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Identification of two loci for resistance to clubroot (*Plasmodiophora brassicae* Woronin) in *Brassica rapa* L.

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Abstract In an analysis of 114 F_2 individuals from a cross between clubroot-resistant and susceptible lines of *Brassica rapa* L., 'G004' and 'Hakusai Chukanbohon Nou 7' (A9709), respectively, we identified two loci, *Crr1* and *Crr2*, for clubroot (caused by *Plasmodiophora brassicae* Woronin) resistance. Each locus segregated independently among the F_2 population, indicating that the loci reside on a different region of chromosomes or on different chromosomes. Genetic analysis showed that each locus had little effect on clubroot resistance by itself, indicating that these two loci are complementary for clubroot resistance. The resistance to clubroot was much stronger when both loci were homozygous for resistant alleles than when they were heterozygous. These results indicate that clubroot resistance in *B. rapa* is under oligogenic control and at least two loci are necessary for resistance.

Keywords *Brassica rapa* · Clubroot · Microsatellites (SSRs) · *Plasmodiophora brassicae* · Disease resistance

Introduction

Clubroot caused by *Plasmodiophora brassicae* Woronin is one of the most serious diseases of *Brassica rapa* L. and other crucifers (Crute et al. 1980). *P. brassicae* is a soil-borne, obligate plant pathogen with a two-phase infection cycle. The primary phase occurs in root hairs, and the secondary phase occurs in cells of the cortex and stele of the root. During the latter phase, multinucleate plasmodia induce clubs on roots, leading to inhibition of nutrient and water transport. As a result, infected plants grow abnormally and yield is decreased. Because the organism survives as resting spores in the soil for a long period and has wide pathogenicity within crucifers, serious damage is hard to prevent by cultural and chemical practices (Karling 1968; Voorrips 1995).

Clubroot resistance is one of the most-desirable traits in *Brassica* breeding. Chiang et al. (1980) introduced resistance into *Brassica oleracea* from *Brassica napus*. Yoshikawa et al. (1981) found clubroot-resistant (CR) lines in European fodder turnips and used them as sources for breeding CR Chinese cabbages. More than 50 CR F_1 hybrid cultivars of Chinese cabbage have been released in Japan. However, expression of resistance is often quantitative and the genetic basis of the resistance to clubroot in *B. rapa* is not clear. Moreover, the breakdown of disease resistance caused by genetic variability of the pathogen has been reported (Kuginuki et al. 1999). The distribution of multiple pathogenic races in a single field and mixed infection by multiple races have also been reported (Buczacki et al. 1975; Jones et al. 1982). Therefore, breeding of CR cultivars has often been futile and the identification of the complete system of resistance to clubroot is required for the establishment of a CR breeding system.

Some researchers have reported genetic analyses of clubroot resistance in *B. oleracea* (Landry et al. 1992; Figdore et al. 1993; Grandclément and Thomas 1996; Voorrips et al. 1997). Fuchs and Sacristán (1996) indicated that a dominant allele of a single nuclear gene controlled the resistance of clubroot in *Arabidopsis thaliana*. In *B. rapa* and *B. napus*, it has been considered that a single locus or gene was sufficient to express clubroot resistance (Fuchs and Sacristán 1996). Yoshikawa (1981), however, suggested the existence of a major

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gene and some genes with a minor effect on clubroot resistance in *B. rapa*. Indeed, Kuginuki et al. (1997) identified a major locus resistant to race 2, but it required an additional genetic element(s) in order to exhibit complete resistance. Nevertheless, no additional locus responsible for clubroot resistance has been identified to-date. All these findings suggest that the genetic mechanisms of clubroot resistance are not simple among *B. rapa* and other crucifers.

It has been widely recognized that DNA markers linked to desirable traits, such as disease resistance and morphological and physiological characters, can be useful tools in the genetic analysis of large numbers of individuals, especially for polygenic traits. Moreover, DNA markers are useful tools unaffected by environmental effects (Ford et al. 1998; Kandemir et al. 2000; Parker and Langridge 2000). Indirect selection based on them, therefore, would have a great advantage in plant breeding.

Recently, microsatellites, or simple sequence repeats (SSRs), have been used as DNA markers in various analyses, such as marker-assisted selection (MAS), linkage mapping and population analysis in various species (Gupta and Varshney 2000). SSRs are repeated nucleotide motifs (1–6 bp) existing throughout the genome, and are highly polymorphic owing to variations in the number of repeats (Tautz and Renz 1984; Hancock 1995; Dib et al. 1996; Dietrich et al. 1996). Moreover they are inherited in a co-dominant manner (Morgante and Olivieri 1993) and can be analyzed by convenient PCR-based methods. Hence, SSRs are more preferable as DNA markers than others, such as RFLPs, AFLPs and RAPDs. In crucifers, SSR markers have been developed in *B. napus* (Kresovich et al. 1995; Uzunova and Ecke 1999), *B. nigra* (Westman and Kresovich 1999), *B. rapa* (Suwabe et al. 2002) and *A. thaliana* (Bell and Ecker 1994). They are powerful tools in various analyses and would be useful in genetic analysis for *Brassica* breeding as well.

In this study, we report the identification of two loci for resistance to clubroot by the use of SSR markers. Our results indicate that clubroot resistance in *B. rapa* is under oligogenic control.

Materials and methods

Plant materials

A CR doubled-haploid (DH) line, ‘G004’, and a clubroot-susceptible (CS) DH line, ‘Hakusai Chukanbohon Nou 7’ (A9709), were obtained by microspore culture (Kuginuki et al. 1997). G004 is a DH line derived from a cross between ‘Homei P09’, a line with a high ability at regenerating from microspores, and ‘Siloga S2’, a line derived by selfing of a CR cultivar of European fodder turnip, ‘Siloga’. A9709, another DH line with a high ability at regenerating from microspores, is derived from a cross between ‘Homei P09’ and ‘Nozaki 2’, an OP cultivar of Chinese cabbage. An F_2 population was derived from a cross of A9709 × G004, and 94 individual plants were randomly selected for primary analysis. Another 20 F_2 plants from the same cross were selected by SSR markers to certify characterization of the resistance loci (see Results). F_3 seeds for a clubroot resistance test were obtained from bud self-pollination of each F_2 individual. Three cultivars of Chinese cabbage, ‘Ryutoku’, ‘Utage 70’ (CR although they show breakdown of resistance to ‘Wakayama-01’, see paragraph ‘Pathogen’ in Materials and methods) and ‘Muso’ (CS), were used as controls in that test. All plants were grown in a greenhouse.

Fig. 1 Classification of clubroot symptoms: 0, no symptoms; 1, a few small, separate globular clubs; 2, intermediate symptoms; 3, severe clubs on main roots. *Arrows* indicate the club(s) on roots

Pathogen

P. brassicae isolate ‘Wakayama-01’ was used in this study. This isolate showed the highest pathogenicity in our pathogen stock. It could infect CR Ryutoku, CR Utage 70 and CS Muso, as the same as ‘Date-01’ which was classified as race 4 (Kuginuki et al. 1999). In Japan, classification of *P. brassicae* isolate has been carried out by these cultivars and the isolate which infect CR Ryutoku and CR Utage 70 is the most-severely one. Pathogenicity of the isolate was checked for its uniformity using positive and negative controls every time. Preparation of the inoculum was carried out as described by Yoshikawa (1981). The inoculum was propagated throughout the infection test and clubs in infected roots were stored at –20 °C until required. Resting spores were purified from the infected clubs and used in the test for clubroot resistance.

Test for clubroot resistance

The test for clubroot resistance was carried out according to Yoshikawa (1981) and Kuginuki et al. (1997). Eight F_3 seeds from a single F_2 individual were sown in a pot containing 5×10^6 spores per gram of dry soil. Two pots (16 seedlings from the same F_2 plant) were used in a single resistance test. Resistance tests were carried out in a growth chamber at 25/20 °C (day/night) with a photoperiod of 14 h at a light intensity of 200 μmol·m^{-2}·s^{-1}. At 6 weeks from sowing, the root symptoms of each plant were evaluated as follows: grade 0, no symptoms; grade 1, a few small, separate globular clubs on lateral roots; grade 2, intermediate symptoms; grade 3, severe clubs on main roots (Fig. 1). A disease index (ID) was evaluated from the results as the mean grade for 16 F_3 seedlings, and the mean ID for each F_2 individual was expressed as the mean from two independent tests (a total of 32 seedlings). In the analysis of quantitative traits, the phenotype of the F_2 individual can be evaluated from the mean phenotype of its progeny (Hayashi and Ukai 1999) and this model enhances the ability of genetic analysis. Each test included control-resistant and susceptible cultivars.

SSR markers

The procedure for the development of SSR markers is described by Suwabe et al. (2002). In brief, small-insert genomic libraries were constructed by using a ZAP Express lambda phage vector (Stratagene) with genomic DNA digested by *Sau*3A I. SSR clones were isolated by filter hybridization using digoxygenin-labeled $(GA)_{15}$ and $(GT)_{15}$ oligonucleotides as probes. After screening and sequencing, primer pairs were designed for amplifying each SSR locus.

Genetic and phenotypic analyses

DNA extraction and SSR analysis were performed as described by Suwabe et al. (2002) with some modifications. Each SSR locus was amplified with 2 ng of template DNA in a 10-μl reaction volume containing 250 nM of each primer, 0.25 mM of dNTPs, 1× reaction buffer (Takara, Japan) and 1 unit of *Taq* polymerase (Takara). Thermal cycling conditions comprised denaturation at 94 °C for 1 min, followed by 35 cycles of 94 °C for 1 min, optimum temperature of each SSR primer for 1 min and 72 °C for 1 min, and final extension at 72 °C for 4 min in a GeneAmp PCR system 9700 (Applied Biosystems). The PCR product was run on a 2.5% agarose gel and/or a 4% denaturing polyacrylamide gel.

The association between the genotypes of each SSR locus in an F_2 population and the mean ID of each F_2 individual was evaluated manually. By phenotypic analysis, F_2 individuals which showed resistance to ‘Wakayama-01’ were selected from the F_2 population. Among them, SSR loci related to the resistance were selected. In the F_2 population, the differences among nine genotype groups categorized by two SSR markers (see ‘Results’) were assessed statistically with the Kruskal-Wallis nonparametric test and Scheffe’s multiple comparisons.

Results

Test for clubroot resistance

In all experiments, the resistant parent, G004, showed nearly complete resistance (mean ID = 0.5). This value is close to that of ‘Siloga S2’, the resistant parent of G004. The susceptible parent, A9709, had a mean ID rating comparable to that of the susceptible control. All CR cultivars susceptible to Wakayama-01 and the CS cultivar had mean ID values of 3.0 (data not shown). These results indicated that the scoring system used in the experiment had reliable reproducibility in the test for clubroot resistance. The ID of F_2 individuals deduced from the F_3 progeny tests ranged from ‘very susceptible’ to ‘resistant’, but ‘very susceptible’ individuals were more frequent than ‘resistant’ individuals (Fig. 2).

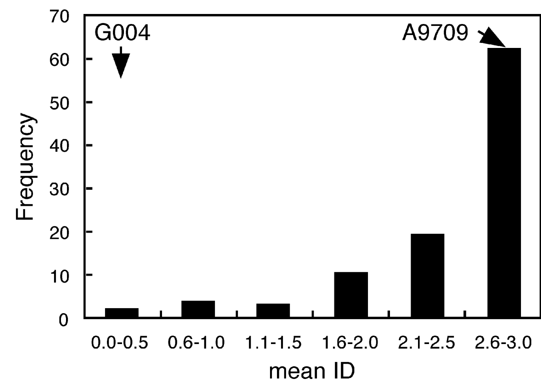

Fig. 2 Frequency distribution of mean ID of clubroot resistance in an F_2 population

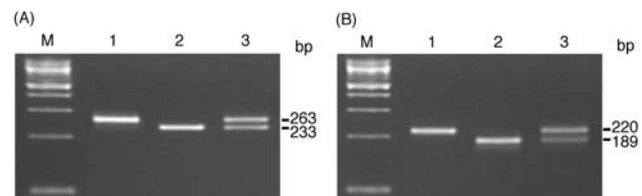

Fig. 3 SSR loci linked to clubroot resistance (2.5% agarose gel). (**A**) BRMS-088, (**B**) BRMS-096. *Lane 1*, G004; *lane 2*, A9709; *lane 3*, heterozygous line; *M*, 100-bp ladder

Associations between SSR markers and clubroot resistance

We developed 56 new SSR markers in addition to the 38 reported by Suwabe et al. (2002). These 94 markers were tested for polymorphism; 42 were found to be polymorphic between the two parental lines and were used for segregation analysis of the F_2 population. From the genetic and phenotypic analyses, we found two CR loci, designated *Crr1* (linked to BRMS-088) and *Crr2* (linked to BRMS-096) (Table 1, Fig. 3). Because each locus segregated independently among the F_2 population, they are genetically independent of each other. Based on the segregation analysis in the F_2 population, they were linked to each resistant gene within 1.75 and 0.88 cM, respectively. The genetic analysis revealed that the alleles derived from the resistant parent (G004) were essential at both loci for expression of resistance to clubroot isolate ‘Wakayama-01’ (Table 2). Moreover, the co-existence of homozygous resistant alleles at both loci gave high resistance. When the alleles derived from the susceptible

Table 1 SSR markers linked to the locus of clubroot resistance

SSR marker	Primer sequence (5′ to 3′)	Product size (bp)[a]	Annealing temp. (°C)
BRMS-088	TATCGGTACTGATTCGCTCTTCAAC ATCGGTTGTTATTTGAGAGCAGATT	233	60
BRMS-096	AGTCGAGATCTCGTTCGTGTCTCCC TGAAGAAGGATTGAAGCTGTTGTG	189	60

[a] Calculated from the sequence in A9709

Table 2 Classification of clubroot resistance into nine genotypes categorized by SSR markers, BRMS-088 and BRMS-096, in an F_2 population

Group no.	Marker[a]		No. of individuals	Mean ID[b]
	BRMS-088	BRMS-096		
1	AA	AA	5	2.98
2		AG	20	2.99
3		GG	15	2.91
4	AG	AA	10	2.99
5		AG	28	2.70
6		GG	22	2.01
7	GG	AA	4	2.94
8		AG	3	2.23
9		GG	7	0.80

[a] AA, homozygous A9709; AG, heterozygous; GG, homozygous G004
[b] Significant difference was deduced among the nine genotypes due to Kruskal-Wallis analysis (of variance by ranks) at the 1% level. Pairing comparisons between groups 1–6, 2–5 and 1–9 were significantly different at the 5% level. Those between groups 2–6, 2–9, 3–6, 3–9, 4–6 and 4–9 were significantly different at the 1% level

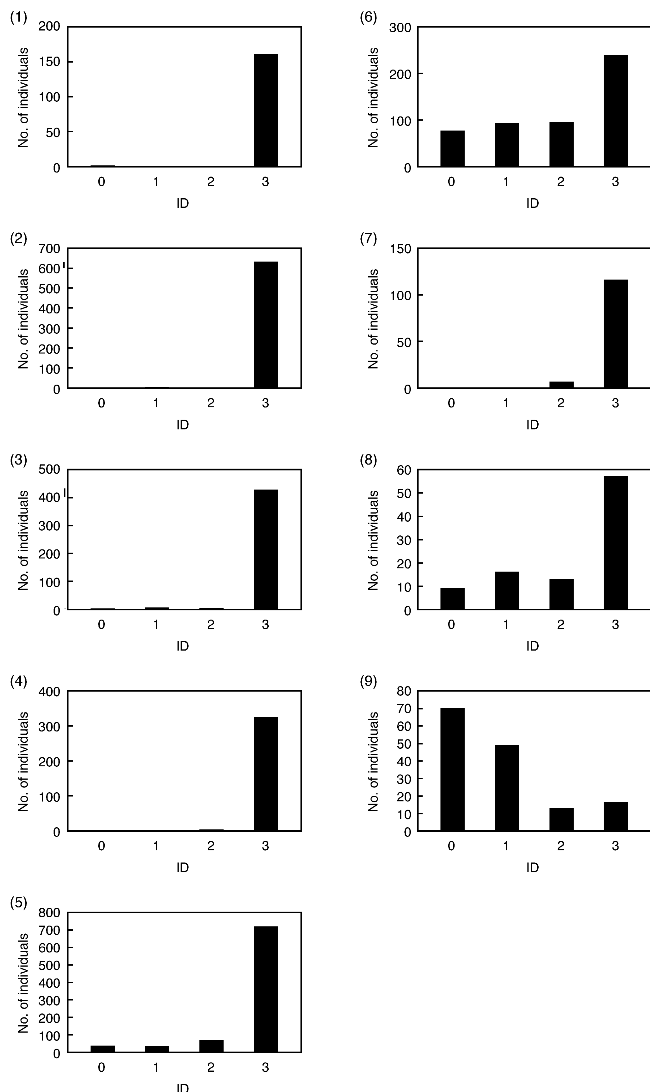

Fig. 4 Frequency distribution of ID in F_3 progenies. (1)–(9) nine genotype groups classified in Table 2

parent (A9709) were homozygous at one or both loci, almost no resistance was observed. In the primary analysis using 94 F_2 plants, those plants which were homozygous for the G004 allele at *Crr1* were too few to analyze statistically. We found only two individuals that were homozygous for the resistant alleles at both loci and showed high resistance, with a mean ID of 1.03. In order to verify the result, 20 additional F_2 plants were selected from the same F_2 population pool by using SSR markers and were added to the analysis. As shown in Table 2, Kruskal-Wallis analysis revealed a significant difference among the nine genotype groups at the 1% level. Multiple comparisons showed that the group that were homozygous for the resistant alleles at both loci (group 9) was significantly different from three groups that were homozygous for the susceptible allele at *Crr1* (group 1, 2 and 3) and from one group that was heterozygous at *Crr1* and homozygous for the susceptible allele at *Crr2* (group 4). Because the group that was homozygous for the resistant allele at *Crr1* and heterozygous at *Crr2* (group 8) contained relatively few individuals, no significant differences from other groups were found, although we would expect differences to exist.

The distribution of ID in each genotype group of F_3 progenies did not agree with a qualitative segregation manner of two genes (Fig. 4).

Discussion

Despite many reports of the genetic analysis of clubroot resistance, little information is available to allow a comprehensive understanding of the resistance. A dominant allele of a single nuclear gene was reported to control resistance to clubroot isolate e in *A. thaliana* (Fuchs and Sacristán 1996). In *B. oleracea*, two QTLs for clubroot resistance have been identified by genetic mapping (Landry et al. 1992; Voorrips et al. 1997). Fuchs and Sacristán (1996) reported that clubroot resistance in *B. rapa* and *B. napus* was controlled monogenically, although Yoshikawa (1981) suggested from the genetic analysis of European fodder turnips, that clubroot

resistance in *B. rapa* is controlled by a major gene and some additional minor genes. Kuginuki et al. (1997) found a locus of resistance to clubroot race 2, but did not explain the complete resistance. These results prompt speculation of a polygenic system of resistance to clubroot in *B. rapa*. In this study we identified two loci, *Crr1* and *Crr2*, for resistance to clubroot isolate 'Wakayama-01' in *B. rapa*. This is the first report that clubroot resistance in *B. rapa* is controlled polygenically, corresponding in part to the genetic system reported in *B. oleracea*.

Neither *Crr1* nor *Crr2* conferred its own clubroot resistance (Table 2). Resistance occurred only when resistance alleles co-existed at both loci. These results clearly indicate that both loci are involved in clubroot resistance and the cooperation of both is essential for this resistance.

We previously reported an STS marker, RA12-75A, which is linked to a locus with a major effect on resistance to clubroot race 2 (Kuginuki et al. 1997; Kikuchi et al. 1999). Among the F_2 population, segregation of BRMS-088 corresponded almost exactly to that of RA12-75A. Although RA12-75A is a dominant DNA marker, by which homo- and hetero-zygotes cannot be distinguished, the recessive allele can be identified in the same way as co-dominant markers. In the F_2 population, the individuals with the recessive allele of RA12-75A corresponded to those with the recessive allele of BRMS-088. All but two plants with the dominant allele of RA12-75A were separated into homo- and hetero-zygotes by BRMS-088. These results indicated that BRMS-088 and RA12-75A are closely linked to each other. This suggests that *Crr1* and the locus for resistance to race 2, linked to RA12-75A, are the same locus, which is a common and major locus controlling resistance to various *P. brassicae* races. In some cases, one resistance locus or gene in host plants has identical specificity to different pathogen races (Crute and Pink 1996). For example, in *A. thaliana, RPP13-Nd* confers resistance to five different isolates of *Peronospora parasitica*, the causal agent of downy mildew (Bittner-Eddy et al. 2000). It is likely that the gene product recognizes the same avirulence determinant in the five *P. parasitica* isolates. Clubroot resistance in *B. rapa* may also have a common locus system among different pathogen races. However, another possibility because of the limited resolution of our present analysis is that *Crr1* and the locus for resistance to race 2 are different loci in the same region of a chromosome. The clustering of disease resistance genes has been reported in *A. thaliana* (Botella et al. 1997; Speulman et al. 1998). The clustering of resistance loci, or genes, has advantages of preserving resistance and allowing the evolution of novel specificities. The precise molecular mapping of two SSR markers is now under way in order to clarify the identity of the two loci. Further analysis will provide detailed information on the relationship between *Crr1* and the locus for resistance to race 2.

In *B. oleracea*, two QTLs, *pb-3* and *pb-4*, for resistance to race 2 have already been identified (Voorrips et al. 1997). *Pb-3* is a major locus controlling clubroot resistance, and explains 54% of the resistance. *Pb-4* has a small effect, and explains an estimated 6% of the resistance. These loci are located in linkage groups 3 and 1, respectively. Although the genetic relationship of *pb-3* and *pb-4* is unclear, the nature of *Crr1* and *Crr2* seems to be consistent with that of *pb-3* and *pb-4*, respectively. Further studies are in progress to identify the chromosomal locations of *Crr1* and *Crr2* to confirm this hypothesis.

RPB1 has been identified as the locus in *A. thaliana* for resistance to clubroot isolate e, and is located at the top of chromosome 1 (Fuchs and Sacristán 1996). Clubroot resistance in *A. thaliana* is considered to be inherited monogenically. It leads to a hypersensitive reaction, which involves the lignification of cell walls to inhibit the spread of the pathogen. As shown in our study here, clubroot resistance in *B. rapa* is oligogenic, with at least two loci, suggesting a different resistance mechanism to that in *A. thaliana*. However, it is not clear whether *Crr1* is orthologous to *RPB1*. It also remains unclear whether these differences are due to the pathotype of *P. brassicae* or the different resistance systems of *A. thaliana* and *B. rapa*. SSR markers developed in *B. rapa* can be used widely as DNA markers within crucifers (Suwabe et al. 2002). Genetic analysis of clubroot resistance using SSR markers common to *A. thaliana* and *B. rapa* would lead to insights into these hypotheses.

The pathogenicity of isolate 'Wakayama-01' could not be classified clearly according to the classification system of Williams (1966). The pathogenicity of this isolate seemed to correspond to 'Date-01', a race 4 population (Kuginuki et al. 1999). However, ambiguity of pathogenicity against Williams' differential hosts was observed (Kuginuki, personal communication). The differential hosts of Williams (1996) are commonly used to classify populations with respect to differential pathogenicity mainly in Europe and North America, although they cannot classify clearly the Japanese populations as reported by Kuginuki et al. (1999). Establishment of a reliable classification system is required for the Japanese populations.

In this study, we identified two independent loci controlling clubroot resistance in *B. rapa*. The loci were marked by convenient co-dominant SSR markers, BRMS-088 and BRMS-096. These findings will help in developing disease resistance breeding technologies in *B. rapa* and molecular cloning of the genes involved. However, F_2 lines having both *Crr1* and *Crr2* did not exhibit resistance at strictly the same level as that of the resistant parent, G004 (Table 2), even though they showed clear resistance against isolate 'Wakayama-01'. In F_3 progenies, the segregation of resistance/susceptible individuals in each genotype group did not agree with the qualitative segregation manner of two genes (Fig. 4). Therefore, we assume at least a third locus is involved in the expression of clubroot resistance. The existence of other loci for specific races of *P. brassicae* is possible as well. High-resolution molecular mapping and genome-wide investi-

gation with precise QTL analysis using various races of the pathogen would provide us with further detailed information on the genetics and molecular basis of clubroot resistance in *B. rapa*.

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