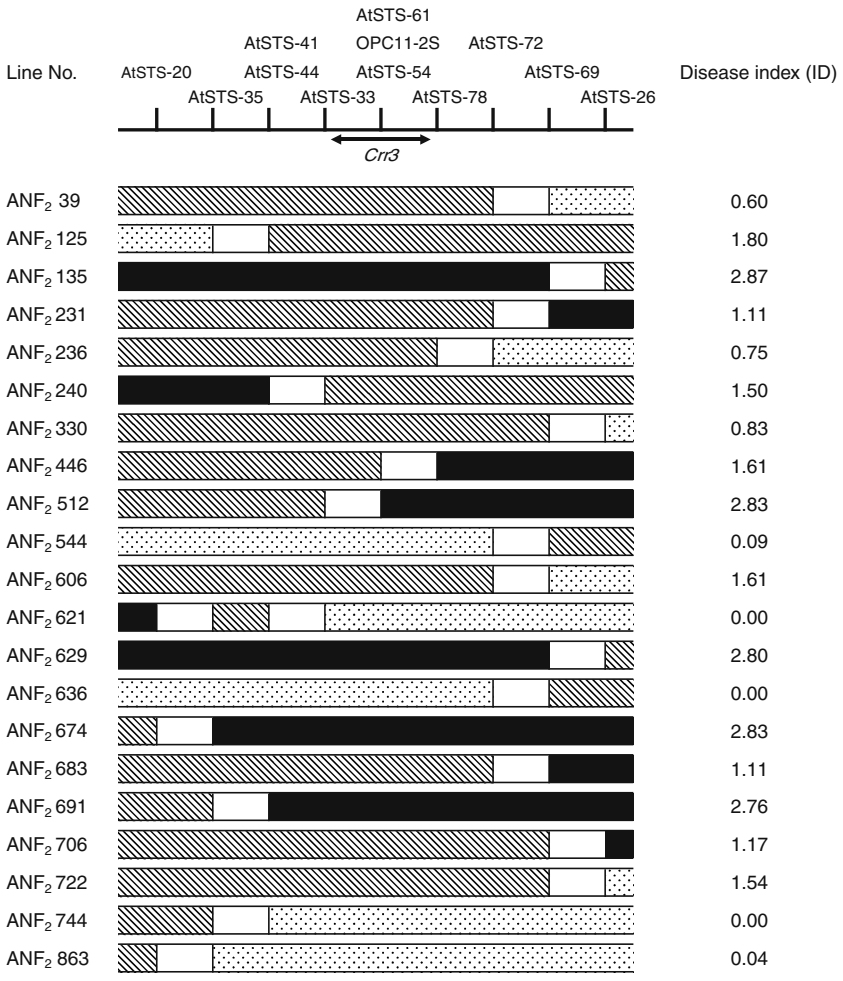

Fig. 4 Graphical genotype of the selected recombinants and their clubroot disease index (ID) in the F_2 population. Name of lines and IDs are shown at the left and right, respectively. Marker names are indicated at the top of the column. Homozygotes of the resistant allele (N-WMR-3), homozygotes of the susceptible allele (A9709) and heterozygotes, are represented by *dotted*, *filled* and *hatched* boxes, respectively. Regions of unknown genotype due to marker intervals are shown by *open boxes*. The putative location of the *Crr3* gene is indicated with *arrow*s

chromosomal region from *Arabidopsis*. These markers did not fall into LG 2 when the minimum LOD score was lowered to 3.0 during map construction (data not shown). Therefore, the three LGs seem to be independent, although their detailed chromosomal assignments remain incomplete. Some loci have been duplicated and are scattered throughout the *Brassica* genome during evolution. We may have picked up polymorphisms of such paralogous loci, resulting in them being mapped to LGs different from LG 2. Alternatively, duplication and loss may have occurred in some loci during the evolution of the *Brassica* genome. Further study using more gene-specific markers could clarify this issue.

The origin of CR loci in *B. rapa*

Earlier studies of CR in turnips suggested that there were three independent CR genes in *B. rapa* (Buczacki et al. 1975; Toxopeus and Janssen 1975). However, five CR loci have recently been identified in *B. rapa* using molecular markers, of which the sequence information has been disclosed for four (Piao et al. 2004; Suwabe et al. 2006; this study). In this study, we mapped linkage markers for *CRb* and *Crr3* to the same linkage group. The linkage group should be R3, because of common markers (Suwabe et al. 2006). Linkage markers for *Crr1* and *Crr2* show homology to the central part of the long arm of *Arabidopsis* chromosome 4 (Suwabe et al. 2006). We also found that linkage markers for *CRb* (TCR05-R and BrSTS-406) show homology to the same part of the *Arabidopsis* genome (Table 1). Therefore, these three CR genes may have originated from the same part of the ancestral genome. In this fine mapping study, we clearly show that the genomic region around *Crr3* has synteny to the top of the long arm of *Arabidopsis* chromosome 3. Therefore, the origin of *Crr3* seems to be different from that of the other three CR loci, *Crr1*, *Crr2* and *CRb*. The map position of another CR locus, *CRa*, is not yet known, because sequence information of its linkage markers has not been disclosed (Matsumoto et al. 1998). Fuchs and Sacristán (1996) reported a CR locus (*RPB1*) in *Arabidopsis* chromosome 1. Therefore, at least three

parts of the ancestral genome may be involved in the evolution of CR genes in crucifer plants. Although many studies have reported the mapping of CR genes in *B. oleracea*, no sequence information is available (Grandclément and Thomas 1996; Voorrips et al. 1997; Nomura et al. 2005). Therefore, the origin of CR genes in *B. oleracea* remains unknown. Reciprocal mapping of anchor markers to the previous linkage maps as well as our map will be necessary to clarify the correspondence of each CR locus in detail.

In addition, for further understanding of the evolution of the CR loci, it will be necessary to clone these CR genes and compare them at the nucleotide level. We have developed a number of high-density linkage markers to *Crr3*, which would enable the map-based cloning of *Crr3*.

Breeding of CR cultivars using DNA markers in *B. rapa*

Breeding of CR Chinese cabbage cultivars was based on the assumption that CR is controlled by a single dominant locus (Yoshikawa 1993). In this context, a simple backcross was an effective way to introduce one CR locus to the Chinese cabbage. However, infection of some CR cultivars has been reported in some production areas in Japan (Kuginuki et al. 1999), indicating a breakdown of the introduced CR trait. Breeding of more resistant CR cultivars is therefore desirable. Most of the present CR Chinese cabbage cultivars in Japan are F_1 hybrids between CR and clubroot-susceptible parents. Therefore, they would be heterozygous at the CR locus. Some CR loci show partial dominance and CR homozygotes are more resistant than heterozygotes (Suwabe et al. 2006). In addition, considering the variation in the pathogen, a single CR gene would not be enough to protect *Brassica* crops from some virulent populations of *P. brassicae* (Hatakeyama et al. 2004). Breeding of more resistant CR cultivars would require the accumulation of more than two CR genes in a single cultivar. Pyramiding CR genes with MAS may be an ideal strategy for this complex breeding procedure. The linkage markers of *Crr3* developed in the present study are codominant and PCR-based markers. Their polymorphisms are easily detectable with agarose gel electrophoresis. Thus, they will be an effective tool in marker-assisted pyramiding of CR genes in Chinese cabbage and other *Brassica* crops.

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