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ORIGINAL PAPER

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SCAR and CAPS mapping of *CRb*, a gene conferring resistance to *Plasmodiophora brassicae* in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*)

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Abstract Clubroot disease, caused by *Plasmodiophora brassicae* Wor., is highly damaging for Chinese cabbage. The CR (clubroot resistant) Shinki DH (doubled haploid) line of Chinese cabbage carries a single dominant gene, *CRb*, which confers resistance to the *P. brassicae* races 2, 4, and 8. An F_2 population derived from a cross between the CR Shinki DH line and a susceptible line, 94SK, was used to map the *CRb* gene. Inoculation of F_3 families with SSI (single-spore isolate) resulted in a 1:2:1 segregation ratio. Use of the AFLP technique combined with bulked segregant analysis allowed five co-dominant AFLP markers, and four and seven dominant AFLP markers linked in coupling and repulsion, respectively, to be identified. Six of the 16 AFLP markers showing low frequencies of recombination with the *CRb* locus among 138 F_2 lines were cloned. A reliable conversion procedure allowed five AFLP markers to be successfully converted into CAPS and SCAR markers. An F_2 population (143 plants) was analyzed with these markers and a previously identified SCAR marker, and a genetic map around *CRb* covering a total distance of 6.75 cM was constructed. One dominant marker, TCR09, was located 0.78 cM from *CRb*. The remaining markers (TCR05, TCR01, TCR10, TCR08, and TCR03) were located on the other side of *CRb*, and the nearest of these was TCR05, at a distance of 1.92 cM.

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Introduction

Clubroot disease, caused by a soil-borne, obligate plant pathogen (*Plasmodiophora brassicae* Wor.), afflicts Chinese cabbage and other *Brassica* crops. Infected root tissue undergoes undifferentiated proliferation to produce galls (clubs) that impair the efficiency of the root system, reducing crop quality and rendering the product unmarketable. The disease is difficult to control, because chemical treatments are ineffective and/or expensive (Voorrips 1995), and the breeding of resistant varieties is an effective approach to eliminate the use of fungicides and to minimize crop loss.

Resistant sources have been identified in *B. rapa, B. oleracea*, and *B. napus* (Crute et al. 1983; Crisp et al. 1989). However, the breeding of clubroot resistance in Chinese cabbage has been impeded, as most cultivars appear to be highly susceptible to the disease. To develop resistant cultivars, clubroot resistance genes from CR European fodder turnip (*B. rapa* ssp. *rapifera*) have been introduced into Chinese cabbage (Yoshikawa 1981) and a number of resistant F_1 hybrid varieties subsequently released. Yoshikawa (1981) reported that there is a major resistance locus in European fodder turnips with additional minor genes. Among turnip genotypes, clubroot resistance is under the control of three independent dominant genes, each conferring resistance to a different *P. brassicae* pathotype (Wit 1964; Tjallingii 1965; Toxopeus and Janssen 1975; James et al. 1978; Crute et al. 1980). Chiang et al. (1977) suggested that the clubroot resistance genes in *B. napus* were located in the A genome derived from *B. rapa.* Genetic analysis of clubroot resistance has also been carried out for *B. oleracea* and *B. napus* (Chiang and Crête 1970, 1976; Crute et al. 1980, 1983; Gustafsson and Fält 1986). Although different hosts were used in these studies, the same conclusion that a dominant gene controls clubroot resistance was obtained.

DNA markers linked to clubroot resistance loci in *B. oleracea* have been developed by several research groups (Landry et al. 1992; Figdore et al. 1993; Grandclément

and Thomas 1996; Voorrips et al. 1997). A number of random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers linked to the major resistance gene of *B. rapa* have been identified (Kuginuki et al. 1997; Matsumoto et al. 1998). Kikuchi et al. (1999) converted RAPD markers into sequence-tagged site (STS) markers. Recently, Manzanares-Dauleux et al. (2000) identified RAPD markers linked to a major gene and to quantitative trait loci (QTLs) involved in *B. napus* clubroot resistance. Piao et al. (2002) developed a sequence characterized amplified region (SCAR) marker from an amplified fragment length polymorphism (AFLP) marker.

DNA markers linked to a trait of interest in plants can be rapidly identified by bulked segregant analysis (BSA) (Michelmore et al. 1991). Furthermore, the AFLP technique (Vos et al. 1995), which allows a large number of locus-specific markers to be studied simultaneously, has been widely used to target specific plant loci. However, due to the complexity and high cost of the technique, AFLP markers are not well suited for high-throughput selection, either for marker-assisted selection (MAS) or map-based cloning, compared to simple, PCR-based markers such as SCARs. The conversion of AFLP markers into SCAR markers would greatly improve their usefulness. Although such conversion has been successfully attempted (Bradeen and Simon 1998; Xu et al. 2001; Piao et al. 2002), difficulty has also been reported in some cases (Shan et al. 1999; Negi et al. 2000; Miftahudin et al. 2002).

In the present study, we focused on (1) the use of SSI of *P. brassicae* in a genetic analysis of clubroot resistance, (2) the identification of AFLP markers that map near the resistance gene *CRb*, and (3) the development of a reliable method for converting AFLP markers into SCAR markers. The application of SCAR markers to MAS is also discussed.

Materials and methods

Materials

One hundred and forty three F_2 plants derived from a cross between the 94SK and CR Shinki DH lines were used as a mapping population. 94SK is an inbred line highly susceptible to clubroot disease. The CR Shinki DH line, derived by anther culture of the Chinese cabbage cultivar CR Shinki, is resistant to races 2, 4, and 8 (Piao et al. 2002). Each F_2 plant was selfed to obtain F_3 seeds. For construction of two bulks, 40 F_2 plants obtained by selfing another F_1 plant were used. Of these 40 plants, only those showing resistance to clubroot were selfed to obtain F_3 seeds.

Clubroot disease resistance tests

Single-spore isolate detected to be race 4 of clubroot on the basis of Williams' classification (Williams 1966) was used to evaluate the responses of F_3 families. The galls from Chinese cabbage for maintaining SSI were collected and stored at −20°C until use. Inoculum was prepared according to the method of Williams (1966). The number of spores was counted and adjusted to 1×10^6 spores/ml. To determine the genotype of each F_2 individual, 16 seeds from the corresponding F_3 family were planted in 16-well multipots and maintained in the greenhouse at 20–25°C and under a 16-h photoperiod. Two parental lines were used as resistant and susceptible controls. Also, 40 F_2 plants used for construction of two bulks were inoculated. The soil was kept moist for 4 weeks. Three-day-old seedlings were inoculated by applying 10 ml of spore suspension to each well. Symptoms of disease were scored on the previously described 0–3 scale (Buczacki et al. 1975), with an additional category (2^+): 0 = no visible root swelling; 1 = very slight swelling usually confined to lateral roots; 2 = moderate swelling of lateral roots; 2^+ = severe clubs on all roots; and 3 = absence of roots, presence of a large gall. Plants in classes 0 and 1 were considered resistant and those in classes 2 and 3 as susceptible.

DNA isolation and BSA

Total DNA was isolated from young leaves of parents and F_2 individuals according to the method described by Pierre and Maréchal-Drouard (1992), with minor modifications. First, 40 F_2 plants (whose genotypes had been determined not only for F_2 generation but also for F_3 families of resistant F_2 plants) were surveyed with a previously described co-dominant marker, TCR01 (Piao et al. 2002). DNA from ten resistant plants and six susceptible plants for which the TCR01 marker showed a homozygous banding pattern were pooled to construct resistant and susceptible bulks, respectively. The parental DNA and the two bulks were used for BSA (Michelmore et al. 1991) combined with the AFLP technique.

AFLP analysis

The AFLP technique was performed using the method described by Vos et al. (1995), with minor modifications. Genomic DNA (500 ng) was digested with *Pst*I and *Tru9*I in a total volume of 25 μl at 37°C overnight. After inactivation of restriction enzymes at 75°C, adapters were ligated in a total volume of 50 μl at 37°C for 3 h. A 5 μl aliquot of a tenfold dilution of the restriction/ligation mixture was used as template DNA in a preamplification reaction containing 2.5 mM dNTP, 1× PCR buffer (15 mM $MgCl_2$), 27 ng each of *Pst*I+G/*Mse*I+C, and 1 U *Taq* DNA polymerase. The amplicon was diluted 50-fold and used as a template DNA in selective PCR. Two hundred fifty-six primer combinations derived from 16 *Pst*I+GNN and 16 *Mse*I+CNN primers were employed to identify markers. Selective PCR was performed with 1× PCR buffer (15 mM $MgCl_2$), 2.5 mM dNTP, 30 ng *Mse*I+CNN, 15 ng *Pst*I+GNN, and 0.4 U *Taq* DNA polymerase. All amplifications were performed in a 9600 Perkin-Elmer thermocycler with the PCR conditions described by Vos et al. (1995). PCR products were mixed with an equal volume of loading dye (98% formamide, 10 mM EDTA, 0.001% each of xylene cyanol and bromphenol blue). Samples were denatured at 94°C for 5 min and separated on a Long Ranger gel (FMC) at 85 W. After electrophoresis, the gel was developed using a silver staining kit (Bioneer).

Cloning of AFLP markers closely linked to clubroot resistance gene

Of 16 markers identified in this study, six AFLP markers (P2, P3, P5, P8, P9, and P10) were selected for conversion. The gel pieces containing these markers were sliced and boiled 5 min in 100 μl sterile water. After centrifugation, a 5-μl aliquot of the supernatant was amplified with the corresponding selective primer combination and the same PCR conditions. The amplicons were separated on a 1.0% agarose gel, followed by elution using a gel extraction kit (Qiagen). The eluted AFLP fragments were cloned into the pGEM-T Easy Vector system I (Promega). Two steps were employed to confirm clones containing the correct AFLP markers. First, the size selection was performed as follows: Plasmid DNA isolated from more than ten clones was amplified with the corresponding selective primer combination, followed by electropho-

resis on a Long Ranger gel. Clones with inserts of the same size as that of the AFLP marker were selected. Second, Southern analysis was performed to verify the selected colonies with the ECL Direct Nucleic Acid Labelling and Detection System (Amersham). The selective PCR products amplified from the two parents, and the two bulks were electrophoresed on a 1.5% agarose gel, and probes were prepared from the selected clones. Clones, for which a hybridization signal was detected only for the resistant parent and bulk but not from the susceptible parent and bulk, were chosen for sequencing.

Conversion of AFLP markers to SCAR or CAPS markers

Selected clones were sequenced from both ends using an ABI PRISM 377 automated sequencer. Primer pairs for developing SCAR markers were designed based on the two terminal sequences using the Primer3 program (http://www.broad.mit.edu/genome_software/other/primer3.html). PCR conditions were optimized with pre-amplification products of the two parents and the two bulks as templates. Subsequently, these conditions were used to amplify genomic DNA from the 16 F_2 plants that constituted the two bulks. PCR products were separated on a 2% agarose gel for dominant markers. PCR products of TCR02 were digested with restriction endonucleases (*Hae*III, *Hpa*II, *Taq*I, and *Xho*I) and separated on a 2% agarose gel. Polyacrylamide gel electrophoresis was utilized to separate other co-dominant markers. If the fragment amplified with the primer pairs and under the optimized condition showed polymorphism between resistant and susceptible plants as AFLP marker analysis, SCAR markers were considered to be successfully developed. The primer sequences and PCR conditions are listed in Table 2.

Linkage analysis

The developed CAPS and SCAR markers, including the previously identified marker TCR01, were used to survey 143 F_2 plants. Linkage analysis for the molecular markers and the clubroot resistance gene was performed with JoinMap 3.0 (van Ooijen and Voorrips 2001). A genetic map was constructed based on a minimum LOD score of 3 and a maximum recombination fraction of 0.4. Map distances were calculated using the Kosambi function (Kosambi 1944).

Results

Segregation of the clubroot resistance gene

A clear inoculation result could be obtained by evaluating the mapping population with SSI. Among the 143 F_2 plants, 32 and 36 gave rise to only resistant or susceptible plants in the next generation (F_3), respectively, and were therefore scored as homozygous for resistance (RR) or susceptibility (rr). The remaining F_2 plants, which gave rise to both resistant and susceptible progeny, were designated as heterozygotes (Rr). The RR:Rr:rr segregation ratio was not significantly different from the 1:2:1 ratio expected for the segregation of a single gene (χ^2=0.57, P>0.75), indicating that a single dominant locus specifies clubroot resistance in the CR Shinki DH line, further confirming the results reported by Piao et al. (2002). The resistance locus was designated *CRb* following the nomenclature established by Matsumoto et al. (1998) with the *CRa* locus.

Identification of AFLP markers linked to the *CRb* gene

The use of the previously developed co-dominant marker TCR01 allowed us to efficiently construct two bulks from homozygous resistant and susceptible plants. From 40 F_2 plants, ten resistant and six susceptible plants were selected for the preparation of DNA pools designated as resistant (Br) and susceptible (Bs) bulks, respectively. The genotypes of resistant plants determined by TCR01 genotyping were confirmed to be identical to the result of the clubroot tests in their F_3 families. BSA was carried out with 256 (16 *Pst*I+GNN × 16 *Mse*I+CNN) AFLP primer combinations. Three primer combinations that failed to amplify products from one or more templates were not used further. The *Pst*I+GTA primer in combination with any of the 16 *Mse*I+CNN primers also did not yield a product. Each of the remaining 237 primer combinations allowed for amplification of an average of 55 fragments. The frequency of polymorphism between the two parents was about 24%, with an overall range of 4–31%.

Of the 3,099 polymorphic loci detected by analysis of the two parents, 30 (defined by 24 primer combinations) displayed polymorphism with respect to two bulks. Among the 30 candidate markers, nine were amplified from the resistant parent and bulk, and nine were amplified from the susceptible parent and bulk. Six primer combinations (including one that identified a previously described marker, Piao et al. 2002) generated both resistant- and susceptible-specific fragments, which were scored as co-dominant markers. After verification of these candidate markers in the F_2 individuals derived from the two bulks, four linked in coupling, seven in repulsion, and five co-dominant markers were identified (Table 1). To reduce error in estimating genetic distance, markers linked in repulsion were not surveyed in the mapping population. Only the four markers linked in coupling, the five co-dominant markers, and TCR01 were finally used to evaluate 138 F_2 plants. Within the mapping population, all these markers were linked to *CRb*. However, several plants were found to be recombinants. The number of recombinants varied between 2 and 17. For example, there were two recombinants between P10 and *CRb* and 17 between P4 and *CRb*. We could localize these AFLP markers and TCR01 to an 8.14-cM interval by analysis of segregation data with JoinMap 3 (Fig. 2A). *CRb* was found to be flanked by the P9 and P5 markers at genetic distances of 1.24 cM and 1.34 cM, respectively.

Conversion of AFLP markers into CAPS and SCAR markers

Several features of the AFLP technique limit its application in large-scale screens for map-based cloning or MAS. To overcome this problem, AFLP markers closely linked to *CRb* (P2, P3, P5, P8, P9, and P10) were excised from polyacrylamide gels and cloned. However, there was a probability of cloning undesirable fragments due to the presence of more than one fragment in the same location

Table 1 AFLP primers for the identification of AFLP markers

Marker	AFLP primers	Marker type[a]	Marker	AFLP primers	Marker type[a]
P2	P+gag/M+cag	Co	P10	P+gga/M+ctc	Dr
P3	P+gtg/M+cta	Co	P11	P+gat/M+cat	Ds
P4	P+gct/M+cta	Co	P12	P+gat/M+cct	Ds
P5	P+gct/M+cca	Co	P13	P+gaa/M+cac	Ds
P6	P+gcg/M+caa	Co	P14	P+gaa/M+ctt	Ds
P7	P+ggt/M+ctc	Dr	P15	P+gag/M+cgc	Ds
P8	P+gaa/M+cga	Dr	P16	P+gct/M+cgg	Ds
P9	P+gca/M+cca	Dr	P17	P+gga/M+cat	Ds

[a] *Co* Co-dominant marker, *Dr* dominant marker linked in coupling, *Ds* dominant marker linked in repulsion

Table 2 Converted SCAR and CAPS markers linked to *CRb*

AFLP marker	SCAR and CAPS markers				
	Name	Primers (5′>3′)[b]	Size	PCR conditions[c]	
P2	TCR02[a]	GCTCCATTCAGTTACGGTGA and GCAGAGAATTTTGGAAGAGGA	447 bp	62°C, 60 s	72°C, 90 s
P5	TCR05	AGAATCATGACCGGGGAAAT and GCAGCTAAGTCATCGACCAA	279 bp	62°C, 60 s	72°C, 90 s
P8	TCR08	GCAGAATTATAACCTGAGCGTGT and ATTACCGGAGTATGCGATCC	131 bp	62°C, 60 s	72°C, 60 s
P9	TCR09	GCAGCAACCGATAATATAAGGA and AACCAGAAGAAGAAAAA-CAAAAA	101 bp	53°C, 60 s	72°C, 60 s
P10	TCR10	AACTCTTGAAGAAAGCAAAGAAGC and GCAGGAATAAGAAGGAA-CACCA	170 bp	58°C, 60 s	72°C, 60 s

[a] PCR products were digested with *Hpa*II
[b] Forward and reverse primers are listed in that order
[c] PCR conditions for all primers were 5 min at 94°C, 35 cycles of 30 s at 94°C

(Vos et al. 1995; Piao at al. 2002). During re-amplifying of the AFLP fragment, there was also the possibility of amplifying fragments of different sizes (data not shown). To verify the cloned fragments as AFLP markers, we performed two experimental steps as described in "Materials and methods." After electrophoresis on a polyacrylamide gel, we selected several candidate clones whose inserts exhibited the same mobility as that of the corresponding AFLP marker. Subsequently, Southern analysis allowed us to identify correct clones containing the desired P5, P8, P9, and P10 fragments on the basis of polymorphism between Br and Bs. Only one clone from each of markers was sequenced from both ends. For the co-dominant markers, however, two clones were selected and sequenced from both ends after the size selection. The comparison between two sequences confirmed that they were identical.

In all, six primer pairs were designed using Primer3 software (Table 2). To obtain the longest possible PCR product, the three selective base pairs and the *Pst*I and *Mse*I recognition sites were included in primers that were specific to the 20–24 base pairs at each end of the AFLP marker. For P3, however, we could not design an appropriate primer from one end due to high AT content. Such a primer has to be designed from the sequences having optimum GC content near an AT-rich end sequence. PCR conditions were optimized using pre-amplification products from each of the two parents and from the two bulks and further confirmed with genomic DNA prepared from F_2 individuals of the two bulks as templates. The results of this analysis indicated that all of these AFLP markers were successfully converted into

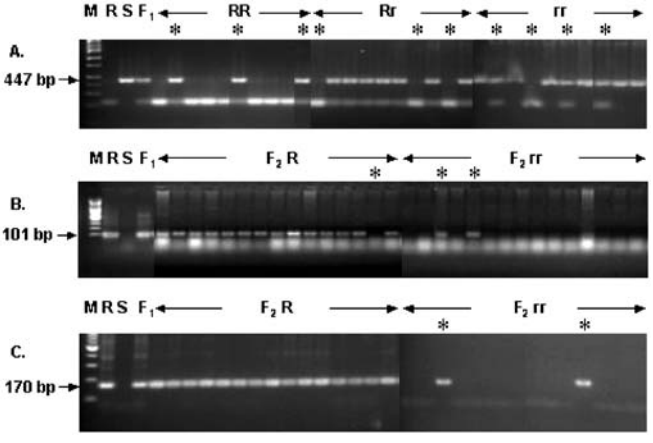

Fig. 1A–C Examples of SCAR markers screened in F_2 plants. **A** Agarose gel electrophoresis of PCR products digested with *Hpa*II revealing CAPS marker TCR02. *RR* Homozygous resistant, *Rr* heterozygous resistant, *rr* homozygous susceptible. **B** Agarose gel electrophoresis of PCR products revealing SCAR marker TCR09. *R* Resistant parent, *S* susceptible parent, F_2 *R* resistant, F_2 *rr* susceptible. **C** Agarose gel electrophoresis of PCR products revealing SCAR marker TCR10. *M* 100-bp ladder. SCAR and CAPS markers are shown by *arrows*. Recombinants are shown by *asterisks*

CAPS or SCAR markers except for P3. TCR08, TCR09, and TCR10 were dominant markers converted from P8, P9, and P10 (Fig. 1), respectively. TCR05, converted from P5, was a co-dominant marker as expected. Primers based on the P2 sequence amplified an identical band from both resistant and susceptible individuals. PCR

Table 3 Segregation of SCAR and CAPS markers in F_2 populations

Marker	Number of F_2 plants with the markers[a]			Expected ratio	χ^2	*P*-value
	RR	Rr	rr			
CRb	32	75	36	1:2:1	0.57	>0.750
TCR01	36	71	36	1:2:1	0.01	>0.995
TCR02	38	74	31	1:2:1	0.86	>0.500
TCR05	35	72	36	1:2:1	0.02	>0.990
TCR08	110	-	33	3:1	0.28	>0.500
TCR09	108	-	35	3:1	0.02	>0.750
TCR10	109	-	34	3:1	0.12	>0.500

[a] The number of F_2 plants showing homozygous and heterozygous band: *RR* resistant homozygous, *Rr* resistant heterozygous, *rr* susceptible homozygous. The numbers for dominant markers represent the total number of RR+Rr

products were, therefore, digested with several restriction endonucleases (see Materials and methods). Only *Hpa*II identified a polymorphism between resistant and susceptible plants. Products from resistant plants were digested into two fragments of the same mobility on agarose gels while those from susceptible plants were undigested (Fig. 1A). This CAPS marker was designated as TCR02.

Mapping of the *CRb* gene with CAPS and SCAR markers

A total of 143 F_2 plants were screened with converted CAPS and SCAR markers. Resistant-specific markers were found to be present in most resistant plants but absent from susceptible plants. In particular, we did not detect recombination between the two co-dominant markers TCR01 and TCR05 among any of the susceptible plants. Some examples showing segregation of SCAR and CAPS markers are illustrated in Fig. 1. Three plants exhibiting recombination between TCR09 and the *CRb* gene were identified (Fig. 1B). Recombination between TCR10 and the *CRb* gene was detected in only two plants (Fig. 1C). For the co-dominant marker TCR01, eight recombinants were detected. These markers were also found to segregate normally within the mapping population (Table 3). Linkage analysis of the segregation data for these markers using JoinMap 3 indicated that they all mapped to the same linkage group with a minimum LOD score of 3 (Fig. 2B). The different distance between two maps is due to the addition of five individuals in the map constructed with SCAR and CAPS markers and the number of markers used.

A dominant marker, TCR09, and a co-dominant marker, TCR05, were found to closely flank the *CRb* gene at distances of 0.74 cM and 1.97 cM, respectively. One recombination event was detected between TCR01 and TCR05, which were separated by 0.18 cM. TCR10, TCR08, and TCR02 were located on the same side of *CRb* as TCR05 and TCR01, at distances of 3.84, 4.67, and 7.8 cM from the gene, respectively.

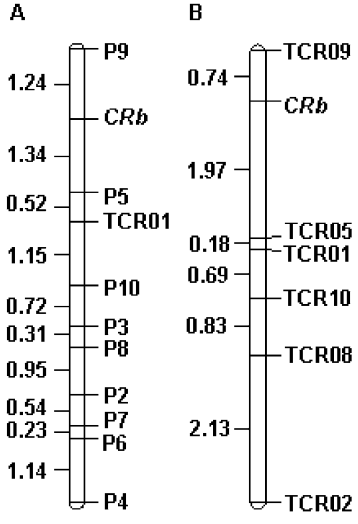

Fig. 2A, B Genetic mapping of the *CRb* gene. **A** Linkage map showing AFLP markers and the previously identified SCAR marker, TCR01, based on 138 F_2 plants. **B** A linkage map showing SCAR and CAPS markers, based on 143 F_2 plants. Genetic distances were calculated in centiMorgans with the JoinMap 3 program (van Ooijen and Voorrips 2001) using the Kosambi mapping function (Kosambi 1944)

Discussion

In this study, we used SSI to determine the genotypes of F_2 individuals in corresponding F_3 families. In the mapping analysis of a simply inherited gene, an exact determination of genotypes of the trait is generally needed. However, the evaluation of resistance to fungus is generally affected by environmental factors. In the clubroot test, both the humidity level in the soil and the temperature can influence the outcome of inoculation (Hamilton and Crete 1978). We observed that 1 out of 40 F_2 plants that were only used to construct the two bulks showed resistance when inoculation was performed on F_2 progeny. However, all progeny derived from this plant showed a susceptible phenotype, indicating that the genotype of an individual F_2 plant cannot be precisely scored. In general, this problem could be resolved by the use of F_3 families.

Different results were observed when SSI and contaminated isolate were used. For example, plants of the CR Shinki DH line inoculated with contaminated field isolate showed either high or intermediate resistance (data not shown). Instead, all of them showed high resistance when SSI was used, indicating that contaminated field isolate could not reliably be used to evaluate resistance in the segregating population. Manzanares-Dauleux et al. (2000) have also demonstrated that clubroot resistance is expressed at high and intermediate levels, and that it is inherited as a qualitative or quantitative trait in *B. napus*, based on the results of inoculating with SSI from different clubroot races. Also, interactions between different clubroot isolates can obscure virulence factors (Jones and Ingram 1982). Hence, the use of SSI is a pre-requisite for a better understanding of host-pathogen interactions and for the exact scoring of clubroot resistance. To our knowledge, SSI was first adopted in constructing a local map of the region around the clubroot resistance gene in Chinese cabbage.

In this study, the AFLP technique was combined with BSA to identify DNA markers linked to the *CRb* gene by using the frequently cutting enzyme *Mse*I and the rarely cutting enzyme *Pst*I (which has a GC-rich recognition sequence). BSA is a powerful technique for detecting markers linked to any specific locus or genomic region (Michelmore et al. 1991; Meksem et al. 1995; Li et al. 1998). However, the relative frequency of homozygous and heterozygous individuals in a bulk can diminish the probability of detection of a co-dominant marker or a dominant marker linked in repulsion. Prior to determining the exact genotype of F_2 plants, a survey of these plants with the co-dominant marker TCR01 allowed us to assemble DNA pools for each of the two homozygous bulks and to identify both co-dominant markers and dominant markers linked in repulsion. An F_2 segregating population is widely used to target a specific locus because it is easily established. To make DNA pools from homozygous plants, the precise genotypes of individual plants must be determined by analysis of F_3 progeny. However, this approach is time-consuming. The successful use of a co-dominant marker in constructing homozygous bulks suggests that the identification of new markers can be simultaneously done with the determination of genotypes. With regard to the construction of homozygous bulks, this method can shorten experimental time by at least half a year, as in the case of Chinese cabbage.

Due to limitations of the AFLP technique, AFLP markers cannot be conveniently used for large-scale selection. In contrast, the conversion of these markers into simple, PCR-based markers will expand the usefulness of the technique. So far, several conversion procedures have been presented by others (Shan et al. 1999; Negi et al. 2000; Miftahudin et al. 2002), but not all AFLP markers were successfully converted. The improved conversion procedure presented here was confirmed to be reliable. In comparison with other conversion procedures, our method combines size selection, Southern analysis, and sequence comparison (Pierre et al. 2000; Xu et al. 2001). Size selection was performed both before and after cloning of the AFLP markers. Prior to the cloning of AFLP markers, however, fragments larger or smaller than AFLP markers appeared during re-amplification. A comparison of the sizes of AFLP markers and cloned inserts allowed us to easily select candidate clones. Southern analysis of the candidate clones also allowed us to select the cloned dominant AFLP markers P8, P9, and P10, and the co-dominant marker P5. These markers were successfully converted into SCAR markers, although some of them ranged in size from 101 bp to 170 bp. In the case of P10, we observed that the probe prepared from the candidate clone could not detect polymorphism between the two bulks (data not shown). This result is in agreement with what was reported by Piao et al. (2002) and strongly suggests that an accurate clone cannot be selected only on the basis of size. For co-dominant markers P2 and P3, the probe did not detect any polymorphism between two bulks, since two bands displaying polymorphism could not be clearly separated on an agarose gel. Therefore, sequence comparison between two selected clones was performed to verify whether they were identical. The identical sequence was used to design a primer pair. The marker P2 was converted into a CAPS marker. However, P3 could not be converted. This might suggest that an insertion/deletion had occurred in the AT rich region at the end of the P3 between resistant and susceptible plants. Also, a primer could not be designed on this region.

The difficulties encountered in the conversion of AFLP markers were most likely due to contamination by undesirable AFLP fragments during their isolation from gels. As demonstrated by Xu et al. (2001), the following factors probably caused this contamination. First, the different mobility between a visible, single ^{33}P-labelled-DNA strand and its invisible, complimentary DNA strand (Vos et al. 1995) results in a possibility of contamination of other invisible AFLP bands when isolating a desired AFLP marker. The second is the presence of neighboring AFLP fragments. Instead of a ^{33}P-labelled-primer, we used the silver staining method to visualize AFLP fragments. This allowed both DNA strands to be observed and avoided the isolation of undesirable fragments. These results indicate that the most likely source of contamination is neighboring AFLP fragments. However, we have also observed that larger fragments far away from the AFLP marker in Long Ranger gel and the AFLP marker were simultaneously amplified during re-amplification of AFLP markers. The reason for this is unclear, but it can affect the isolation of a desired AFLP marker.

The identification of co-dominant markers allowed us to accurately construct a local map around the *CRb* gene and to estimate genetic distances. Linkage analysis of the developed SCAR and CAPS markers revealed that *CRb* is located in an approximately 2.7-cM interval. The two flanking markers TCR09 and TCR05 are closely linked to the resistance locus at distances of 0.74 cM and 1.97 cM,

respectively. Although only two susceptible plants (nos. 15 and 66) exhibited recombination between TCR10 and *CRb*, this marker was mapped further away from the gene than was TCR05. This could be explained by the following. First, both TCR05 and TCR01 are co-dominant markers. Also, no susceptible plants were found to be recombinant. Furthermore, in addition to eight recombination events detected between TCR01 and the gene, three susceptible plants (nos. 15, 18, and 66) were among additional plants that exhibited recombination between TCR02 and the gene. Second, the dominance of TCR10 prevents it from distinguishing heterozygotes and homozygotes. We also identified an additional marker located on the other side of the gene. Two susceptible plants (nos. 58 and 75) and one resistant plant (no. 126) were TCR09-*CRb* recombinants, suggesting that TCR09 is on the other side of *CRb*.

So far, RAPD and RFLP markers linked to the *B. rapa* resistance locus have been identified and mapped (Kuginuki et al. 1997; Matsumoto et al. 1998). Kuginuki et al. (1997) identified three RAPD markers using 36 DH lines derived from five F_1 plants. STS markers converted from these markers (Kikuchi et al. 1999) were tested for mapping in our segregating population. However, the results of this attempt indicated that they are monomorphic. Matsumoto et al. (1998) also mapped the clubroot resistance gene *CRa* to an interval of about 34 cM between two RFLP markers on linkage group 3 using ECD02 (European Clubroot Differential) as a resistant source. As demonstrated by Piao et al. (2002), *CRb* is most likely derived from ECD01 or from the same source based on a survey of ECD01 to ECD05 with TCR01. A survey of these accessions with TCR09 and TCR05 also confirmed this result (data not shown). It is also worthwhile to determine by further analysis whether the gene mapped here is identical to any of the clubroot resistance genes previously reported for *B. rapa*.

The SCAR markers developed in this study should be suitable for use in MAS programs to introduce resistance to clubroot disease into Chinese cabbage without the need for inoculation and detection of symptoms, thereby avoiding genetic variation among races of *P. brassicae*. TCR09 and TCR10, which flank the *CRb* gene, detected only three and two recombinants among 143 F_2 plants, respectively, suggesting that they could correctly predict the genotype in all but 2.1% and 1.4% of analyses when they are separately used. However, the error of predicting the selection of resistant plants is decreased to 0.0029% if they are used in combination. Further, they can be easily detected on agarose gels. Therefore, these two dominant markers can be successfully used in backcrosses to breed resistant lines carrying a resistance gene. Also, the co-dominant markers TCR01 and TCR05 can distinguish homozygous and heterozygous plants in the last selfing generation. Due to the genetic variation of *P. brassicae* and to the presence of a mixture of several races in a single field, the current clubroot resistant cultivars are confronted with breakdown (Kikuchi et al. 1999; Piao et al. 2002). However, CR European fodder turnips including ECD01 to ECD04, which have been used as a resistant source in breeding Chinese cabbage resistant lines (Yoshikawa 1981; Kuginuki et al. 1994), are still resistant to field isolates (Kuginuki et al. 1997; Kikuchi et al. 1999; Piao et al. 2002), suggesting that there are genes for resistance to other races. The combination of these genes in a single line can improve resistance to a broad spectrum of races (Huang et al. 1997). The markers developed here, combined with markers linked to other resistance genes (Matsumoto et al. 1998), would enable these genes to be channeled into a single Chinese cabbage line.

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