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A novel locus for clubroot resistance in *Brassica rapa* and its linkage markers

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Abstract An inbred turnip (*Brassica rapa* syn. *campestris*) line, N-WMR-3, which carries the trait of clubroot resistance (CR) from a European turnip, Milan White, was crossed with a clubroot-susceptible doubled haploid line, A9709. A segregating F_3 population was obtained by single-seed descent of F_2 plants and used for a genetic analysis. Segregation of CR in the F_3 population suggested that CR is controlled by a major gene. Two RAPD markers, OPC11-1 and OPC11-2, were obtained as candidates of linkage markers by bulked segregant analysis. These were converted to sequence-tagged site markers, by cloning and sequencing of the polymorphic bands, and named OPC11-1S and OPC11-2S, respectively. The specific primer pairs for OPC11-1S amplified a clear dominant band, while the primer pairs for OPC11-2S resulted in co-dominant bands. Frequency distributions and statistical analyses indicate the presence of a major dominant CR gene linked to these two markers. The present marker for CR was independent of the previously found CR loci, *Crr1* and*Crr2*. Genotypic distribution and statistical analyses did not show any evidence of CR alleles on *Crr1* and*Crr2* loci in N-WMR-3. The present study clearly demonstrates that *B. rapa* has at least three CR loci. Therefore, the new CR locus was named *Crr3*. The present locus may be useful in breeding CR Chinese cabbage cultivars to overcome the decay of present CR cultivars.

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Introduction

Clubroot disease is caused by an obligate parasite, *Plasmodiophora brassicae*, and is one of the most serious diseases of *Brassica* crops worldwide. It was known as early as the 15th century in Spain and subsequently recognized in the mid-18th century in England and mid-19th century in Scotland (Karling 1968). The disease was first recorded in Japan in the 1890s (Ikegami et al. 1981) and is now one of the major problems in cabbage and Chinese cabbage production in Japan and Korea. The infection is thought to be a two-phase process. Following the cultivation of *Brassica* species, the resting spores in the soil germinate, and the resultant zoospores attack root hairs where they grow into multi-nucleate plasmodia. The plasmodia cleave and then form secondary zoospores that migrate to root cortical tissue where they induce abnormal growth that ultimately takes the form of a distorted massive gall, called a club (Ikegami et al. 1981). This growth prevents the roots from taking up water and nutrition and results in slow growth of the host plant and a severe reduction of crop production. Upon the decay of the clubs, numerous resting spores are released into the soil, where they can survive for many years.

Some European turnips have been reported to be resistant to the pathogen (Buczacki et al. 1975; Wit 1964). Yoshikawa (1993) reported that the resistance in most European fodder turnips is controlled by a single dominant gene with some minor genes. Based on his monogenic assumption, a number of Chinese cabbage cultivars showing clubroot resistance (CR) were bred in Japan using European fodder turnips as sources of resistance genes. However, these CR cultivars have recently become susceptible to clubroot disease in some of the production areas. Evolution of the pathogen population is thought to be a cause of the decay of resistance shown by these CR cultivars. European CR

turnips are thought to have more than two resistant loci, since the CR turnips are still resistant to pathogens collected from the field where CR Chinese cabbage cultivars are severely infected. The loss of resistance genes during breeding is an expected phenomenon (Kuginuki et al. 1999).

Kuginuki et al. (1997) found linkage markers for a CR locus in *Brassica rapa*. Recently two CR loci, *Crr1* and *Crr2*, were identified in a doubled-haploid (DH) line, G004 (Suwabe et al. 2003). The former locus corresponds to Kuginuki's CR locus because the linkage marker RA12-75 is tightly linked to BRMS-088, a marker for *Crr1*. These two studies were done on CR traits derived from a European fodder turnip, Siloga. The results suggest a complex genetic nature of CR in *B. rapa*. For breeding of more resistant Chinese cabbage cultivars, more information about the CR loci of turnips is needed. Of the CR turnips studied by Yoshikawa (1993), one, Milan White, was found to have polygenic resistance. To elucidate the genetics of CR of this turnip, in the investigation reported here we analyzed an inbred line having the resistant trait from Milan White.

Materials and methods

Plant materials

The CR turnip (*Brassica rapa* syn. *campestris*) inbred line N-WMR-3 is a parental line of the F_1 hybrid CR turnip cultivar, Shinano. The line has a CR trait derived from Milan White, a European fodder turnip (Otani et al 1982). A Chinese cabbage DH line, A9709, is susceptible to the clubroot pathogen. A population of 67 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 analysis. The F_4 plants obtained by the selfing of F_3 plants were used for scoring CR. The rate of the infected plants was used as the phenotype of each F_3 line.

Pathogen and infection

The Ano-01 isolate of the clubroot pathogen (*Plasmodiophora brassicae*), which was maintained by infection of a Chinese cabbage cultivar, Muso (Takii Seeds, Kyoto) 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).

Resting spores were isolated from clubs and suspended in a 65% (w/v) sucrose solution. The suspension was centrifuged at 440 *g* for 10 min. The supernatant was diluted four times by distilled water and then centrifuged at 1,000 *g* for 5 min. The sedimented resting spores were used as inoculum. The soil used was a mixture of peat moss (Theriault Hachey Peat Moss, N.B., Canada), Japanese acid clay (Wako Pure Chemical Industries, Osaka), Pearlite (Nenisanso No. 2; Mitsui Mining and Smelting, Tokyo) and $CaCO_3$ in a ratio of 5:5:15:1 (dry weight). The pH of the mixture was 6.3. The infected soil was made by mixing 10^9 resting spores, 20 ml of 1:2,000-diluted Hyponex (Hyponex Japan, Osaka) and 200 g of the autoclaved soil. An 8-cm diameter Jiffy pot (Nippon Jiffy Pot Products, Yokohama) was initially filled with non-infected autoclaved soil to a depth of approximately 5 cm; this was then topped with an approximately 2-cm-thick layer of the infected soil. Pots were sown with eight to ten seeds each, placed in a refrigerator for 1–2 days and then transferred to a greenhouse maintained at 30°C under natural day length. After 6 weeks, the disease incidence (DI)—the rate at which the plants were diseased—was determined. The means of duplicated tests were used for a subsequent genetic analysis. Disease index was also scored (Suwabe et al. 2003).

DNA polymorphism

A total of 81 individuals of the F_3 population were used for detecting DNA polymorphism. Genomic DNA was isolated from freeze-dried leaves of each F_3 plant as described (Murray and Thompson 1980). For the detection of random amplified polymorphic DNA (RAPD), 10 ng genomic DNA, 1× buffer (supplied by Takara Bio, Otsu), 0.5 U *Taq* DNA polymerase (Takara Bio), 2 nmol of each dNTP and 5 pmol of arbitrary primer (Operon Technologies, Alameda, Calif.), were used in a total volume of 10 μl. The reaction was performed using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, Calif.) with the following parameters: 1 cycle of 94°C for 30 s; 45 cycles of 94°C for 30 s, 40°C for 1 min and 72°C for 2 min; an extra extension at 72°C for 7 min. Microsatellite polymorphism was scored with primer pairs as described by Suwabe et al. (2002).

For a bulked segregant analysis (Michelmore et al. 1991), DNA samples of seven F_3 lines showing high resistance (DI=0) and seven F_3 lines showing no resistance (DI=1.0) were mixed respectively and used for the survey of RAPDs.

Cloning of the amplified bands

The amplified bands were cut from the gel, extracted using a QIAquick gel extraction kit (QIAGEN, Valencia, Calif.), and ligated to a pCR-XL-TOPO vector (Invitrogen, Carlsbad, Calif.). The resultant plasmids were sequenced by an ABI 310 Genetic analyzer (Applied Biosystems).

Results

The duplicated clubroot tests showed similar scores and segregation patterns in the F_3 families. The resistant parent, N-WMR-3, showed nearly no symptoms (DI=0.04) and had a disease index of 0.04, while the susceptible parent, A9709, showed severe symptoms (DI=1.00) and had a disease index of 3.00. The F_1 showed no symptoms (DI=0.00) (disease index=0.00). The majority of the F_3 lines were either highly resistant or highly susceptible (Fig. 1). These results suggest that a small number of major genes are involved in the resistance.

Since preliminary surveys using microsatellite markers developed by Suwabe et al. (2002) and RAPD did not show any association with the CR traits, a bulked segregant analysis (Michelmore et al. 1991) was conducted. As a result, two markers, OPC11-1 (1.4 kb) and OPC11-2 (1.0 kb), amplified with an Operon primer, OPC11, were obtained as linkage marker candidates. These were then converted to sequence-tagged sites (STS) by cloning and sequencing and named OPC11-1S and OPC11-2S, respectively. Specific primer pairs were synthesized on the basis of the sequence data (Table 1). The PCR analysis using the primer pair for OPC11-1S resulted in one clear dominant band, while two clear co-dominant bands—a 1.0-kb band for the susceptibility allele and a 1.3-kb band for the resistance allele—were amplified with the primer pair for OPC11-2S (Fig. 2). These two marker loci were linked to each other at a

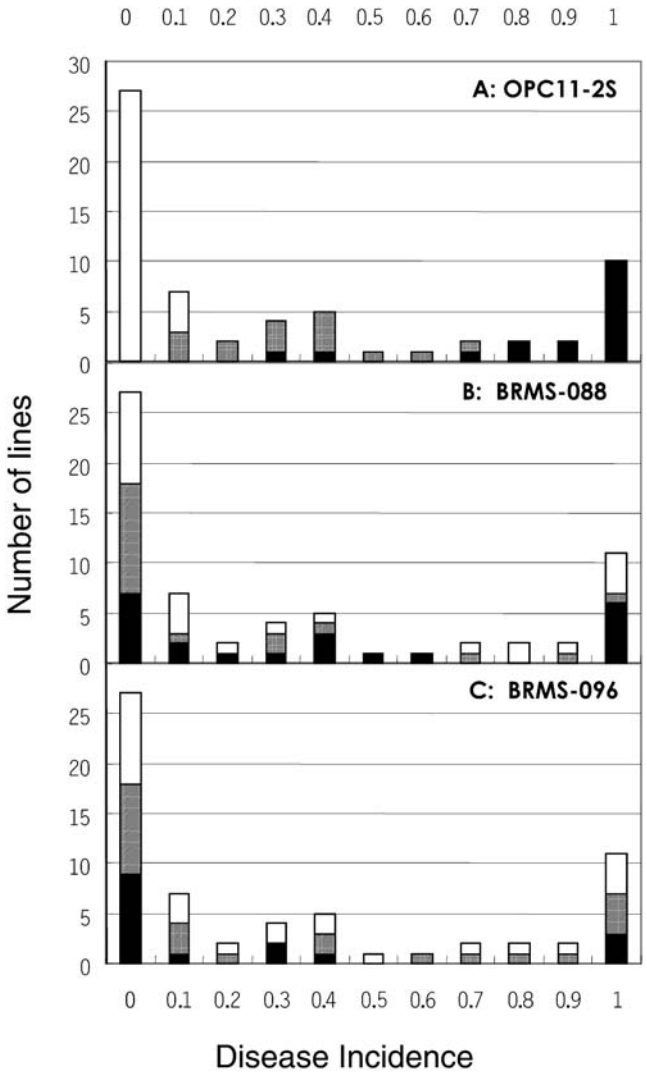

Fig. 1A–C Frequency distribution of disease incidence in the F_3 population. **A** OPC11-2S locus, **B** BRMS-088 locus, **C** BRMS-096 locus. *Black box* Homozygotes of A9709 allele, *open box* homozygotes of N-WMR-3 allele, *striped box* heterozygotes. *Ordinate* Number of lines, *abscissa* disease incidence

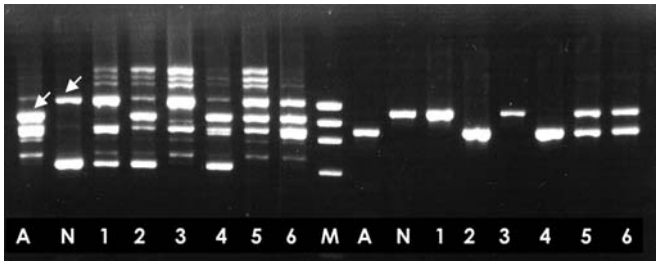

Fig. 2 DNA amplification using arbitrary primer OPC11 (*left*) or a specific primer pair, OPC11-2F and OPC11-2R (*right*). *Lanes*: *A* A9709, *N* N-WMR-3, *1–6* segregating F_3 plants, *M* molecular-weight standard φx174 DNA digested with *Hae*III. *Arrows* indicate cloned bands (1.4 kb, OPC11-1; 1.0 kb, OPC11-2)

distance of approximately 10 cM. The frequency distribution of DI among OPC11-2S genotypes in the F_3 population clearly showed that a gene closely linked to OPC11-2S had a large effect on the CR of N-MWR-3 (Fig. 1A). A non-parametric test showed that the difference in CR among the three genotypes of OPC11-2S was significant at the 1% level. The difference in CR between OPC11-1S genotypes was also significant (Table 2). Chi-square tests revealed that these two markers were independent of previously reported linkage markers for CR loci, BRMS-088 for *Crr1* and BRMS-096 for *Crr2* (Suwabe et al. 2003) (results not shown). The present CR locus was, therefore, named *Crr3*. The resistance allele is most likely to be dominant because the F_1 plants were resistant to the pathogen. The effects of *Crr1* and *Crr2* on the CR trait of this population were examined using the above-mentioned linkage markers (Suwabe et al. 2003). Non-parametric Kruskal-Wallis tests did not show a significant difference in CR among the BRMS-088 genotypes or among the BRMS-096 genotypes, respectively (Table 2). In addition, frequency distributions did not show any effect of these two loci (Fig. 1B, C).

Table 1 Specific primers used in this study

Target band	Size[a] (kb)	Primer name	Sequence (5′–3′)
OPC11-1S	1.4	OPC11-1F	TTACAGCTGGACCAAGAACATAG
		OPC11-1R	ATCGATGTTTGTGAGTCTCTACT
OPC11-2S	1.0	OPC11-2F	GTAACTTGGTACAGAACAGCATAG
		OPC11-2R	ACTTGTCTAATGAATGATGATGG

[a] The size of the band was estimated by agarose gel electrophoresis

Table 2 Disease incidence in the F_3 population. Average disease incidences for each genotype are shown

Genotype[a]	OPC11-1S	OPC11-2S	BRMS-088	BRMS-096
A	0.74	0.84	0.24	0.38
H	-	0.29	0.33	0.18
B	-	0.01	0.33	0.33
H+B	0.13	-	-	-
H [b]	-	52.1**	0.96	3.69
z [c]	−5.40**	-	-	-

**Significant at 1% level
[a] A, A9709 genotype; B, N-WMR-3 genotype; H, heterozygous
[b] *H*, Values of Kruskal-Wallis test among the three genotypes
[c] *z*, Value of Mann-Whitney test

Discussion

Occurrence of three CR loci in *B. rapa*

Our results indicate that *B. rapa* has a novel CR locus, which we named *Crr3*. Although the precise map position of *Crr3* is unknown, the two markers described in this report can be used to detect this locus. The present locus has been shown to be independent of *Crr1* and *Crr2*, which have recently been identified (Suwabe et al. 2003). Matsumoto et al. (1998) reported a CR locus, *Cr-A,* using a fodder turnip, ECD 02 (Buczacki et al. 1975), as a source of CR. It is unknown whether *Cr-A* corresponds to one of the above three loci or is another independent CR locus. The precise relationship of the CR loci should be studied using common linkage markers, such as microsatellite markers (Suwabe et al 2002).

The occurrence of three CR loci in *B. rapa* is not so surprising because earlier studies of CR in turnips have suggested the occurrence of three independent genes in *B. rapa* (Buczacki et al. 1975; Crute et al. 1980; Toxopeus and Janssen 1975; Williams 1966; Wit 1964). At least two CR loci have also been reported using DNA markers in another diploid species, *B. oleracea* (Figdore et al. 1993; Grandclément and Thomas 1996; Landry et al. 1992; Voorrips et al. 1997), whose genome size and structure are similar to those of *B. rapa*. Genome sizes of diploid *Brassica* species are about three- to fourfold that of *Arabidopsis* (Arumuganathan and Earle 1991). In a study of comparative mapping of loci controlling the flowering time, Lagercrantz et al. (1996) reported three homologues of an *Arabidopsis* contig in *B. nigra* and emphasized the extensive triplicated nature of diploid *Brassica* genomes. Because one CR locus has been reported in *Arabidopsis thaliana* (Fuchs and Sacristán 1996), it is reasonable to assume that the CR loci in diploid *Brassica* species are derived by duplication. It remains to be determined whether the CR loci found in the crucifer genomes are homologous to each other. Several studies have examined the synteny of crucifer genomes (Axelsson et al. 2001; Li et al. 2003; Osborn et al. 1997; Truco et al. 1996). The present linkage markers, in addition to the previous microsatellite markers (Suwabe et al. 2003), will be useful tools for the comparative mapping of CR loci in crucifer genomes.

Complex nature of clubroot resistance loci in *B. rapa*

The CR inbred parental line N-WMR-3 used in this study was obtained by crossing the turnip Milan White with a clubroot-susceptible local turnip, Nozawana, and then the backcrossed generation (BC_2S_3) was selfed several times. CR tests were carried out in every generation. Despite the assumption that CR is controlled by polygenes (Yoshikawa 1993), Otani et al. (1982) reported the nearly monogenic nature of CR of Milan White. Since no molecular markers were used to support the selection for CR, it is reasonable to assume that only the most effective CR locus remains in N-WMR-3. Therefore, *Crr3* in N-WMR-3 is thought to be the most effective CR locus in Milan While. In a recent study (Suwabe et al 2003), a DH line, derived from the CR turnip Siloga (G004) was used as the resistant parent and was found to have two CR loci, *Crr1* and *Crr2*. The DH line had a resistance to clubroot that was similar to that of Siloga. Therefore, the *Crr3* locus in Siloga may have an allele having only a minor effect on the clubroot pathogen, or a susceptibility allele. A number of European turnips are reported to have CR. Several of these are now used as a genetic source of CR for the breeding of *B. rapa* crops in Japan and Korea. The present investigation and previous studies (Kikuchi et al. 1999; Kuginuki et al. 1997; Suwabe et al. 2003) have described the genetics of CR traits of Siloga and Milan White. Further information on the CR loci of other CR turnips and the effect of each allele is needed in order to breed more resistant Chinese cabbage cultivars.

CR genes in breeding of *B. rapa* crops

Pyramiding of disease-resistant genes using DNA markers is one of the most promising fields in marker-assisted breeding (Huang et al. 1997). The identification of a novel CR locus and its linkage markers found in this study will provide valuable tools for breeding CR cultivars in *B. rapa* crops, including Chinese cabbage. Earlier breeding efforts were based on an assumption that CR is controlled by a single dominant locus (Yoshikawa 1993). A simple backcross is an effective way to introduce one CR locus to Chinese cabbage. However, recent variation in the virulence of *Plasmodiophora brassicae* suggests that attempts to suppress more virulent pathogens with a single CR gene will be unsuccessful (Kuginuki et al. 1999). Marker-assisted pyramiding of CR loci from different turnip lines into a single cultivar may be one of the ideal strategies to overcome the decay of CR Chinese cabbage cultivars. The other ideal strategy may be the rotation of cultivars having different resistance genes. However, the breeding of a number of resistant Chinese cabbage cultivars having very similar phenotypes may require complex breeding techniques. We believe that the identification of three CR loci may increase the chance of pyramiding CR loci and the rotation of resistant cultivars and that the linkage markers found here will be useful for these strategies.

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