Note

Identificaiton of a clubroot resistance locus conferring resistance to a *Plasmodiophora brassicae* classified into pathotype group 3 in Chinese cabbage (*Brassica rapa* L.)

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In Chinese cabbage (*Brassica rapa*), the clubroot resistance (CR) genes *Crr1* and *Crr2* are effective against the mild *Plasmodiophora brassicae* isolate Ano-01 and the more virulent isolate Wakayama-01, but not against isolate No. 14, classified into pathotype group 3. ‘Akiriso’, a clubroot-resistant F₁ cultivar, showed resistance to isolate No. 14. To increase the durability of resistance, we attempted to identify the CR locus in ‘Akiriso’. CR in ‘Akiriso’ segregated as a single dominant gene and was linked to several molecular markers that were also linked to *CRb*, a CR locus from cultivar ‘CR Shinki’. We developed additional markers around *CRb* and constructed partial genetic maps of this region in ‘Akiriso’ and ‘CR Shinki’. The positions and order of markers in the genetic maps of the two cultivars were very similar. The segregation ratios for resistance to isolate No. 14 in F₂ populations derived from each of the two cultivars were also very similar. These results suggest that the CR locus in ‘Akiriso’ is *CRb* or a tightly linked locus. The newly developed markers in this study were more closely linked to *CRb* than previously reported markers and will be useful for marker-assisted selection of *CRb* in Chinese cabbage breeding.

Key Words: clubroot, disease resistance, *Plasmodiophora brassicae*, linkage map, *CRb*, simple sequence repeat (SSR) markers, *Brassica rapa*.

Introduction

Clubroot disease, which is caused by the soil-borne plant pathogen *Plasmodiophora brassicae* Woronin, is the most serious disease in cultivated *Brassica* species such as *B. rapa*, *B. oleracea* and *B. napus*. The pathogen causes abnormal cell enlargement and cell division of infected roots that produces characteristic deforming galls (the “clubs”). The disease causes substantial reductions in crop quality and yield. The ability of this pathogen to survive in soil as resting spores for several years makes it difficult to prevent clubroot disease by cultural practices (Voorrips 1995). Although fungicide application is sometimes used as a means of disease control in Chinese cabbage and cabbage grown in Japan, breeding for resistance is the most effective approach to minimize crop losses. The clubroot resistance (CR) genes from European fodder turnip (*B. rapa*) cultivars such as ‘Gelria R’, ‘Siloga’, ‘Debra’ and ‘Milan White’ have been introduced into Chinese cabbage (Yoshikawa 1981), and a

number of clubroot-resistant F₁ cultivars have been subsequently released. Recently, however, breakdown of the clubroot resistance of several clubroot-resistant cultivars has been reported in some production areas in Japan (Hatakeyama *et al.* 2004, Kuginuki *et al.* 1999). Many of the present clubroot-resistant F₁ cultivars appear to be heterozygous for a single dominant resistance gene. Because *P. brassicae* is a highly variable pathogen, the likelihood that it could overcome CR controlled by a single gene is high. Therefore, breeding for additional resistant cultivars is needed, and pyramiding of different CR genes into a single line is necessary to confer resistance to the broad spectrum of races of *P. brassicae* (Hirai 2006).

Many mapping studies for CR loci have been reported in *Brassica*, and at least eight CR loci have been identified in *B. rapa* (Diederichsen *et al.* 2009, Hirai 2006). *Crr1*, *Crr2* and *Crr4* were mapped to *B. rapa* linkage groups R8, R1 and R6, respectively (Suwabe *et al.* 2006) and *CRc* was mapped to R2 (Sakamoto *et al.* 2008). Four CR loci, *CRa* (Hayashida *et al.* 2008), *CRb* (Piao *et al.* 2004), *CRk* (Sakamoto *et al.* 2008) and *Crr3* (Saito *et al.* 2006), were mapped to R3. Although *CRk* and *CRc* have been reported to confer resistance to different races of the pathogen

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(Sakamoto *et al.* 2008), knowledge of the relationship between CR loci and pathogenicity is limited. We have developed the CR Chinese cabbage parental line 'Chukanbohon Nou 9 gou' (PL9) containing two CR genes, *Crr1* and *Crr2*, introduced by marker-assisted selection (MAS) (in preparation). We demonstrated that plants having *Crr1* conferred resistance to the mild strain Ano-01, whereas both *Crr1* and *Crr2* were necessary for resistance to the more virulent strain Wakayama-01 (Suwabe *et al.* 2003, 2006). Four pathotypes (group 1 to group 4) were identified in Japanese field isolates through the use of two commercial CR F₁ cultivars of Chinese cabbage (Hatakeyama *et al.* 2004, Kuginuki *et al.* 1999). PL9 showed resistance to isolates of pathotype groups 1, 2 and 4, but not group 3. Thus, the resistant cultivars against isolates of pathotype groups 1, 2 and 4, could be developed by introduction of *Crr1* and *Crr2* with MAS. Although several F₁ cultivars have been reported to show complete and partial resistance to pathotype group 3 (Hatakeyama *et al.* 2004), the genetic basis of CR locus effective to pathotype group 3 is unknown.

To increase the durability of resistance, it is necessary to identify CR gene against this pathotype and develop the useful markers for MAS. 'Akiriso', a *Verticillium* yellows resistant F₁ Chinese cabbage cultivar, is resistant to pathotype group 3. Therefore, we performed genetic analysis for the resistance in 'Akiriso' and compared the CR locus in this cultivar with previously identified CR loci.

Material and Methods

Plant materials

We used two CR F₁ cultivars of Chinese cabbage, 'Akiriso' (Nippon Norin Seed Co.) and 'CR Shinki' (Takii & Co., Ltd.) and the parental lines of 'Akiriso', called T-line and V-line. Populations of 285 and 172 F₂ plants were obtained by self-pollinating 'Akiriso' and 'CR Shinki', respectively. Of these, 189 of the 285 plants in the 'Akiriso' population and all of the plants in the 'CR Shinki' population were used for CR tests and construction of linkage maps. The remaining 96 F₂ plants derived from 'Akiriso' were used to analyze the correlation between resistance and the genotypes of previously reported markers linked to CR loci.

Pathogen and CR test

For CR testing, we used *Plasmodiophora brassicae* field isolate No. 14, which is classified into group 3 (Hatakeyama *et al.* 2004). The CR test was performed as previously described (Suwabe *et al.* 2003). The disease index (ID) was scored according to Hatakeyama *et al.* (2004) on a scale of 0 to 3: 0, no symptoms; 1, a few small, separate globular clubs on the lateral roots; 2, no clubs of main root, but moderate clubbing of lateral roots; 3, absence of normal roots, presence of large gall. Plants with ID scores of 0 to 2 were categorized as resistant (which included partial resistance); those with a score of 3 were categorized as susceptible, because healthy roots were observed in the former, but not in the latter.

Developing markers and linkage analysis

DNA markers were designed based on genome sequencing information from the *B. rapa* Genome Sequencing Project (BrGSP) (<http://www.brassica-rapa.org/BRGP/index.jsp>) (Mun *et al.* 2008, 2010). We developed SSR markers based on BAC end sequences of KBr libraries in BrGSP using the read2Marker program (Fukuoka *et al.* 2005). To investigate the relationship between the CR locus of 'Akiriso', here named *CRaki*, and known CR loci, we used markers reported by Hayashida *et al.* (2008), Piao *et al.* (2004), Saito *et al.* (2006), Sakamoto *et al.* (2008) and Suwabe *et al.* (2006). In addition, we converted the *CRb*-linked CAPS marker TCR02 (Piao *et al.* 2004) to an SSR marker, designated TCR02-F, based on the sequence of the PCR product of TCR02. The primers designed in this study are listed in Table 1. PCR amplification was carried out in 10- μ l reaction mixtures containing 10 ng genomic DNA, 4 pmol of each primer and 2 $\times$ Quick Taq HS Dye Mix (TOYOBO, Osaka, Japan) in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA). The reaction was performed with the following parameters: 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 30 s and 68°C for 30 s; and final extension at 68°C for 5 min. Detection of polymorphisms was performed as described previously (Hatakeyama *et al.* 2010). To construct a linkage map of 'Akiriso', markers showing polymorphism between the two parental lines, T-line and V-line, were used for segregation analysis in an F₂ population obtained by selfing 'Akiriso'. Segregation analysis of resistance in an F₂ population obtained by selfing 'CR Shinki' suggested that 'CR Shinki' was heterozygous for a single dominant CR gene (see Results and Discussion), so we concluded that 'CR Shinki' was a hybrid of a CR parent and a clubroot-susceptible parent. Because the parental lines of 'CR Shinki' are proprietary and not available to us, we could not determine the marker genotypes of the parental lines. Instead, we selected three resistant and three susceptible F₂ plants that were homozygous for genotypes of two *CRb*-linked markers, TCR05 and TCR02-F, to represent the genotypes of the postulated CR parent and clubroot-susceptible parent, respectively. To construct a linkage map of 'CR Shinki', we searched for polymorphisms between the selected resistant and susceptible F₂ plants. The linkage maps of 'Akiriso' and 'CR Shinki' were constructed using JoinMap ver. 4.0 (van Ooijen 2006).

Results and Discussion

Association between molecular markers and clubroot resistance

We evaluated the clubroot resistance of the parental lines of 'Akiriso' (T-line and V-line), 'Akiriso' (F₁) and 189 F₂ plants by inoculation with *P. brassicae* isolate No. 14. The frequency distribution of resistance in the F₂ population is shown in Fig. 1A and Table 2. The parental lines, T-line and V-line, were resistant (mean ID = 1.08, $n = 26$) and

Table 1. Primer sequences for detection of genomic SSRs and PCR product sizes in ‘Akiriso’ and ‘CR Shinki’

Marker name	Repeat motif ^a	Forward primer (5' to 3') Reverse primer (5' to 3')	Size of PCR product (bp) ^b			
			Akiriso		CR Shinki	
			T-line	V-line	R	r
KBrH129J08R ^c	TA	ATGAGATTGAAGAGGGAAACACAA GTTTCCAATGGTGAAACCAATCCTA	234	241	234	241
KBrH059N21F	CT	ATCGACGCCGTTTATTAGAAGCTCG GTTTACGCCACGTCAGCTCACTAACTC	218	236	218	236
KBrH129J18R	TC	AGAGCAGAGTGAAACAGAACT GTTTCAGTTCAGTCAGGTTTTGTCAG	254	194	254	194
KBrB091M11R	AG	ACTTAAAGCACGAGAATGCAAA GTTTGGTGTCGAAGCTATGTGTG	750	177	750	177
TCR02-F	CTA	AGCTCCATTTCAGTTACGGTGA GTAAAGAACTTGCAGAACTCG	203	207	203	207

^a SSRs found in genomic sequence information from the *B. rapa* Genome Sequencing Project (<http://www.brassica-rapa.org/BRGP/index.jsp>).

^b PCR product sizes of T-line and V-line in ‘Akiriso’, and resistant (R) and susceptible alleles (r) detected in homozygous F₂ progeny of ‘CR Shinki’.

^c This marker was reported by Kakizaki *et al.* (2011).

susceptible (mean ID = 2.96, $n = 25$), respectively (Fig. 1A). ‘Akiriso’ showed resistance (mean ID = 1.49, $n = 37$). In the F₂ population, 140 plants (74%) were scored as resistant and 49 (26%) as susceptible (Table 2). This segregation ratio fitted a 3 : 1 Mendelian ratio ($P = 0.79$ for χ^2). These results indicate that the clubroot resistance of ‘Akiriso’ is conferred by a single dominant gene derived from T-line.

To examine whether the CR gene detected in ‘Akiriso’ is an allele of one of the eight known CR genes in *B. rapa*, we analyzed the correlation between the resistance phenotypes in the F₂ population and genotypes of markers linked to each of the eight known CR loci. Among the markers tested, TCR10 and TCR02-F, both of which are linked to *CRb* (Piao *et al.* 2004) (Fig. 2A), were also linked to resistance in a subset of the ‘Akiriso’ F₂ population ($n = 96$) (Table 3). F₂ plants with a band amplified by the TCR10 primer set showed resistance (mean ID = 1.18), whereas F₂ plants with no bands were susceptible (mean ID = 2.88). In addition, plants homozygous for the resistant and susceptible alleles of TCR02-F showed resistance (mean ID = 0.88) and susceptibility (mean ID = 2.86), respectively, and heterozygous plants showed intermediate resistance (mean ID = 1.21). Other TCR markers (TCR01, TCR05, TCR08 and TCR09) linked to *CRb* (Piao *et al.* 2004) did not show polymorphism between T-line and V-line. There was no correlation between resistance and genotypes of markers linked to other CR loci, including OPC11-2S (*Crr3* and *CRk*) (Saito *et al.* 2006), BRMS-088 (*Crr1*) (Suwabe *et al.* 2006), BRMS-096 (*Crr2*) (Suwabe *et al.* 2006) and BRMS-125 (*Crr4*) (Suwabe *et al.* 2006) (data not shown), and no polymorphisms were detected between the parental lines for markers HC352b-SCAR (*CRa*) (Hayashida *et al.* 2008) and m6R (*CRc*) (Sakamoto *et al.* 2008). These results suggest that *CRaki* is linked to TCR10 and TCR02-F, which are linked to *CRb*, thus raising the possibility that *CRaki* and *CRb* are the same locus.

Table 2. Segregation of resistance to isolate No. 14 in F₂ populations derived from two F₁ cultivars

Cultivar	Phenotype ^a		χ^2 ^b	<i>P</i> -value
	R	S		
Akiriso	140	49	0.07	0.79
CR Shinki	126	46	0.24	0.62

^a R, number of resistant plants (ID = 0, 1, 2); S, number of susceptible plants (ID = 3). Fig. 1. shows the data broken down by ID score.

^b Test of 3 : 1 expected ratio.

Table 3. Correlation between genotypes of two *CRb*-linked markers and resistance to isolate No. 14 in F₂ plants derived from ‘Akiriso’ ($n = 96$)

Marker	Genotypes ^a	No. of plants	Mean ID ^c
TCR10	<i>RR</i> , <i>Rr</i> ^b	79	1.18 a
	<i>rr</i>	17	2.88 b
TCR02-F	<i>RR</i>	29	0.88 a
	<i>Rr</i>	45	1.21 a
	<i>rr</i>	22	2.86 b

^a *RR*, genotype of T-line (CR); *rr*, genotype of V-line (clubroot-susceptible); *Rr*, heterozygous genotype.

^b TCR10 is a dominant marker, so the value represents the total number of *RR* + *Rr* plants.

^c Significant differences of the two (TCR10) and three (TCR02-F) genotype groups were assessed using Mann-Whitney *U*-test and the Mann-Whitney *U*-test with Bonferroni's correction following Kruskal-Wallis analysis, respectively. The same letter is not significantly different by the 5% level.

Development of DNA markers and construction of linkage maps

To investigate whether *CRaki* is the same locus as *CRb*, we attempted to construct detailed linkage maps around *CRaki*. We assumed that *CRb* was located in the long arm of chromosome R3 because TCR05-R, which is linked to *CRb*,

Fig. 1. Frequency distribution of disease index (ID) for clubroot resistance in F_2 populations derived from F_1 cultivars 'Akiriso' (A) and 'CR Shinki' (B). 0 = complete resistance; 3 = susceptible. The ID scores of T-line and V-line, the parents of 'Akiriso', 'Akiriso' and 'CR Shinki' are indicated by arrowheads.

is located between BRMS-124 and BRMS-206 in this chromosome region (Saito *et al.* 2006; Fig. 2B). These two markers were located at positions 12 cM and 30–40 cM on the long arm of chromosome R3, respectively (Saito *et al.* 2006, Suwabe *et al.* 2006). In this region, marker KBrH129J08R, which was developed from the end sequence of BAC clone KBrH129J08 was located on the linkage map reported by Kakizaki *et al.* (2011) (Fig. 2C). Therefore, we developed three SSR markers based on the sequences of BAC clones assembled around KBrH129J08 by using genome sequence information from BrGSP (Mun *et al.* 2008, 2010) (Fig. 2). Partial linkage maps were constructed for 'Akiriso' (Fig. 2D). Four newly developed markers (KBrH059N21F, KBrH129J18R, KBrB091M11R and TCR02-F) and KBrH129J08R (Table 1) were mapped around *CRaki*. In particular, KBrH059N21F and KBrH129J18R were localized 0.3 cM distal and 0.4 cM proximal to *CRaki*, respectively.

We also constructed a linkage map around *CRb* using an F_2 population derived from the selfing of 'CR Shinki' (Fig. 2E). Five markers linked to *CRaki* were also linked to *CRb* (Fig. 2D, 2E), and the positions of these markers and

TCR05 on genome sequence of chromosome R3 were determined (Fig. 2F). Markers KBrH059N21F and KBrH129J18R were localized 0.2 cM distal and 0.4 cM proximal to *CRb*, respectively. These two markers were closer to *CRb* than TCR05 (Piao *et al.* 2004), which was located 1.4 cM from *CRb* in this experiment. The sizes of the PCR products, positions and order of the five common markers were almost identical in 'CR Shinki' and 'Akiriso' (Table 1 and Fig. 2D, 2E). These results suggest that the CR loci of these two cultivars are identical and that *CRaki* is an allele of *CRb*, but we cannot yet rule out the possibility that *CRb* and *CRaki* are distinct but tightly linked loci.

CRb was originally identified through genetic analysis of a resistant doubled haploid line derived from 'CR Shinki' using a single spore isolate with race 4 on the basis of Williams' (1966) classification (Piao *et al.* 2004). We performed CR tests with isolate No. 14 on 172 plants from the F_2 population derived from 'CR Shinki' and compared the results with the results from 'Akiriso' (Fig. 1 and Table 2). 'CR Shinki' showed resistance (mean ID = 1.25, $n = 15$). In the F_2 population, resistant and susceptible plants were scored in a ratio of 126 (73%) to 46 (27%), respectively, which fit a 3 : 1 Mendelian ratio (Table 2). Thus, the segregation of resistance observed in the F_2 population derived from 'CR Shinki' (Fig. 1B) was very similar to that in 'Akiriso' (Fig. 1A). These results suggested that the resistance of 'CR Shinki' to pathotype group 3 is controlled by a single gene encoded at *CRb*.

We also demonstrated that the resistance responses of 'Akiriso' to four other pathotypes (data not shown) were quite similar to those reported by Hatakeyama *et al.* (2004) for 'CR Shinki'. It is unknown whether the pathogenicity of isolate No. 14 used in this study is the same as that of the isolate used in Piao *et al.* (2004) because we did not perform the CR test using Williams' hosts (Williams 1966) with No. 14. However, isolate No. 2 classified into pathotype group 3 was found to be race 4 (Hatakeyama *et al.* 2004), suggesting that the pathogenicity of both isolates was very similar.

CRb was previously reported to be distal to TCR05 on R3 (Piao *et al.* 2004, Mun *et al.* 2010), but our mapping data placed *CRb* proximal to TCR05. This difference may be due to the difference in marker types used for the two analyses. In the present study, we used co-dominant markers to construct the linkage map, whereas three (TCR08, TCR09, TCR10) of the six markers used in Piao *et al.* (2004) were dominant markers. Co-dominant markers are highly informative: they can discriminate between homozygous and heterozygous genotypes and have greater reproducibility than dominant markers (Jones *et al.* 1997). In mapping studies, the characteristics of the markers used will influence the precision of the linkage map. Although TCR09 was previously reported to be the closest marker to *CRb* (Piao *et al.* 2004) (Fig. 2A), TCR05 has been used as the selection marker for *CRb* by many researchers (Diederichsen *et al.* 2009, Nagaoka *et al.* 2010, Saito *et al.* 2006, Sakamoto *et al.* 2008). Fluorescent in situ hybridization (FISH) analysis

Fig. 2. Genetic linkage maps of the long arm of *B. rapa* chromosome R3 showing positions of CR loci. Marker names and genetic distances (cM) are indicated on the right and left sides, respectively, of each linkage map. (A) Linkage map of the *CRb* region reported by Piao *et al.* (2004). (B) A part of the linkage map of the region containing *Crr3* (Saito *et al.* 2006). (C) Linkage map constructed by Kakizaki *et al.* (2011). (D, E) Linkage maps containing the CR loci of ‘Akiriso’ (*CRaki*) (D) and *CRb* of ‘CR Shinki’ (E) constructed in the present study. (F) Positions of markers on the *B. rapa* genome sequence of chromosome R3 (*BrR3*) based on information from the *Brassica* database (BRAD) (<http://brassicadb.org/brad/>) are shown. Markers in common between linkage maps are connected by dashed lines. Maps are not drawn to scale.

revealed that BAC clones containing TCR09 and TCR05 were localized in different regions of the same chromosome (Piao *et al.* 2009), indicating that they might not be as closely linked as previously reported (Piao *et al.* 2004). In this study, the presence of TCR09 did not correlate with resistance in the F_2 population derived from ‘CR Shinki’. In addition, TCR10 was located more than 13 cM apart from *CRb* (data not shown), which was a different position on R3 than was described by Piao *et al.* (2004). Therefore it is possible that difficulty in using these two markers (TCR09 and TCR10) can affect the apparent order of the markers and the position of *CRb*. On the other hand, order of 4 markers developed in this study and 2 markers (TCR02-F and TCR05) was consistent with *B. rapa* genome sequence (Fig. 2F).

In conclusion, we demonstrated that the segregation of resistance to *P. brassicae* isolate No. 14 in the F_2 progeny and the order of SSR markers around the CR loci were quite similar between ‘Akiriso’ and ‘CR Shinki’, both of which show resistance to isolate No. 14. Our results indicate that the CR locus controlling resistance to isolate No. 14 in ‘Akiriso’ is tightly linked to *CRb* and is likely to be the same locus. Two SSR markers developed in this study, KBrH059N21F and KBrH129J18R, were closely linked to *CRb* and can be scored by agarose gel electrophoresis. These two markers will be useful for introducing *CRb* into other cultivars using marker-assisted selection.

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