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ORIGINAL PAPER

Identification of AFLP markers linked to the male fertility restorer gene of CMS (*Moricandia arvensis*) *Brassica juncea* and conversion to SCAR marker

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Abstract We have developed a cytoplasmic male sterile (CMS) line of *Brassica juncea* through somatic hybridization with *Moricandia arvensis* and introgressed the fertility restorer gene into *B. juncea*. This fertility restorer locus is unique in that it is capable of restoring male fertility to two other alloplasmic CMS systems of *B. juncea*. As a first step toward cloning of this restorer gene we attempted molecular tagging of the *Rf* locus using the amplified fragment length polymorphism (AFLP) technique. A BC_1F_1 population segregating for male sterility/fertility was used for tagging using the bulk segregant analysis method. Out of 64 primer combinations tested in the bulks, 5 combinations gave polymorphic amplification patterns. Further testing of these primers in individual plants showed four amplicons associated with the male fertility trait. Polymorphic amplicons were cloned and used for designing SCAR primers. One of the SCAR primers generated amplicons mostly in the fertile plants. Linkage analysis using MAPMAKER showed two AFLP and one SCAR markers linked to the male fertility gene with a map distance ranging from 0.6 to 2.9 cM. All the markers are located on one side of the *Rf* locus.

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Introduction

Cytoplasmic male sterility (CMS) is one of the few traits of angiosperms that are governed by nuclear and mitochondrial gene interactions. CMS is often associated with the expression of novel, chimeric open reading frames (*orfs*) encoded by the mitochondrial genome. The chimeric *orfs* differ among CMS systems, but often carry recognizable segments derived from the coding or flanking sequences of essential mitochondrial gene(s). These *orfs* encode novel proteins, which are either cytotoxic or interfere with the expression of the normal mitochondrial gene (Schnable and Wise 1998). Nuclear genes can restore male sterility to the CMS plants, and are usually found in the cytoplasmic donor species. Thus CMS systems serve as excellent models to study nuclear and cytoplasmic interactions.

A number of alloplasmic male sterile lines of *Brassica juncea* have been developed following either sexual or somatic hybridizations with wild species (Prakash 2001). These CMS lines carry either unaltered or recombined mitochondrial genomes. A stable CMS line of *B. juncea* was derived from the somatic hybrid *Moricandia arvensis* + *B. juncea* following back crosses with *B. juncea* (Prakash et al. 1998). The fertility restorer gene for this CMS was introgressed into *B. juncea* from *M. arvensis*. Subsequently, it was found that this *Rf* locus is capable of restoring male fertility to two other CMS systems of *B. juncea* carrying *Diplotaxis catholica* or *Diplotaxis erucoides* cytoplasm (Bhat et al. 2005, 2006).

While considerable information is available about the mitochondrial genes responsible for male sterility, molecular details of male fertility restoration are limited. Isolation of fertility restorer genes remained

elusive for a long time and only in recent years successes were registered. Maize *Rf2* gene, the first fertility restorer gene to be cloned was found to encode for an aldehyde dehydrogenase (Cui et al. 1996). However, all restorer genes cloned subsequently have been found to belong to the pentatricopepetide repeat (PPR) family of proteins that are implicated in processing of organellar transcripts (Small and Peeters 2000). This is true of the restorer genes of CMS systems of petunia (Bentolila et al. 2002), radish (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003), rice (Kazama and Toriyama 2003) and sorghum (Klein et al. 2005). It is assumed that they suppress or compensate for mitochondrial dysfunction during male gamete development.

In almost all cases, isolation of *Rf* genes has been accomplished using linked molecular markers. For tagging of traits, molecular markers such as random amplified polymorphic DNA (RAPD, Williams et al. 1990) and amplified fragment length polymorphism (AFLP, Vos et al. 1995) have been effectively used in combination with near isogenic lines (NILs; Muehlbauer et al. 1988) or bulk segregant analysis (BSA; Michelmore et al. 1991). Delourme et al. (1994) employed BSA approach and identified four RAPD markers linked to the fertility restorer locus (*Rfo*) of (*ogu*) *Brassica napus*. These markers were cloned and sequenced to develop co-dominant SCAR markers. The region around the *Rfo* gene was further saturated (Delourme et al. 1998) and a marker RAPD-13, was identified that co-segregated with the *Rfo* locus. Similarly, Hanson et al. (1997) have also reported a RAPD marker tightly linked to the *Rfo* locus. Imai et al. (2003) identified four RAPD-STS and six AFLP-STS markers linked to the fertility restorer *Rfk1* of CMS Kosena radish. AFLP technology combined with BSA enabled identification and positional cloning of the *Rfk1* gene (Koizuka et al. 2003). Similarly, Jean et al. (1997) identified 11 DNA markers (10 RFLP and 1 RAPD marker) linked to the *Rfp1* locus of *pol* CMS *B. napus* by BSA. For (*tour*) *B. napus* restorer gene, Trendelkamp et al. (1999) identified 11 AFLP markers linked to the restorer gene through BSA method, whereas Janeja et al. (2003b) found 2 AFLP markers using NILs. Likewise, two RAPD markers flanking the *Rf1* locus of (*Enarthrocarpus lyratus*) *B. napus* were identified (Janeja et al. 2003a).

DNA-based molecular markers tightly linked to fertility restorer genes have also been identified in several other species such as rice (Akagi et al. 1996; Ichikawa et al. 1997; Zhang et al. 1997), petunia (Bentolila et al. 1998; Bentolila and Hanson 2001), sorghum (Klein et al. 2001; Wen et al. 2002), barley (Matsui et al. 2001), sunflower (Horn et al. 2003), cotton (*Gossypium hirsutum*; Liu et al. 2003), wheat (Zhou et al. 2005) and chili pepper (Kim et al. 2006).

The objective of the present study was to tag the fertility restorer gene of CMS (*M. arvensis*) *B. juncea* and identify closely linked markers for its positional cloning. We mapped this restorer locus using the AFLP technique in combination with BSA and one of the AFLP markers linked to the *Rf* locus has been converted to SCAR marker.

Materials and methods

Plant materials and development of mapping population

The CMS and fertility restorer lines used in this study have been described earlier (Prakash et al. 1998). A single F_1 plant derived from the cross between the CMS line and the fertility restorer was backcrossed with the maintainer euplasmic line *B. juncea* cv. Pusa Bold. The BC_1F_1 population segregating for male sterility and fertility was used for the present study.

Plants were examined at flowering for male sterility/fertility through visual examination and by staining the pollen with 2% acetocarmine. Male fertility was further confirmed based on the ability of plants to set seed on selfing.

DNA extraction and preparation of bulks

Genomic DNA was isolated from fresh leaves, using CTAB (cetyl trimethyl ammonium bromide) procedure as described earlier (Kirti et al. 1995). DNA quantification was done with spectrophotometer. Fertile and sterile bulks were prepared for BSA by pooling equal quantity of DNA from ten fertile (*Rr*) and ten sterile (*rr*) individuals.

AFLP analysis

Amplified fragment length polymorphism analysis was performed using kits following manufacturer's instructions (Life Technologies, USA). Sixty-four primer combinations were tested for polymorphism analysis between the bulks. Genomic DNA (180 ng) was restricted with *Eco*RI and *Mse*I enzymes (2.5 U each) for 2 h at 37°C in 25 µl of reaction volume and the enzymes were heat-inactivated by incubating tubes at 70°C for 15 min. The DNA fragments were ligated to appropriate adapters using T4 DNA ligase (1 U) and 24 µl of *Eco*RI and *Mse*I adapter ligation mixture by

incubating at 20°C for 2 h. Ligated DNA mixture was pre-amplified using adapter primers with A- and C-selective nucleotides (i.e., *Eco*RI+A and *Mse*I+C) in a final volume of 50 µl. The PCR cycling parameters were 20 cycles of 94°C for 30 s, 56°C for 60 s and 72°C for 60 s.

For selective amplification, *Eco*RI primers with three selective nucleotides at their 3′ ends were end labeled with [γ-^{33}P] ATP (2,000 Ci/mmol) using T_4 polynucleotide kinase. Labeled *Eco*RI (+3) primers (0.5 µl) were mixed with 5 µl of 50-fold diluted pre-amplified DNA, PCR buffer and the *Mse*I (+3) primers in a final volume of 20 µl. The following PCR cycling parameters were used for selective amplification: First cycle of 94°C for 30 s, 65°C for 30 s, 72°C for 60 s. During the next 12 cycles, the annealing temperature was lowered by 0.7°C per cycle. The temperature conditions for the next 23 cycles were 94°C for 30 s, 56°C for 30 s and 72°C for 60 s. The amplified products were analyzed on 6% denaturing polyacrylamide gel in 1× TBE buffer. Gels were run using a Thermo EC (EC4000P) power pack at a constant power of 60 W and maximum voltage of 2,000 V. After electrophoresis, the gel cast was dismantled and the gel was lifted onto a Whatman paper sheet, covered with saran wrap and dried on a gel dryer for 2 h at 80°C. The dried gel was placed in a cassette and exposed to Kodak SB film at −80°C for 12–16 h.

Conversion of AFLP markers to SCAR markers

The positions of polymorphic AFLP bands were first marked on the dried polyacrylamide gel by aligning the autoradiogram with the gel. The marked gel piece was excised and the DNA from the gel fragment was isolated following Ausubel et al. (1994). The excised bands were re-hydrated in TE for 1 h. A 2 µl of supernatant was used as template for PCR amplification using appropriate primers. PCR conditions were the same as used for selective amplification reaction. The amplified products were electrophoresed on 3% MetaPhor agarose (Cambrex, USA). Remaining DNA was precipitated and dissolved in 10 µl of H_2O.

Re-amplified products (3–5 µl) were cloned into pGEMT vector as per manufacturer's instructions (Promega, USA) and used for transformation of *Escherichia coli* DH5α. The cloning of the amplicons was checked by restriction digestion with *Eco*RI.

The AFLP clones were sequenced using automated DNA sequencer (ABI-prism 310). Based on the sequence information, PCR primers were designed to amplify the genomic DNA. The PCR products were electrophoresed on 3% MetaPhor agarose or 6% polyacrylamide gel at a constant voltage of 2 V/cm for 5 h. The amplicons were visualized under UV after staining with ethidium bromide.

Linkage analysis

Linkage analysis of markers and trait was carried out using MAPMAKER version 3.0 software (Lander et al. 1987). The linkage group was established with an LOD-score threshold of 3.0 and the maximum distance of 30 cM, using the mapping function of Kosambi (1944).

Results

Phenotyping of male fertility and sterility

For tagging of the restorer gene, a BC_1F_1 population was used. Only two phenotypes, male sterile and partially fertile, were observed. Since fertility restoration in this CMS system is gametophytic, fertility restored plants (with Rf/- genotype) are expected to show about 50% pollen fertility. Male sterile and partially fertile plants could be distinguished unambiguously by visual examination. However, for confirmation, data of both acetocarmine staining of pollen grains and seed set upon selfing were also used. The BC_1F_1 population (124 plants) segregated for male sterility (61) and fertility (63) as per the expected 1:1 ratio for a monogenic inheritance.

AFLP analysis

To identify markers linked to the restorer gene, AFLP analysis was carried out with BSA. Sixty-four *Eco*RI/*Mse*I primer combinations were tested in the two bulks for DNA amplification polymorphism. In AFLP approximately 4,500 bands (70 bands per primer combination) were produced. Nineteen primer combinations generated a total 29 polymorphic bands between the bulks; 16 of these polymorphic bands were associated with fertility and 13 with sterility. The size of the amplified fragments ranged from 50 to 450 bases. Primer combinations yielding polymorphic amplification pattern between the bulks were further examined in individual BC_1F_1 plants used to prepare the bulks. A total of 40 individuals (20 of each type) were examined. Only five primer combinations gave polymorphic amplification pattern in individual plants and fragment size of polymorphic amplicons ranged between 103 and 300 bp (Table 1). The polymorphic bands are designated with the primer combination and the fragment

size. For example, EAAC/MCTA$_{118}$ indicates that the polymorphic fragment is of 118 bp size and is generated using the primers EAAC and MCTA. Except for the marker EAAC/MCTG$_{248}$, other five markers segregated in a 1:1 ratio in the BC$_1$F$_1$ population (Table 1). Primers EAAC/MCTA and EACC/MCAC yielded one polymorphic band each that co-segregated with male fertility (Fig. 1a, b). The primer combination EACT/MCAT gave two polymorphic amplicons, one associated with male fertility (Fig. 1c) and the other with male sterility. The primer combination EACT/MCAC gave a polymorphic band associated with male sterility.

Conversion of AFLP markers into SCAR markers

The polymorphic fragments were eluted from the gel, re-amplified with the respective primers and ligated with cloning vector and used for transforming *E. coli*. Cloning was confirmed through restriction digestion. The polymorphic AFLP markers were cloned and sequenced. However, the EACC/MCAC$_{300}$ fragment could not be cloned due to its weak intensity. The sequences were subjected to BLAST analysis. Only the EACT/MCAT$_{214}$ clone showed significant homology with the *Brassica rapa* subsp. *pekinensis* genome sequence (AC155340).

Based on sequence information of the AFLP clones, SCAR primers were designed for PCR amplification. Details of SCAR primer sequences and PCR reaction conditions are given in Table 2. SCAR1 and SCAR2 primers gave, respectively, 90 and 250 bp long amplified products in all 40 BC$_1$F$_1$ fertile and sterile plants. The banding pattern was monomorphic. SCAR3 primers designed based on sequence of the EACT/MCAT$_{214}$ clone gave two amplicons of 200 and 400 bp size in

Table 1 Segregation of AFLP markers in BC$_1$F$_1$ population

S. no.	Primer name	Trait associated with polymorphic band	Segregation pattern of markers		χ^2 1:1
			Present	Absent	
1	EAAC/MCTA$_{118}$	Fertility	17	23	0.9
2	EAAC/MCTG$_{248}$	Fertility	8	32	14.4
3	EACC/MCAC$_{300}$	Fertility	18	22	0.4
4	EACT/MCAT$_{214}$	Fertility	20	20	0.0
5	EACT/MCAT$_{103}$	Sterility	16	24	1.6
6	EACT/MCAC$_{205}$	Sterility	24	16	1.6

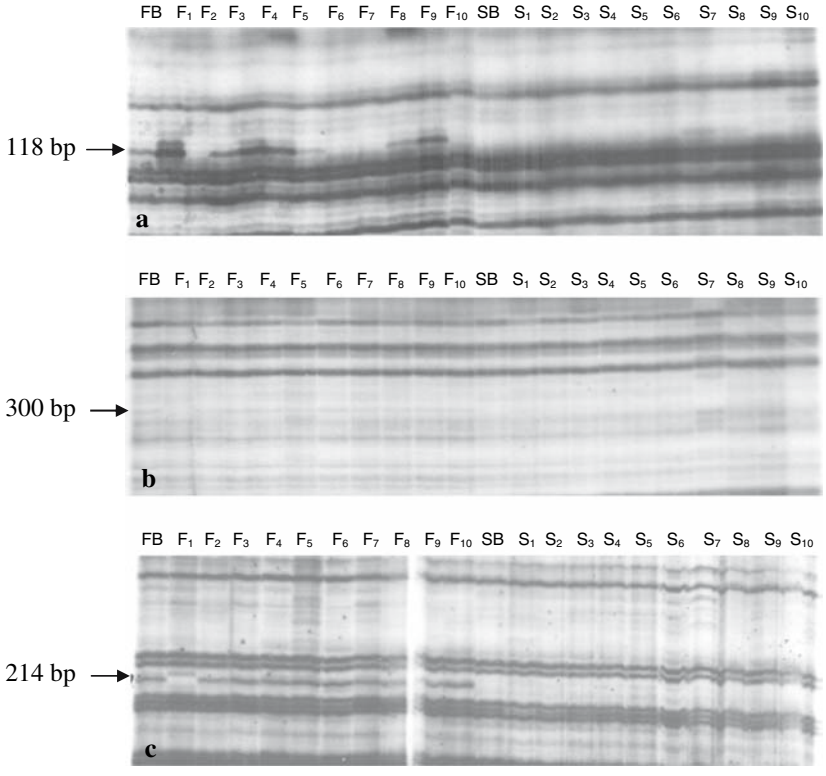

Fig. 1 AFLP profiles showing polymorphic amplification patterns associated with male fertility trait obtained with primer combinations **a** EAAC/MCTA, **b** EACC/MCAC and **c** EACT/MCAT (size and position of polymorphic band is indicated with an *arrow*). *FB* fertile bulk, *SB* sterile bulk, *F* fertile individual, *S* sterile individual

Table 2 Details of PCR conditions and SCAR primers for amplifying male fertility-associated AFLP markers

AFLP marker	SCAR			PCR conditions
	Name	Size (bp)	Primer (5′–3′)	
EAAC/MCTA$_{118}$	SCAR1	~90	GACTGCGTACCAATTCAACATC ATGAGTCCTGAGTAACTAAAG	94°C for 30 s, 56°C for 60 s, 72°C for 60 s
EAAC/MCTG$_{248}$	SCAR2	~250	TAACTGGGATTTGTTGCAGTAG ATTCAACAGAGGTGACTTTAGA	94°C for 30 s, 55°C for 60 s, 72°C for 60 s
EACT/MCAT$_{214}$	SCAR3	~200 ~400	TCACTAAAGATCGAGATAGTACC TAACATCTTCAACGTTTCGGTG	94°C for 60 s 54°C for 60 s 72°C for 60 s

PCR. These bands were present almost exclusively in fertile individuals and absent in sterile individuals (Fig. 2). When 124 BC_1F_1 individuals were tested with SCAR3 primers, the 2 amplicons were obtained in all 63 fertile plants whereas only 1 out of 61 sterile plants showed the amplicons. Thus a strong association was found between the SCAR3 marker and male fertility.

Linkage analysis

Linkage analysis of AFLP and SCAR markers with the fertility restorer gene and with one another was determined by analyzing the co-segregation data. The linkage map constructed with two AFLP and one SCAR markers is presented in Fig. 3. The markers were found linked to the fertility restorer gene and mapped on one side of the *Rf* locus. The genetic distance between the marker and the *Rf* locus ranged from 0.6 to 2.9 cM. The SCAR3 marker was found to be tightly linked to the *Rf* locus with a map distance of 0.6 cM.

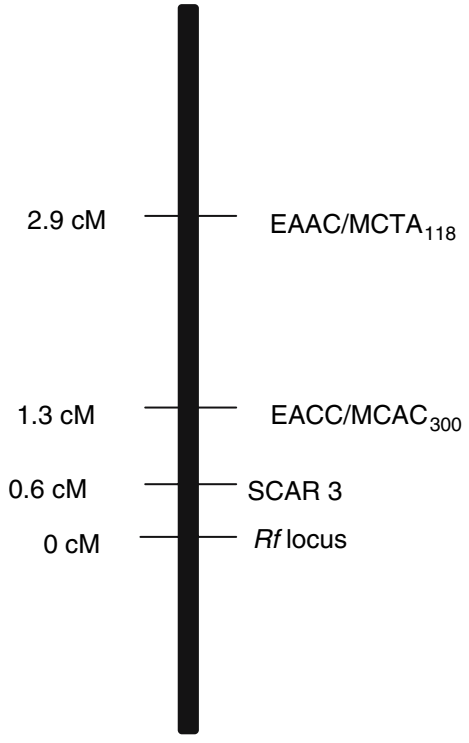

Fig. 3 Linkage map of the restorer gene

Discussion

Among the methods available for molecular tagging of traits, BSA method devised by Michelmore et al. (1991) has been the most widely employed. For tagging qualitative traits that can be easily classified into two or more discreet classes, BSA is particularly robust. The advantage of this approach is that the likelihood of identifying false positive markers is very small (Michelmore et al. 1991). BSA is generally used with PCR-based methods such as RAPD, AFLP for tagging and most of the CMS restorer genes have been tagged following this approach.

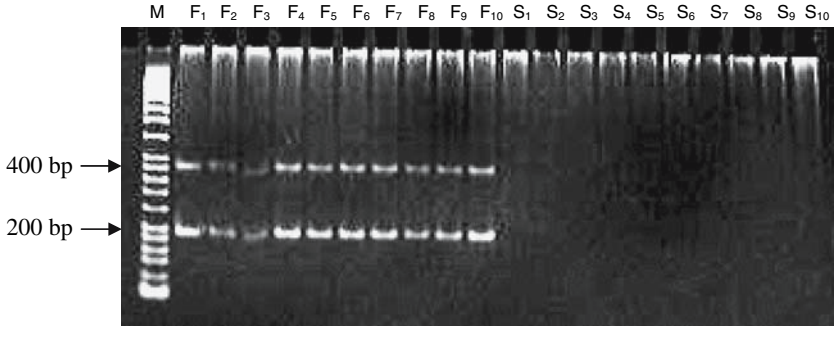

Fig. 2 Segregation of SCAR3 marker in BC_1F_1 individuals. *M* 25 bp DNA ladder, *F* fertile, *S* sterile

In polyploid species such as *B. juncea*, *B. napus*, *G. hirsutum* where RAPD does not yield desired level of polymorphism, AFLP is now frequently used to reveal polymorphisms between closely related varieties/lines and for tagging of genes. Since the number of fragments amplified in an AFLP reaction can be controlled by the choice of primers and conditions of PCR, AFLP offers greater opportunity for tagging of traits. Since our initial attempts to use RAPD revealed low polymorphism (data not shown), we resorted to AFLP technique to tag the *Rf* locus.

In the present study, out of 64 *Eco*RI/*Mse*I primer combinations screened, 19 combinations revealed polymorphism between the bulks. However, when examined in individual BC_1F_1 plants, only five primer combinations gave polymorphism associated with the trait. The discrepancy between the results of bulk and individual plant analyses is not uncommon (Kim et al. 2006) and may be attributed to the composition of the bulk. In BSA using BC_1F_1 it is expected to find markers linked to the dominant allele (*Rf*). However, in practice fragments associated with recessive traits are also observed (Negi et al. 2000). Even in the present study two AFLP bands were found to be associated with sterility. However, further investigation revealed that these were not reliable. This could be due to differences in template DNA concentrations between individuals homozygous and heterozygous for the locus in question. The level of polymorphism detected in this study is comparable to previous reports in *Brassica* (Sabharwal et al. 2004; Yi et al. 2006).

Amplified fragment length polymorphism in conjunction with BSA has been successfully used to identify markers linked to fertility restorer gene in petunia (Bentolila et al. 1998; Bentolila and Hanson 2001), radish (Imai et al. 2003; Koizuka et al. 2003; Murayama et al. 2003), rapeseed (Janeja et al. 2003b), sorghum (Klein et al. 2001), sunflower (Horn et al. 2003) and sugar beet (Touzet et al. 2004; Hagihara et al. 2005). While AFLP technique is efficient for tagging of traits, AFLP markers are not user friendly. They are generally expensive to develop and not amenable for routine and quick screening, may require radioactivity and are dominant in nature. For a broader application, AFLP markers need to be converted into simple SCAR marker. For this, we cloned and sequenced the AFLP marker fragments and based on sequence information, designed primers for PCR amplification of the fragments. Out of three, only one AFLP marker could be converted into SCAR. In general, AFLP sequences are not directly convertible into SCAR because of short sequence information. Sometimes SSCP or CAPS markers can still be derived from AFLP sequences. For example, in sorghum three AFLP markers linked to *rf4* gene were converted to STS/CAPS marker (Wen et al. 2002). However, we could not find any CAPS marker using SCAR1 and SCAR2 amplicons (data not shown). More often, however, genome walking is employed to derive SCAR markers from AFLP sequences (Yi et al. 2006). For example, Negi et al. (2000) converted AFLP marker linked to seed coat color in *B. juncea* into SCAR marker through chromosome walking. Similarly in CMS (*ogura*) radish (Murayama et al. 2003), Kosena radish (Imai et al. 2003) and in barley (Murakami et al. 2005) restorer-linked AFLP markers were successfully converted into STS markers.

All three (two AFLP and one SCAR) markers were found linked to the fertility restorer gene. The genetic distance between markers and the *Rf* locus ranged from 0.6 to 2.9 cM. The SCAR3 marker was found to be the closest to the *Rf* locus with a map distance of 0.6 cM. Since only 40 individuals were used for AFLP mapping, our estimates of linkage between the AFLP markers and the *Rf* locus may be on the higher side. For example, the AFLP marker $EACT/MCAT_{214}$ was initially found to be 1.4 cM away from the *Rf* locus. However, upon its conversion into a SCAR marker and analysis of 124 BC_1F_1 individuals, the map distance between the SCAR3 marker and the *Rf* locus was found to be 0.6 cM. All markers were found to lie on one side of the restorer gene.

Map-based cloning of the restorer genes of radish CMS systems 'Ogura' and 'Kosena' was facilitated by the close synteny existing between radish and *Arabidopsis* genomes (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003). In the present case, the restorer gene is introgressed from the wild species *M. arvensis*, and the size of alien introgression is undetermined. Hence, marker information of the radish restorer is not useful for our purpose. Similarly, sequence information of other restorer genes cloned so far is of limited value in devising strategies for cloning of the restorer gene because PPR motif is a degenerate motif found in more than 200 genes in *Arabidopsis* (Small and Peeters 2000). Hence, conventional approaches will have to be applied to find additional markers. The present study could identify only one closely linked SCAR marker. The 200 bp SCAR3 amplicon was found to share homology with *B. rapa* genome sequence. Further, the SCAR3 primers amplified an additional 400 bp fragment in all the fertile plants which suggests that the two SCAR3 fragments are tightly linked. We are now trying to find additional markers located within the *Brassica* genome to define the introgressed region before attempting cloning of the restorer gene.

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