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ORIGINAL PAPER

# QTL mapping of clubroot resistance in radish (*Raphanus sativus* L.)

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**Abstract** A QTL analysis for clubroot resistance (CR) of radish was performed using an $F_2$ population derived from a crossing of a CR Japanese radish and a clubroot-susceptible (CS) Chinese radish. $F_3$ plants obtained by selfing of $F_2$ plants were used for the CR tests. The potted seedlings were inoculated and the symptom was evaluated 6 weeks thereafter. The mean disease indexes of the $F_3$ plants were used for the phenotype of the $F_2$. The results of two CR tests were analyzed for the presence of QTL. A linkage map was constructed using AFLP and SSR markers; it spanned 554 cM and contained 18 linkage groups. A CR locus was observed in the top region of linkage group 1 in two tests. Therefore, the present results suggest that a large part of radish CR is controlled by a single gene or closely linked genes in this radish population, although minor effects of other genomic areas cannot be ruled out. The CR locus was named *Crs1*. Markers linked to *Crs1* showed sequence homology to the genomic region of the top of chromosome 3 of *Arabidopsis*, as in the case of *Crr3*, a CR locus in *Brassica rapa*. These markers should be useful for breeding CR cultivars of radish. As Japanese radishes are known to be highly resistant or immune to clubroot, these markers may also be useful in the introgression of this CR gene to *Brassica* crops.

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## Introduction

Clubroot disease is caused by an obligate pathogen, *Plasmodiophora brassicae*, and is one of the most serious diseases of cruciferous crops (Hirai 2006). The pathogen infects the roots of plants, causing abnormal growth and, eventually, a massive gall called a club (Ingram and Tommerup 1972). The abnormal growth prevents the root from absorbing water and nutrients, resulting in slow growth of the host plant. Consequently, the disease reduces the quality and commercial value of the products. Because the pathogen survives as resting spores for long periods in the soil, it is hard to control the disease using cultural practices or agrochemicals. Therefore, the breeding of resistant cultivars is one of the most effective approaches for minimizing crop loss caused by infection with this pathogen.

Several mapping studies have been reported for *Brassica* crops. *Brassica rapa* has five major genes associated with clubroot resistance (CR): *CRa* (Matsumoto et al. 1998), *Crr1* (Kuginuki et al. 1997; Suwabe et al. 2003), *Crr2* (Suwabe et al. 2003), *Crr3* (Hirai et al. 2004), and

*CRb* (Piao et al. 2004). The last-mentioned four CR loci have been mapped to a reference map for this species, whereas the mapping position of *CRa* is unclear. Recently, Sakamoto et al. (2008) identified two CR loci in *B. rapa*, one of which seems to be a new CR locus. Many QTLs for CR have been detected in *B. oleracea*; *pb-3* (Voorrips et al. 1997) contributed substantially to CR and *Pb-Bo1* (Rocherieux et al. 2004) was associated with resistance in five isolate of *P. brassicae*, although its locus varied in effectiveness. However, the map positions of these CR loci of *B. oleracea* are unclear because the sequences of the markers linked to CR loci were not disclosed. Three QTLs were detected in *B. napus*, one of which, *Pb-Bn1*, was identified as a major gene (Manzanares-Dauleux et al. 2000). Recently, 19 isolate-dependent QTLs were reported by Werner et al. (2008). In *Arabidopsis thaliana*, a single dominant gene for CR on chromosome 1 induced a hypersensitive reaction (Fuchs and Sacristán 1996). The radish (*Raphanus sativus* L.) is a close relative of the *Brassica* and originated in the Mediterranean region. This species is now cultivated in East Asia as a vegetable crop (Kaneko et al. 2007) and exhibits a diverse morphology in this region. Most radish cultivars and lines are highly resistant to the clubroot pathogen, but some are susceptible to it (Scheijgrond and Vos 1954; Yoshikawa 1993). However, there is no published genetic analysis on CR in the radish.

We conducted a QTL analysis of CR in the radish using a segregating $F_2$ population and identified a genetic region that has a major effect on CR. We also developed some PCR-based markers in this region. Synteny analysis suggests that this region in the radish and the *Crr3* region in *B. rapa* (Saito et al. 2006) originate from the same region of their ancestral genome.

## Materials and methods

### Plant materials

Two cultivars of radish, Koga benimaru (Huang-he hong-wan) (Sakata Seed Co., Yokohama, Japan) and Utsugi-gensuke (Ishikawa Agricultural Research Center, Kanazawa, Japan) ($2n = 18$) were used for seed and pollen parents, respectively. Koga benimaru is a Chinese radish that has a globular root with a red skin and is susceptible to clubroot disease. Utsugi-gensuke is a Japanese radish that has a relatively short, cylindrical root with a white skin and is resistant to clubroot disease (Yoshikawa 1993). The seeds of this cultivar are available from seed companies in Japan. A population of 95 $F_2$ plants was obtained by bud pollination of an $F_1$ plant derived from the crossing. The $F_2$ population was used for genetic and linkage analysis. $F_3$ families were obtained by selfing of the $F_2$ plants. A population of 88 $F_3$ families was used for scoring the disease index (ID) (Suwabe et al. 2003) for the CR trait.

### Pathogen and CR test

*Plasmodiophora brassicae* isolates Ano-01 and Wakayama-01 were used in this study. The Wakayama-01 isolate is highly virulent and has a broad host range, but occasionally loses virulence (Suwabe et al. 2003; Hatakeyama et al. 2004). In contrast, the Ano-01 isolate has one of the lowest virulence of all isolates. However, this isolate shows stable virulence, and has been used in our study as a standard isolate (Hirai et al. 2004; Saito et al. 2006). The infection profile of this isolate was described by Kuginuki et al. (1999). Isolation of resting spores and the CR test were performed as previously described (Hirai et al. 2004; Saito et al. 2006). The CR test was carried out two times with Ano-01 (in October 2005, and September 2006) and three times with Wakayama-01 (in April, September, and November 2007). A total of 88 $F_3$ families obtained by selfing of each $F_2$ line were used in the CR test for Ano-01. Ten selected $F_3$ families were used in the test for Wakayama-01. Six weeks after sowing, the root symptoms of each plant were evaluated as follows: grade 0, no club; grade 1, a few small or separate clubs on lateral roots; grade 2, intermediate symptoms; and grade 3, severe clubs on main roots. The mean score for 12 $F_3$ seedlings for each test was used as an ID for each $F_2$ line.

### Detection of DNA polymorphisms

Total DNA was extracted from young leaves using the Nucleon Phytopure DNA extraction kit (Amersham Biosciences, Buckinghamshire, UK). Detection of AFLP was performed according to Tsuro et al. (2005). SSR markers of *B. rapa* were amplified as previously reported (Suwabe et al. 2002, 2006). SSR primer pairs from *B. rapa* (Suwabe et al. 2002, 2006) and genomic- and EST-derived SSR markers from radish (Wang et al. 2007) were used in this study. Amplified products were electrophoresed on 1.5 or 3% agarose gels or on a 10% polyacrylamide gel. A Beckman CEQ 8800XL sequencer (Beckman Coulter, Fullerton, CA, USA) was used to electrophorese markers with small size differences. The sequence-tagged site (STS) markers linked to *Crr3,* a CR locus of *B. rapa*, were also used (Saito et al. 2006). For PCR-Single-strand conformation polymorphism (PCR-SSCP), a mixture containing 2 µl of PCR products and 10 µl formamide with 1% bromophenol blue and xylene cyanol was denatured at 96°C for 15 min and then electrophoresed in a 12.5% polyacrylamide gel containing 5% glycerol at 15°C. The PCR product was visualized using a silver stain kit (Kanto

Chemical Co. Inc., Tokyo, Japan). AFLP markers linked to QTL for CR were converted into STS markers according to Sakata et al. (2006). The primer sequences of the genetic markers that were linked to the CR locus are listed in Table 1.

### Map construction and QTL analysis

Segregation of each marker in the $F_2$ population ($n = 95$) was scored. Based on this data, a linkage map was constructed using JoinMap ver. 3.0 (Van Ooijen and Voorrips 2001). In the linkage analysis, markers with more than 10 missing data were discarded. A LOD threshold of 3 was used for assignment of linkage groups (LGs). The Kosambi map function (Kosambi 1944) was used to calculate the genetic distance between markers. Because CR symptom was recorded as categorized datum, an interval mapping method developed by Hayashi and Awata (2006) for the analysis of categorical traits was used. In this method, the genomic regions (genetic markers), significantly influencing the probabilities of an individual being classified into some specific categories, are searched in the framework of generalized linear models (McCullagh and Nelder 1989). The $F_3$ families were categorized into two based on median of the ID: $F_3$ families showing ID = 0 were categorized to susceptible, and those showing ID > 0 to resistant. The threshold values of LOD score were determined by permutation test with 1,000 iterations (Churchill and Doerge 1994).

## Results

### Test for CR in radish

The resistant parent, Utsugi-gensuke, exhibited almost complete resistance to the Ano-01 isolate. The ID was 0.25 or less in the two tests. The ID of the CS parent, Koga Benimaru, was 2.54, and 2.75 in the first and second tests, respectively. Therefore, the latter was categorized as a susceptible class (supplementary Fig.). The $F_3$ families varied widely in ID. A large part of $F_3$ families showed ID of 0.5 or less (Fig. 1). The $F_1$ did not show any disease symptoms.

### Development of genetic markers and construction of a linkage map

In this mapping study, we used only PCR-based markers. First, SSR markers developed from the *B. rapa* genome (Suwabe et al. 2002, 2006) were tried, but a large portion of the marker primers did not amplify fragments or amplified multiple fragments and could not be used for mapping. As only 53% of the primer pairs, some of which were monomorphic, amplified clear bands, 17 SSRs were mapped. Most of the mapped SSRs were dominant markers, and only 10 markers detected codominant loci. This suggests that the two genomes are relatively remote. Genetic markers developed for *B. rapa* are also mapped (Saito et al. 2006). The linkage map was constructed using 313 polymorphic loci. A map spanning 554 cM with 18 LGs was constructed with 201 loci using a LOD score of 3. One hundred and twelve loci were unmapped. The length of the LGs ranged from 2 to 83 cM. Nine LGs spanned more than 30 cM, and the remaining LGs were less than 30 cM. Among the LGs, LG1 was the longest and had 37 markers (Fig. 2a). We mapped 24 SSRs for radish and 17 for *B. rapa*.

### QTL analysis of the radish

The QTL analysis was performed using the linkage map. The results of two CR tests with the Ano-01 isolate were independently analyzed. A broad genomic region in LG1 showed LOD scores higher than the threshold values in the two tests. The top region from 0 to ca. 40 cM commonly showed LOD scores higher than the threshold in the two tests (Fig. 2b). The peak LOD scores were as high as 9.95 and 12.64 for first and second test, respectively. The peak positions expressed as one-LOD support intervals

**Table 1** Primer sequences of sequence-tagged site (STS) and simple sequence repeat (SSR) markers linked to *Crs1*

| Marker name | Primer sequence (5′–3′) | | Origin | Motif | Accession no.* |
|---|---|---|---|---|---|
| RSACCCTC4 | F | GTAATCTCCATCGTTGAAAAGACA | AFLP | – | AB508844-45 |
| | R | ATACAATGAATTTCGCTGGTATCG | | | |
| REL24 | F | AGAGAGAAGGAGAGTTGTAG | SSR | [AG]30 | AB520986 |
| | R | TCCTAACATAGCGTGTACTG | | | |
| REL6 | F | GATTAAGGAGAAGCTTCCAGG | SSR | [ATG]7 | AB520985 |
| | R | GGAAAGCTGAAATCAACAAAGG | | | |

* Accession numbers of the sequences for Koga-benimaru and Utsugi-gensuke are shown for RSACCCTC4

(Van Ooijen 1992) were around 7 cM in LG 1 (Fig. 3).This region is named *Crs1* (clubroot resistance locus of *R. sativus* 1). Allele from Utsugi-gensuke on the LOD peak around 7 cM had a clear effect increasing probability of CR as shown Table 2. The probability of the $F_2$ individuals with the allele from Utsugi-gensuke being classified as resistant was near 1, while the probability of resistance was much low (0.36 and 0.11 in the first and second tests, respectively) for the $F_2$ individuals without the allele from Utsugi-gensuke. Therefore, a clubroot resistance gene having a large effect seems to exist in this genomic region. The genomic region around *Crs1* corresponds to LG12 of the previous map (Tsuro et al. 2008). Other than LG1, only one genomic region, a part of LG14 showed LOD score slightly higher than the threshold values in the two tests. The peak LOD scores were 2.19 and 2.14 for first and second test, respectively. No other genomic region showed LOD scores higher than the threshold in both tests.

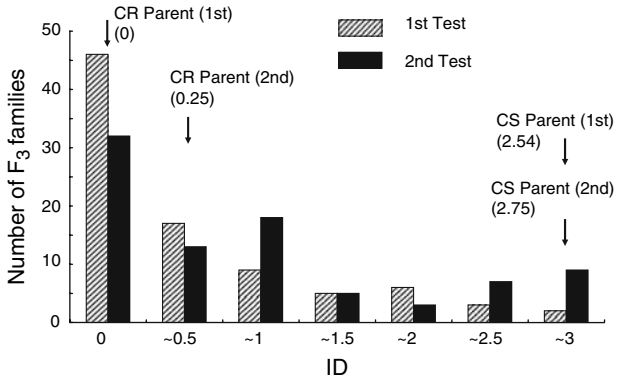


**Fig. 1** Frequency distribution of the disease index (ID) in the $F_2$ population when Ano-01 was used as a pathogen. The IDs of the parents are indicated by *arrows*

## Synteny analysis of the major QTL region

Previously, we demonstrated synteny in the region around the *Crr3*, a CR locus in *B. rapa* with a genomic region of *A. thaliana* chromosome 3 (Saito et al. 2006). During sequencing of the AFLP fragments linked to *Crs1*, we found that an AFLP fragment, ACC/CTC4 that had 75% homology to a segment of the *Arabidopsis* chromosome 3. This marker was converted to a sequence-specific marker, RSACCCTC4 (Table 1). Therefore, the markers linked to *Crr3* were then examined. Among them, BN142, BrSTS-33, BrSTS-61, and BrSTS-72 were mapped in the vicinity of the *Crs1* locus (Fig. 3). The last-mentioned three markers show homology to chromosome 3 of *Arabidopsis* (Saito et al. 2006). Information for these markers has already been shown by Saito et al. (2006). This evidence strongly suggests that a genomic region around the *Crs1* is

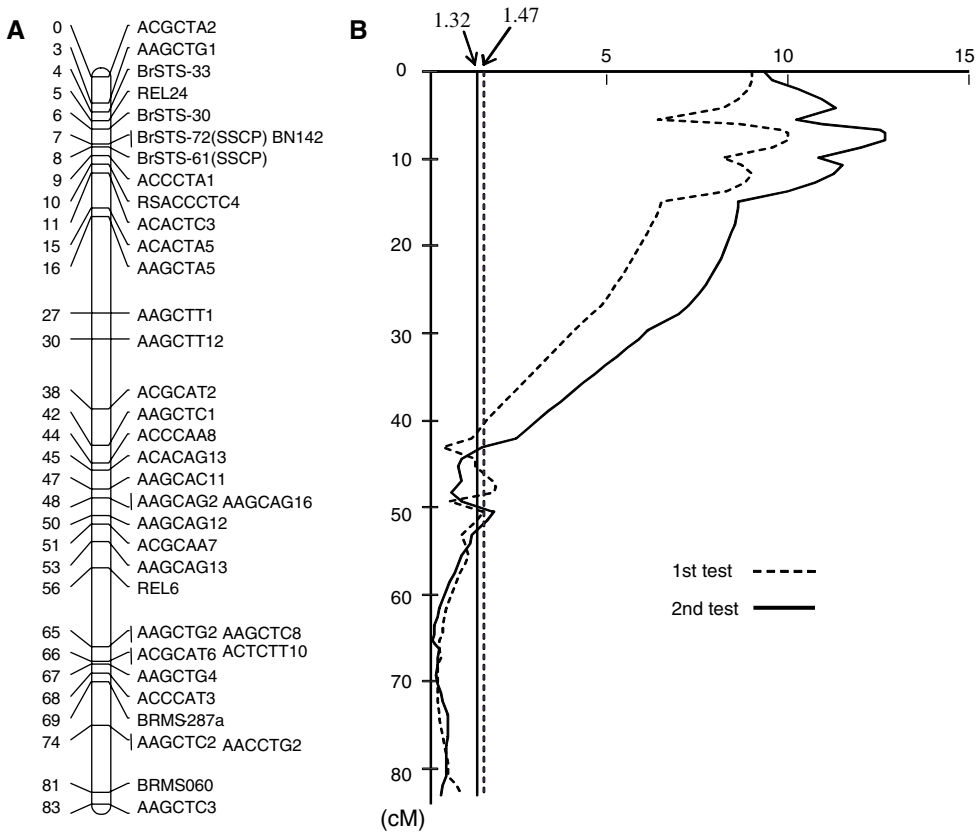


**Fig. 2** A partial linkage map of radish (**a**) and QTL analysis (**b**). Only LG1 is shown. Map distances (cM) and marker names are shown to the left and right of the map, respectively. Markers named by six nucleotides plus one or two digits are AFLP markers. Markers prefixed with BrSTS or BN are those developed for *B. rapa* (Saito et al. 2006). BRMS markers are shown previously (Suwabe et al. 2006). LOD score profiles of the first and second tests are shown as *dashed* and *solid lines*, respectively. The threshold value for each test is indicated by a *straight line*

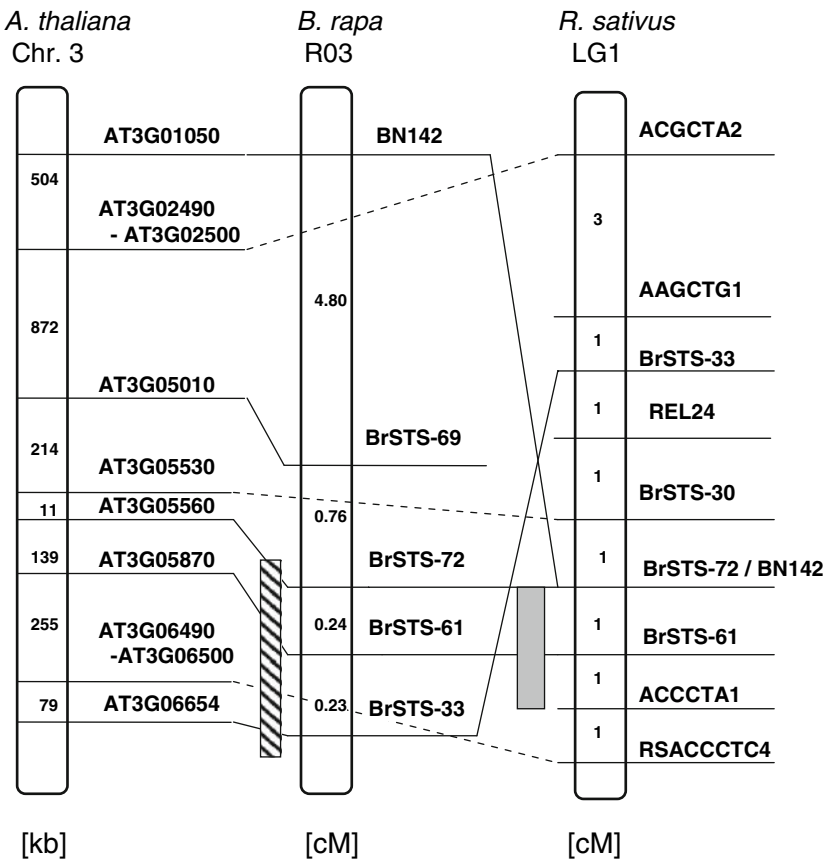


**Fig. 3** Synteny of the radish genomic region around the clubroot resistance locus, *Crs1*. The top of chromosome 3 of *Arabidopsis* (*left*), the *Crr3* region of linkage group (LG) R03 of *Brassica rapa* (*middle*), and the *Crs1* region of LG 1 of *Raphanus sativus* (*right*) are shown. The *bar* to the left of LG R03 indicates the expected genomic region of *Crr3* (Saito et al. 2006). *Bars* to the left of radish LG1 indicate one-LOD support intervals (Van Ooijen 1992). Only approximate marker distances are shown in the figure

**Table 2** Quantitative trait loci (QTL) detected on linkage group 1 affecting clubroot resistance (CR)

| CR test | Proximal marker | Position (cM) | LOD score[a] | Probability of CR[b] | | |
|---|---|---|---|---|---|---|
| | | | | QQ | Qq | qq |
| 1 | BrSTS-61 | 7.8 | 9.95 (1.47) | 1.00 | 1.00 | 0.36 |
| 2 | BN142 | 7.0 | 12.64 (1.32) | 0.96 | 0.95 | 0.11 |

[a] The figure in the parenthesis indicates a threshold value of genome-wide 1% significance level for LOD score, which is obtained by a permutation test with 1,000 replications

[b] Prediction probability of plants being resistant to clubroot given QTL genotypes, QQ, Qq, and qq, where Q and q indicate QTL alleles derived from Utsugi-gensuke and Koga-benimaru, respectively

syntenic to the *Arabidopsis* chromosome 3, as in the case of *Crr3* in *B. rapa*.

### CR test using a virulent isolate of *P. brassicae*

As radish is known to be highly resistant to the clubroot pathogen, we challenged it with a more virulent isolate, Wakayama-01. For this CR test, 10 $F_3$ families were selected on the basis of the genotype of a marker, BN142. This marker was mapped in the vicinity of *Crs1* (Fig. 3). Because two-way analysis of variance showed no significant differences among the three tests (data not shown), total number of plants showing each ID score was shown in supplementary Table. The $F_3$ families homozygous for the allele of the resistant parent at BN142 locus showed entirely no symptoms. The homozygotes of the susceptible allele showed susceptibility. The most seedlings of the heterozygous families showed no symptom, but a part of them showed some symptoms (supplementary Table).

## Discussion

### QTL analysis of CR in radish

The top region of LG1 showed the highest LOD score in the two tests (Fig. 2). Genotype of the peak region effectively discriminate resistant phenotypes (Table 2). These results suggest that a large part of CR of radish is controlled by this QTL, *Crs1*, when Ano-01 was used as a pathogen. Since LOD profiles were rather broad, precise position of the resistance gene remained to be studied. In addition, the presence of two or more resistance genes linked to each other in this region cannot be ruled out. However, region around 7 cM is the most plausible to have the resistance

gene. The present results are in accordance with the recent report by Akaba et al. (2009), in which CR tests using monosomic addition line showed that the CR is largely controlled by one chromosome of radish. The present study could not deny the presence of CR genes having minor effects outside LG1. Subsequent CR tests with the pathogen isolate with a wide virulence spectrum, Wakayama-01, were done using the selected $F_3$ families. Families homozygous for resistant parent allele at the BN142 locus showed no symptoms in any of the three tests (supplementary Table). Although an effect of another locus or other loci cannot be ruled out, the results suggest that the CR locus, *Crs1*, expressed resistance not only to the Ano-01 isolate but also to the virulent isolate, Wakayama-01. The latter isolate infects most commercial CR Chinese cabbage cultivars (Hatakeyama et al. 2004). Our previous study on *B. rapa* showed that resistance to the Wakayama-01 isolate requires the coexistence of two CR loci, *Crr1* and *Crr2* (Suwabe et al. 2003). Therefore, the *Crs1* locus may be effective against a wide range of field isolates of the clubroot pathogen and may be responsible for the high resistance of radish against this pathogen (Yoshikawa 1993).

## Synteny of the genomic region around *Crs1*

In this study, six codominant markers, BrSTS-30, BN142, BrSTS-72, BrSTS-61, REL24, and BrSTS-33 were found in regions with reliable LOD scores. Among them, four (BN142, BrSTS-72, BrSTS-61, and BrSTS-33) are common to the *Crr3* region of *B. rapa*. This region has synteny to chromosome 3 of *Arabidopsis* (Saito et al. 2006). *Crr3* is located in a 0.35 cM segment between BrSTS-78 and BrSTS-33 (Saito et al. 2006). The LOD score peaks of *Crs1* are located in the vicinity of BrSTS-61. This marker is located between BrSTS-78 and BrSTS-33 in *B. rapa*. Therefore, it is suggested that the genomic region around *Crs1* of the *Raphanus* genome and that around *Crr3* of the *B. rapa* genome evolved from the same region of the common ancestral genome. Suwabe et al. (2006) pointed out that two CR regions in *B. rapa*, the *Crr1* and *Crr2* regions, show synteny to the bottom arm of chromosome 4 of *Arabidopsis*. *CRb*, a CR locus that has been mapped in detail for *B. rapa* (Piao et al. 2004) shows synteny to chromosome 4 of *Arabidopsis* (Saito et al. 2006). Therefore, CR genes mapped in *Brassica* and *Raphanus* may have evolved from very limited regions of the ancestral genome.

## Breeding of CR cultivars of *Raphanus* and *Brassica*

Clubroot disease of radish has been reported in the USA and Korea (Thuma et al. 1983; Cho et al. 2003). Although there are no reports on clubroot in radish in China, it is likely that it is a problem in China because some of the radish cultivars in northern China are highly susceptible (Scheijgrond and Vos 1954; Yoshikawa 1993). Although most Japanese radishes are immune to the clubroot pathogen, infection of a few commercial cultivars of radish in Japan has been reported recently (Horikoshi and Tairako 2002). The hosts were modern cultivars. Korean radishes are sometimes used to breed radish in Japan. Some of the Korean radishes are known to be CS. The CR gene may have lost during crossing with Korean radish. Breeders of radish cultivars should be careful to retain the CR gene. We developed several genetic markers in the vicinity of the CR gene, *Crs1*, some of which are codominant. These markers may be useful in marker-assisted selection (MAS) of CR radish cultivars. Transfer of the radish CR gene to other *Brassica* crops may confer high resistance against clubroot. Although some attempts have been made to introduce the CR trait of radish into *Brassica* crops (Xing et al. 1989; Hagimori et al. 1992), fertile, resistant *Brassica* plants have not been produced yet. An introgression study on the Kosena restorer gene for cytoplasmic male sterility suggests that recombination events between the *Brassica* and *Raphanus* genomes are rare (Sakai et al. 1996). The genetic markers identified in our study are useful tools for monitoring this rare event in hybrids.

## Mapping of the *Raphanus* genome

Only a few studies on genetic linkage maps of radish have been reported (Bett and Lydiate 2003; Tsuro et al. 2005, 2008; Kaneko et al. 2007). Two of these maps are based on RFLP and the markers are not useful for MAS. As SSR markers developed from genomic library of *B. rapa* were not so effective in the present radish mapping, more PCR-based markers should be developed from its own genomic or EST sequences to construct a saturated map of *Raphanus*. Effectiveness of genetic markers of *B. oleracea* is remained unexamined in the present study.

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