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Identification of AFLP markers linked to fertility restorer genes for tournefortii cytoplasmic male-sterility system in Brassica napus

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Abstract The *tournefortii* cytoplasmic male-sterility system is being used as a method of pollination control to develop hybrids in *Brassica napus*. Genetic analyses have indicated that two dominant genes, one major (*Rft1*) and another minor (*Rft2*), were required to achieve complete fertility restoration. Though the major gene (*Rft1*) can cause complete fertility restoration on its own, its expression was significantly enhanced in the presence of the minor gene (*Rft2*). In the absence of *Rft1*, *Rft2* caused only partial fertility restoration. We used a pair of near-isogenic lines (NILs), differing for the presence/absence of *Rf* genes, to identify AFLP markers linked to fertility restorer genes. A total of 64 *Eco*RI/*Mse*I primer combinations were surveyed which produced 3,225 bands, of which 19 (0.006%) were polymorphic between parental NILs. Primer combinations which led to the identification of polymorphic bands present in fertile parental NILs were used for assaying a mapping population of 70 F_2 plants for determining the segregation pattern of markers. Initial screening resulted in the identification of five AFLP markers. The recombination analyses of these AFLP markers revealed that at least two (EACC/$MCTT_{105}$, EAAG/$MCTC_{80}$) were present in the same linkage group along with the *Rf* loci. Marker EACC/$MCTT_{105}$ was separated from the major gene (*Rft1*) by a distance of 18.1 cM, while it was 33.2 cM away from the minor fertility restorer gene (*Rft2*). Another marker EAAG/$MCTC_{80}$ was also located adjacent to *Rft1* at a distance of 18.1 cM, but on other side. Identification of flanking markers (EACC/$MCTT_{105}$, EAAG/$MCTC_{80}$) for the major fertility restorer gene (*Rft1*) provides a crucial component for marker-assisted selection and map-based cloning of the restorer genes, and can hence be used to construct elite restorer genotypes.

Keywords Cytoplasmic male sterility · Fertility restorer gene · AFLP markers · *Tournefortii* CMS · *Brassica napus*

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Introduction

Cytoplasmic male sterility (CMS) is an important genetic tool to facilitate hybrid seed production in hermaphrodite crops. It is caused by chimeric genes, which arise by rearrangements of mitochondrial DNA sequences (Kaul 1998). Nuclear genes, termed as fertility restorer (*Rf*) genes, can restore male fertility by selectively modulating the expression of CMS-associated regions without influencing the other mitochondrial genes. The mechanism through which it is achieved appears to vary among different systems as is indicated from CMS specific fertility restoration by a nuclear gene or genes. The four main CMS sources identified in oilseed rape (*Brassica napus*) are *nap*, *pol*, *tour*, and *ogura* (Fu and Yang 1998). Fertility restoring genes for *pol* and *tour* CMS occur in native *B. napus* germplasm (Fang and McVetty 1989; Banga et al. 1994; Sodhi et al. 1994), whereas *Rf* genes have to be introgressed from cytoplasm donor species for the *ogura* system (Pelletier et al. 1987). *Rf* genes always act as dominant, and the genetic determination is mostly monogenic or sometimes digenic. Although *Rf* genes can be easily scored in an alloplasmic background due to their male-fertility restoring expression, these can not be traced in euplasmic genotypes without resorting to test-crossing with the CMS line. This can prolong the breeding procedures to transfer *Rf* genes into the male parent of a potentially heterotic group in a hybrid-breeding programme. In addition, any mutation in the restorer gene, when present in a euplasmic background, can go unnoticed resulting in unacceptable levels of male-sterile contaminants. These problems can be obviated by the deployment of *Rf* genes in male-sterile alloplasm. However, pollen production and dispersal from the alloplasmic

male parent is almost always lower than the euplasmic parent which necessitates a greater frequency of male (alloplasmic fertility restorer) rows in hybrid seed-production blocks, resulting in an increased cost of seed production. DNA markers tightly linked to a restorer gene are expected to result in a fairly straightforward breeding protocol for *Rf* gene-transfer irrespective of the cytoplasmic background, besides providing a check of any mutation affecting the fertility restorer gene(s). DNA markers, especially flanking markers, also impart efficiency to breeding efforts directed at reducing the size of alien chromosome fragments accompanying the restorer gene during interspecific or intergeneric *Rf* gene transfer. To achieve this, several research laboratories are striving to generate markers within the genomic region of interest using such marker techniques as RAPD, RFLP and AFLP. Four RAPD markers, OPC 02_{1150}, OPD 02_{1000}, OPF 06_{1200} and OPG 02_{700}, linked to the *Rfo* locus for the *ogura* CMS system (Delourme et al. 1994) have been identified. These have been cloned and sequenced to develop co-dominant sequence-characterized amplified region (SCAR) markers. The region around the *Rfo* gene was further saturated (Delourme et al. 1998) and a RAPD marker, namely RAPD 13, has been identified which co-segregates with the *Rfo* locus. Hansen et al. (1997) have also reported a RAPD marker tightly linked (1.9 cM) to the *Rfo* locus. In the *pol* CMS system, 11 molecular markers (10 RFLP markers and 1 RAPD marker) directly linked to the *Rtp1* locus have been reported (Jean et al. 1997).

The primary objective of this study was to identify AFLP markers linked to genes responsible for fertility restoration that will facilitate restorer breeding for the *tour* CMS system. It may be mentioned here that the *tour* CMS system in *B. napus* was developed through the transfer of male-sterile cytoplasm from *Brassica juncea*, in which it was initially discovered (Rawat and Anand 1979), by a backcross programme. A rapeseed hybrid (PGSH 51), based on *tour* CMS, was later commercialized in India following the discovery of *Rf* genes in spring rapeseed germplasm (Banga et al. 1994). To pursue the objective of identifying linked DNA markers, a set of NILs that differed for the presence or absence of the *Rf* gene(s) were developed and utilized to generate a F_2 mapping population.

Materials and methods

Plant material and fertility analysis

The *tour* CMS line GSL 8968A and the fertility restorer genotype GSL 8953R, homozygous for two *Rf* genes (*Rft1 Rft1*, *Rft2 Rft2*) were used to develop F_1 and F_2 generations. Plants (*B. napus* L.) were grown to maturity under field conditions, using standard agronomic practices. Fertility was assessed through visual examination of freshly opened flowers for anther size, colour and presence/absence of pollen grains. The sterile plants had small flowers, pale and reduced anthers with little or no pollen grains. Fertile plants had yellow, well-developed anthers with fertile pollen grains. Anthers in partially fertile plants were pale yellow, medium in size and were placed lower than the stigma. In completely restored or euplasmic[1] fertile plants, anthers were positioned above the stigma to facilitate self-seed setting. Segregation pattern for fertile, partially fertile and sterile plants in the F_2 generation was analyzed to understand the genetics of fertility restoration. Goodness of fit to theoretical postulates was determined by the chi-square (χ^2) test.

Parental near-isogenic lines (PNILs) for *tour* CMS were developed by repeated backcrossing of GSL 89 separately with *Rf* and the CMS source. For development of *Rf* NILs, the *tour Rf* genes were transferred from the source population by backcrossing with recipient GSL 89 as a recurrent parent. Backcrossing was continued until the BC_5 generation, and subsequently the fertile line was maintained by selfing. The presence of *Rf* genes was confirmed in every generation of backcrossing or selfing by test-crossing with *tour* CMS. GSL 89 with the restorer gene(s) was termed as GSL 8953R. Backcrossing of GSL 89 with the original CMS source was continued until the BC_8 to develop the male-sterile version of GSL 89 (termed GSL 8968A).

Mapping population and molecular analysis

Parental near-isogenic lines were crossed to develop F_1 plants. All F_1 plants were fully fertile. One F_1 plant was selected randomly and bagged to produce the F_2 segregating population. Seventy F_2 plants were randomly selected for further analysis. Genomic DNA was isolated from leaf material by the CTAB method (Dyole and Dyole 1990) with few modifications. Three to four young leaves (2-cm in size) were ground to powder in a mortar with liquid nitrogen. The powder was transferred to micro-centrifuge tubes containing 900 μl of pre-heated (65 °C) extraction buffer [100 mM of Tris (pH 8.0), 1.4 M of NaCl, 20 mM of EDTA and 2% (w/v) CTAB]. Tubes were incubated at 65 °C for 35 min with regular shaking. Chloroform (900 μl) was added and mixed until it turned dark-green in colour. The resultant mixture was centrifuged for 25 min at 6,000 rpm at room temperature. The supernatant was recovered and RNase A (100 mg/ml) was added. The reaction mix was incubated at 37 °C for 45 min to eliminate RNA. Again chloroform (900 μl) was added and centrifuged for 15 min at 6,000 rpm. The supernatant was transferred to a fresh tube. A two-third volume of ice-chilled isopropanol was added and the DNA was spooled out. DNA was subsequently washed with 70% ethanol and dissolved in 200 μl of 1 × TE buffer (10 mM Tris, 1 mM EDTA).

AFLP analysis was performed as per the technical instructions given by the manufacturer (GIBCO BRL). Since *Brassica* species have a complex genome, AFLP analysis I was employed. AFLP fingerprints were generated based on the protocol described by Vos et al. (1995). Amplification products were separated on 6% denaturing polyacrylamide gels and visualised by autoradiography (Negi et al. 2000).

Marker scoring and linkage analysis

For each primer, the amplified DNA products were sequentially named according to their molecular sizes in decreasing order. The polymorphic primers were identified on the basis of the presence/absence of the amplified product in either of the parent and respective F_2 plants. Linkage was determined by analyzing the data of AFLP markers and fertility restorer genes using MAPMAKER Version 3.0 (Lander et al. 1987). The 40% recombination is generally used as a benchmark for considering markers showing linkage with any trait.

Since the objective was to tag a qualitative trait, i.e. a male-fertility restorer gene(s), markers showing more than 35% recombination frequency were not considered as linked with the *Rf*

[1] The term 'euplasmic' refers to lines containing the native cytoplasm. It is opposite to 'alloplasmic' that refers to the lines containing the nuclear genome of one species and the cytoplasm of the other

Table 1 Genetics of fertility restoration in F_1 and F_2 generations derived from the cross GSL 8968A × GSL 8953R

Generation	No. of plants observed	Observed segregation			χ^2
		Fertile plants	Partial fertile plants	Sterile plants	
F_1	68	68	–	–	–
F_2	276	214	46	16	0.988 (12:3:1)

gene(s). All markers were, therefore, allocated to linkage groups by pair-wise analysis with a threshold LOD score of 3.5. The order of markers on each of the linkage groups (frames) was calculated from multi-point analysis. The final order of the markers on each linkage group was reconfirmed using the ripple command.

Results and discussion

Phenotypic expression and the genetics of the *Rf* gene(s)

Male-sterile flowers in *tour* CMS were characterized by narrow petals, reduced anthers with little or no pollen grains and no seed set was observed on selfing. The F_1 plants with complete male-fertility restoration had normal-sized anthers, but petal size was slightly smaller than the euplasmic fertile plants. Pollen fertility of F_1 plants was normal and it was sufficient to ensure complete seed-set upon selfing. In the F_2 population, the partial male-fertile plants had flowers with intermediate-sized anthers and significantly less pollen load. These were placed lower than the stigma. Seed set by bagging in such plants was less than half that observed in the euplasmic controls.

The F_1 and F_2 generations obtained from the cross between GSL 8968A (*tour* CMS) and GSL 8953R (fertility restorer) were studied to understand the genetics of male-fertility restoration. Dominance of male-fertility restoration was indicated by complete male-fertile F_1 progeny. F_2 segregation data (214 fertile: 46 partially fertile: 16 sterile plants) fitted well to the theoretical expectation of the 12:3:1 ratio of digenic epistatic interaction (Table 1). It was apparent that male-fertility restoration for *tour* CMS was governed by two genes, of which one gene was stronger than the other. The presence of single major gene (*Rft1*), in dominant form (*Rft1-/rf2rf2*), was sufficient to achieve complete fertility restoration of the F_1 hybrids. In the absence of the major dominant gene (*Rft1*), however, the second minor gene (*rft1rft1*, *Rft2*-) resulted only in partial male-fertility restoration. These results are in conformity with our previous studies (Banga et al. 1994; Pahwa et al. 2002), but differ from those of Sodhi et al. (1994), who reported monogenic dominant control of fertility restoration. The results of Sodhi et al. (1994) could also not be confirmed in later studies by Stiewe and Röbbelen (1997), who obtained restored plants with intermediate fertility and observed that the genetic determination was not simple.

Molecular tagging of fertility restorer genes

The amplified fragment length polymorphism (AFLP) technique developed by Vos et al. (1995) was used for the tagging of *Rf* genes for the *tour* CMS system. A mapping population of 70 F_2 plants was used for AFLP analysis to check co-segregation of amplified bands with loci governing *tour* male-fertility restoration. This population was generated by hybridizing BC_5F_4/BC_8 NILs, which offered the advantage of being similar in genetic make up, except for the character in question, i.e. male fertility restoration in the present study.

Detection of polymorphism between parental NILs

Gene-mapping techniques involving NILs provide an effective and convenient way for identifying presumptive linkages between the introgressed gene and the molecular marker. The tagging of genes through NILs involves introgression of a gene from the donor parent to a recurrent parent that results in lines that are theoretically isomorphic to the recurrent parent, except for a small portion of the donor genome which contains the introgressed gene (Muehlbauer et al. 1988). NILs have been developed for a number of crop species (Bernard 1976; Maxon and Ritchie 1983; Shi et al. 2001).

In the present study the *Rf* genes for the *tour* CMS system were tagged using NILs with the aid of AFLP markers. Sixty four *Eco*RI/*Mse*I primer combinations were used for surveying polymorphism between parental NILs and eight F_2 plants, four-fertile and four-sterile each. The 64 primer combinations produced a total of 3,225 bands with approximately 50 bands per primer combination. Nineteen (0.006%) bands were polymorphic between parental NILs and their respective F_2 plants. Nine of these polymorphic bands were present in the fertile and ten in the sterile parental near-isogenic line (PNIL). The size of the amplified segment ranged from 45 to 500 bases. Of the 64 primer combinations employed, only 15 produced polymorphic bands between the parents (Table 2). Since the parents were NILs, only 15 primer combinations produced polymorphism, which is as expected. Primer combinations EAAC/MCAA, EAAC/MCAC, EAAG/MCTA, EACC/MCAA and EAGC/MCTT amplified one band each that was present only in the male-fertile parent and male-fertile F_2 plants, and absent in the male-sterile parent and male-sterile F_2 plants. Primer combination, EACC/MCTT, produced two polymorphic bands that were present in the male-fertile parent but absent in the male-sterile parent. Primer

Table 2 The AFLP primers showing polymorphism between fertile and sterile parental isogenic lines (PNILs) for the *Rf* gene(s)

S. no.	Primer	Total number of bands	Bands in fertile PNIL	Bands in sterile PNIL	Number of polymorphic bands
1	EAAC/MCAA	52	52	51	1
2	EAAC/MCAC	46	46	45	1
3	EAAC/MCAT	53	52	52	2*
4	EAAG/MCAG	42	41	41	2*
5	EAAG/MCTA	44	44	43	1
6	EAAG/MCTC	51	50	50	2*
7	EAAG/MCTT	58	57	58	1
8	EACA/MCAC	51	50	51	1
9	EACC/MCAA	51	51	50	1
10	EACC/MCAC	53	52	53	1
11	EACC/MCTT	53	53	51	2
12	EACT/MCAG	52	51	52	1
13	EAGC/MCAA	51	50	51	1
14	EAGC/MCTT	55	55	54	1
15	EAGG/MCAT	53	52	53	1

* One each in fertile and sterile parental near-isogenic lines

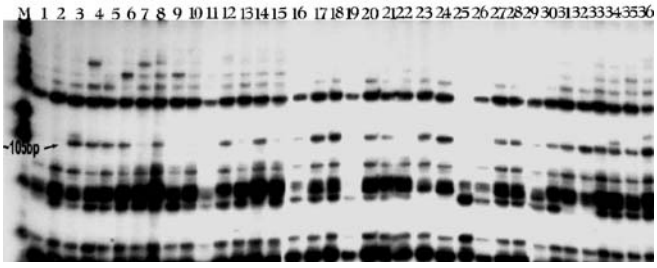

Fig. 1 A representative AFLP fingerprint depicting parental NILs and the F_2 population amplified with primer combination EACC/M-CTT. The 105-bp AFLP marker, indicated with an *arrow*, is linked to the minor fertility restorer gene (*Rft2*). Fertile and sterile pNILs correspond to *lanes 12 and 11*, respectively. Fertile F_2 plants are present in lanes *13 to 36*. Sterile F_2 plants are present in lanes *1, 2, 7, 8, 9 and 10*. Finally, *lanes 3 to 6* correspond to partially fertile plants. *M* indicates the molecular size marker

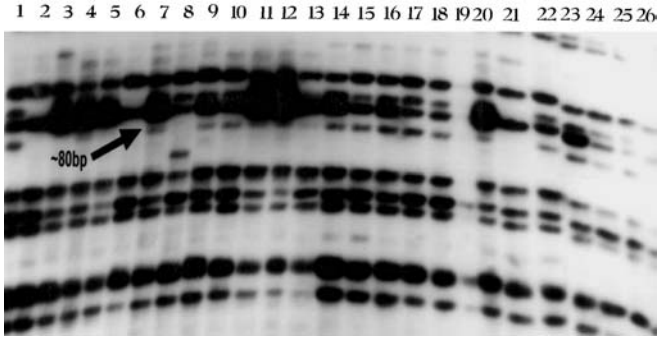

Fig. 2 A representative AFLP profile of parental NILs and the F_2 population generated on using primer combination E-AAG/M-CTC. The 80-bp AFLP marker, indicated with an *arrow*, is linked to restorer gene (*Rft1*). *Lanes 10 and 9* correspond to fertile and sterile pNILs, respectively. Fertile F_2 are present in lanes *1, 10 to 18, 20 and 25*. Sterile F_2 are present in *lanes 2–7* and partial fertile plants correspond to lanes *19, 21 and 26*, respectively

combinations EAAG/MCTC, EAAG/MCAG and EAAC/MCAT produced two polymorphic bands each, one being present in the fertile parent and the other in the male-sterile parent. The polymorphic bands were designated according to their size in base pairs such as EACC/$MCTT_{130}$ and EACC/$MCTT_{105}$.

Segregation of AFLP markers in the F_2 population

Since the objective of the present study was to tag a gene(s) for fertility restoration, only those primer combinations that showed the presence of a band in the fertile parent and absent in the male-sterile parent were used for studying co-segregation in the F_2 population. A set of 70 F_2 plants was analysed for the segregation pattern of the AFLP markers. Four primer combinations, namely EAAG/MCAG, EAAG/MCTA, EAAG/MCTC and EACC/MCTT, that amplified distinct bands, were employed for the analysis of *Rf* genes. The AFLP profile (Fig. 1) revealing the segregating pattern of informative makers in the F_2 population, was generated by employing primer combination EACC/MCTT. The AFLP marker EACC/$MCTT_{105}$ showed a segregation ratio of 5:1 (Table 3) indicating distortion, possibly due to the presence of a complex region around the *Rf* gene(s) which showed differential segregation or linkage to both the genes governing male-fertility restoration. Most of the F_2 plants having partial male-fertility status also showed the presence of this band along with fully fertile F_2 plants

Table 3 Segregation behaviour of AFLP markers for the *tour* CMS-based F_2 tagging population

Marker	Total F_2 plants studied	Polymorphic bands		χ^2
		Present	Absent	
EACC/$MCTT_{105}$	70	58	12	1.941 (5:1)
EACC/$MCTT_{130}$	70	34	38	0.057 (1:1)
EAAG/$MCAG_{125}$	68	30	38	0.514 (1:1)
EAAG/$MCTA_{90}$	68	44	24	3.843 (3:1)
EAAG/$MCTC_{80}$	70	49	21	1.852 (3:1)

suggesting its linkage to both the target genes. Similarly, Fig. 2 shows the segregation pattern of primer combination EAAG/MCTC. The marker EAAG/$MCTC_{80}$ exhibits a segregation ratio of 3:1, which was also observed with another AFLP marker EAAG/$MCTA_{90}$ (data not shown). EACC/$MCTT_{130}$ and EAAG/$MCAG_{125}$ exhibited a 1:1 segregation. It is evident that these two markers were not linked to male-fertility restorer genes.

Tagging of fertility restorer genes

Theoretically, AFLP markers are expected to be evenly distributed in the genome (Zhu et al. 1998). Since our mapping population was derived from near-isogenic lines, differences were expected for only a few linkage groups. Therefore, one of the major limitations of utilizing NILs for gene tagging is that a large number of genetic loci are required to be screened for identifying and detecting polymorphism between them. The probability of obtaining a marker within close proximity to the target region depends on the number of polymorphic primers, the genomic size in map units and the degree of DNA sequence variation around the target region. If the sequence is very similar, a primer is less likely to detect polymorphism even if the priming event takes place near the target gene(s). The distance from the target gene increases as the proportion of detectable polymorphism decreases (Michelmore et al. 1991). An increase in genome size is also proportional to an increase in the estimated distance. Analysis of data on the presence/absence of bands in 70 F_2 individuals was carried out using MAPMAKER (ver. 3.0) to determine linkage between *Rf* genes and AFLP markers. These markers were grouped at a LOD score of 3.5 and a maximum distance of 35 cM (Table 4).

The LOD score based on two-point analysis and the distances between markers based on multi-point analysis are presented in Tables 5 and 6. Of the five AFLP markers analysed, two grouped on the same linkage group along with the male-fertility restorer loci. Marker EACC/$MCTT_{105}$ was separated from the major fertility restorer gene (*Rft1*) at a distance of 18.1 cM (LOD 7.68) and was also linked to the minor fertility gene (*Rft2*) from which it was 33.2 cM away (LOD 3.99). The marker EAAG/$MCTC_{80}$ was located adjacent to the major fertility restorer gene (*Rft1*) at a distance of 18.1 cM with a LOD score of 7.68. The remaining three markers, EACC/$MCTT_{130}$, EAAG/$MCAG_{125}$ and EAAG/$MCTA_{90}$, were found to be unlinked to this linkage group or the male-fertility restorer genes. The two genes (*Rft1*, *Rft2*) controlling male-fertility restoration, that mapped on the same linkage group, were 39.4 cM apart (LOD score 3.07). Failure in finding any molecular marker tightly linked to the target gene(s) may be attributed to low polymorphism on the chromosomal region between the parents and/or possibly a few restriction/annealing sites for the primers in the chromosomal region of interest. It may be possible to find a closely linked marker, if one utilizes different marker techniques such as a microsatellites or a retrotransposons to target the region.

Table 4 Linkage analysis of AFLP markers scored among F_2 individuals (LOD 3.5)

Linkage group	Unlinked loci
EACC/$MCTT_{105}$ EAAG/$MCTC_{80}$	EAAG/$MCAG_{125}$ EACC/$MCTT_{130}$ EAAG/$MCTA_{90}$

Table 5 LOD score and distances between markers (cM) by pairwise analysis with respect to fertility restorer genes in the *tour* CMS system

Marker/gene	*Rft2*	EAAG/$MCTC_{80}$	*Rft1*
EAAG/$MCTC_{80}$	33.20* 3.99		
Rft1	39.40 3.07	18.10 7.68	
EACC/$MCTT_{105}$	33.20 3.99	33.20 3.99	18.10 7.68

* Upper values indicate distance (cM) and lower values indicate LOD score

Table 6 Map distances of different markers with respect to fertility restorer genes in the *tour* system. Log likelihood = –60.13

S. no.	Marker	Distance (cM)
1	*Rft2*	33.2
2	EAAG/$MCTC_{80}$	18.1
3	*Rft1*	18.1
4	EACC/$MCTT_{105}$	
	Total	69.3

NILs, which result from introgression and backcross breeding, have been used efficiently to identify DNA markers linked to the target gene. Their efficiency emanates from the likely presence of residual DNA from the donor parent, scattered randomly in the genome or possibly flanking the introgressed allele. In the present work, if the residual donor DNA fragments located around the *Rft1* gene are considered to be defined by two markers, EACC/$MCTT_{105}$ and EAAG/$MCTC_{80}$, their size can then be estimated to be 36.2 cM. In *B. napus*, with a predicted genomic length of 2,826 cM (Landry et al. 1991), the genomic segment carrying the *Rft1* gene may constitute 1.28% of the donor genome. RFLP/RAPD markers linked to the *Rf* gene(s) for *pol* (Jean et al. 1997) and *ogu* (Delourme et al. 1994) have been reported previously. Jean et al. (1997) also showed that the *pol* restorer gene(s) *Rfp1* and *Rfp2*, previously designated as non-allelic based on elaborate genetic studies, were allelic because one RFLP marker, cRF 16, showed identical polymorphic fragments in both the *Rfp1* and *Rfp2* restorer lines indicating perfect linkage with the marker.

The restorer gene for *tour* CMS, introgressed from the cytoplasm donor species, was previously mapped using the BC_3 population segregating for male-fertility restora-

tion in CMS *tour* 25–143 cytoplasm that had been transferred from *Brassica tournefortii* to *B. napus* (Trendelkamp et al. 1999). Using bulked-segregant analysis, 11 AFLP markers linked to the restorer gene were identified. Together these formed a linkage group spanning 18.3 cM in length. This report differs from our study for two reasons; the *Rf* gene in the study of Trendelkamp et al. (1999) was introgressed from the cytoplasm donor species, *B. tournefortii*. Secondly, the inferences drawn were based on single gene control for fertility restoration, inspite of the occurrence of three distinct classes (male sterile, male fertile and plants with intermediate plant type having less pollen production) in the segregating population. Our earlier studies (Pahwa et al. 2002), using the *Rf* gene(s) source from native germplasm, had clearly shown that the major gene *Rft1* conferred almost complete fertility. However, flower and anther size was somewhat smaller than that of the euplasmic controls. To achieve complete restoration (as good as that of the euplasmic parent), the presence of the second gene *Rft2* was also required. Both genes (*Rft1*-, *Rft2*-) in restored plants resulted in normal or even bigger-sized anthers and flowers. One could easily identify (*Rft1*-, *Rft2*-) segregants by the supra-normal size of anthers and petals, in comparison to the narrow petals and slightly smaller anthers of (*Rft1*-, *rft2 rft2*) plants.

Lack of markers displaying perfect co-segregation with *Rf* genes in the present study, and their distribution across a wider genomic region, indicates normal meiotic recombination in the vicinity of the gene. Our results are, therefore, in contrast to those of the *ogura*-INRA system where recombination around the restorer genes was suppressed (Delourme et al. 1998). Linkage drag may, therefore, prelude the use of these *Rf* genes in hybrid breeding programmes. Molecular markers such as EACC/$MCTT_{105}$ and EAAG/$MCTC_{80}$ linked to the *tour-Rf* gene can be useful for screening a large germplasm breeding-population for developing maintainer and restorer gene pools. The development of the SCAR marker from these linked AFLP markers would make the task of identifying the potential plants carrying the desired *Rf* gene much easier. These markers can also be used to facilitate the transfer of *Rf* genes to other commercially significant species, namely *B. juncea*. A breeding programme based on marker-assisted selection is likely to be more efficient not only in recovering the recurrent genome but can also help in reducing the number of generations required to transfer the restorer genes to diverse genotypes.

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