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M. Jean · G. G. Brown · B. S. Landry

Genetic mapping of nuclear fertility restorer genes for the 'Polima' cytoplasmic male sterility in canola (*Brassica napus* L.) using DNA markers

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Abstract Co-segregation of male fertility with DNA markers selected by targeted mapping approaches as being potentially linked to the *Rfp1* restorer gene for the *pol* cytoplasmic male sterility (CMS) was analyzed using two canola (*Brassica napus* L.) backcross populations. Eleven DNA markers (10 RFLP markers and one RAPD marker) directly linked to the *Rfp1* locus were identified. The linkage group containing the *Rfp1* locus was found to correspond to *B. napus* linkage group 18 of Landry et al. (1991). A similar pattern of co-segregation between DNA markers and male fertility was observed in a backcross population segregating for the *pol* restorer gene *Rfp2* from line 'UM2383'; one RFLP marker, cRF1b, showed perfect linkage with both *Rfp1* and *Rfp2* and detected identical polymorphic fragments in both the *Rfp1* and *Rfp2* restorer lines. Our findings indicate that restoration of *pol* CMS is controlled by a single nuclear genetic locus on linkage group 18 and that *Rfp1* and *Rfp2* are likely allelic.

Key words *Rfp1* restorer gene · *Rfp2* restorer gene · *pol* CMS · RFLP mapping

Introduction

Cytoplasmic male sterility (CMS) is a common trait in angiosperms (Laser and Lersten 1972). This maternally inherited inability to produce pollen results, in general, from mitochondrial DNA rearrangements and is often associated with the presence of unusual mitochondrial open reading frames (Hanson 1991; Williams and Levings 1992; Bonen and Brown 1993). CMS is often considered to be a nuclear-mitochondrial incompatibility since plants with specific nuclear genes, termed restorers of fertility, are male-fertile when carrying a CMS-inducing cytoplasm (Breiman and Galun 1990; Hanson 1991; Braun et al. 1992). In many cases, restorer genes have been found to alter the expression of mitochondrial gene regions implicated in specifying CMS, but the mechanism through which this is achieved appears to vary among different CMS systems.

Major increases in yield have been observed in experimental F_1 hybrids of *Brassica napus* L. (Sernyk and Stefansson 1983; Grant and Beversdof 1985), and the implementation of this heterosis has become a major objective of canola breeders. In species capable of self-pollination, like *B. napus*, the capacity to produce hybrid seeds on a commercial scale depends on the availability of a simple and efficient method to force outcrossing of the parental inbred lines. CMS can be used to genetically emasculate hermaphrodite flowers for commercial hybrid seed production. A dominant restorer gene, however, must be present in the male parent of the cross, since the hybrid must be at least partially male fertile to give adequate yield.

There are three main sources of CMS in *B. napus*: the *nap*, *ogu* and *pol* cytoplasms. The *pol* cytoplasm from the cultivar 'Polima' (Fu 1981) confers complete male-sterility to many *B. napus* cultivars and partial sterility to others (Fan et al. 1986). Two non-allelic dominant genes, each independently capable of restoring this CMS, have been reported: the *Rfp1* gene from the cultivar 'Italy' and the *Rfp2* gene from the breeding line 'UM2353' (Fang and McVetty 1989). The *ogu* cytoplasm from radish (Ogura 1968) confers complete male-sterility to all known *B. napus* cultivars (Fan et al. 1986), and a radish restorer gene for this CMS (*Rfo*) has been introgressed in *B. napus* (Heyn 1976; Pelletier et al.

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M. Jean · G. G. Brown
McGill University, Biology Department, 1205 ave. Doctor Penfield, Montreal, Quebec, Canada, H3A 1B1

B. S. Landry (✉)
DNA LandMarks Inc., 430 boul. Gouin, St-Jean-sur-Richelieu, Quebec, Canada, J3B 3E6

1987; Delourme et al. 1991; Sundberg and Glimelius 1991). Finally, the common *B. napus* (*nap*) cytoplasm (Shiga and Baba 1971, 1973; Thompson 1972) can induce male-sterility in a few *B. napus* cultivars. These cultivars lack a restorer for the *nap* CMS that is present most other varieties (Fan et al. 1986).

The *pol* CMS system is often considered by canola breeders as one of the most promising for hybrid seed production. Indeed, a large number of maintainer lines are available, and the generation of *pol* restorer lines is expected to be a relatively straightforward process involving the introgression of a single dominant trait. DNA markers tightly linked to a restorer gene would be valuable tools for marker-assisted breeding as well as important landmarks in a map-based cloning strategy aimed at isolating a restorer gene. Genetic markers linked to restorer genes have been identified for various maize CMS systems (Sisco 1991; Kamps and Chase 1992; Schnable and Wise 1994; Wise and Schnable 1994), for the *Rfo* restorer of rapeseed *ogu* CMS (Delourme and Eber 1992; Delourme et al. 1994), and for a CMS restorer in bean (He et al. 1995). The *Rf2* gene, one of two genes required for restoration of maize *cms*-T, has been isolated through a transposon tagging approach (Cui et al. 1996).

The study reported here is focused on restorer genes for the *pol* CMS. We report the mapping of the *Rfp1* locus in two BC_1 populations and the identification of 11 DNA markers linked to it, one of which perfectly co-segregates with male-fertility restoration. We also present evidence indicating that the *pol* restorer genes *Rfp1* and *Rfp2* are likely to be allelic and may be the same gene.

Materials and methods

Plant materials and fertility scoring

Brassica napus lines and cultivars used in the present study have been described previously (Singh and Brown 1991; L'Homme and Brown 1993). Cytoplasms and genotypes are designated in italics and between parentheses following the cultivar name. The *pol* restorer sources used in the present study are the BC_6 breeding line 'Westar-Rf' which carries the *Rfp1* restorer gene from the cultivar 'Italy' (Fang and McVetty 1989) in a 'Westar' nuclear background, and the breeding line 'UM2353', which contains the *Rfp2* restorer gene (Fang and McVetty 1989).

Two BC_1 populations segregating at the *Rfp1* locus were generated and used as mapping populations (Table 1). The KW population consisted of 111 BC_1 plants derived from an intervarietal cross between single plants from *pol* CMS 'Karat' (*rfprfp*; *pol*) and *pol* restorer 'Westar-Rf' (*Rfp1Rfp1*; *pol*) lines. The WW population consisted of 64 BC_1 plants derived from an intravarietal cross between single plants from 'Westar' (*rfprfp*; *nap*) and a 'Westar-Rf' (*Rfp1Rfp1*; *pol*) line. Some mapping studies were also performed with a BC_2 population segregating at the *Rfp2* locus (kindly provided by X. Q. Li, McGill University). This KU population (Table 1) was composed of 20 BC_2 plants derived from an intervarietal cross between single plants from *pol* CMS 'Karat' (*rfprfp*; *pol*) and *pol* restorer 'UM2353' (*Rfp2Rfp2*; *pol*) lines.

Plants were grown to maturity in growth chambers or greenhouses under standard conditions (16-h photoperiod, 22°/16°C day/night temperatures). The fertility was assessed by the careful observation of five flowers per plant at least two times during the flowering period. The overall morphology of the flowers was noted as well as the number of anthers producing pollen. The flower morphology was used as the main criteria to determine the fertility of plants segregating for the restoration of the *pol* CMS. Flowers from a male-fertility restored plant look identical to those of a fertile maintainer plant; flowers from a *pol* CMS plant have shrunken petals, and the style of the pistil is longer and often bent. CMS anthers also have shorter filaments (Fan and Stefansson 1986), a poorly developed or absent pollen sac, and no pollen or a reduced amount of pollen is released. The morphological contrast between CMS and normal flowers was sufficient to allow plants carrying a restorer allele to be unambiguously distinguished from those with only maintainer alleles, even though some CMS plants shed a small amount of pollen. Plant genotypes at the *Rfp* locus were therefore assigned as heterozygous *Rfp*/*rfp* for plants with normal flowers, or homozygous recessive *rfp*/*rfp* for those with male-sterile or partially male-sterile flowers.

DNA extraction, purification and analyses

Plant DNA extractions were as in Landry et al. (1991). Random amplified polymorphic DNA (RAPD) analyses were performed as described in Deragon and Landry (1992) with random 10-mer primers (Operon Technologies, Alameda, Calif.; kits A–G). Restriction fragment length polymorphism (RFLP) analyses were performed as described in Landry et al. (1991), with the following modifications. DNA samples (5 µg were digested with each of four restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, and *Hin*dIII, electrophoresed, and transferred onto Hybond™-N + membranes (Amersham Int plc, UK). Southern blots were made by alkali transfer as recommended by the manufacturer. Dextran sulfate (10%) was added to the hybridization solution and membranes were briefly rinsed at room temperature in 2 × SSC (1X SSC = 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0) before the regular low- and

Table 1 Genetic analysis of fertility restoration

Population name	Female parent	Male parent	Number of plants		Chi² (1 : 1 ratio)
			Male-fertile	Male-sterile	
KW	Karat (*rfprfp*; *pol*)	Westar-Rf (*Rfp1Rfp1*; *pol*)	56	55	0.000
WW	Westar-Rf (*Rfp1Rfp1*; *pol*)	Westar (*rfprfp*; *nap*)	30	34	0.141
KU	Karat (*rfprfp*; *pol*)	UM2353 (*Rfp2Rfp2*; *pol*)	13	7	1.250

Table 2 DNA markers that were assessed for linkage with the *Rfp1* locus

Marker name	Marker type	Mode of selection[a]	Marker name	Marker type	Mode of selection[a]
1ND1	RFLP	BSA	5NA2c	RFLP	NILs
1ND1c	RFLP	NILs	5NB8b	RFLP	BSA
1ND4	RFLP	BSA	5NB9b	RFLP	NILs
1NF2c	RFLP	BSA	5NB12	RFLP	NILs
1NF7a	RFLP	BSA	5NE2a	RFLP	BSA
1NF7b	RFLP	BSA	5NE12b	RFLP	BSA
1NH3	RFLP	RM	5NF2	RFLP	NILs
2NB10a	RFLP	BSA	5NF3b	RFLP	BSA
2NB12a	RFLP	RM	5NF4	RFLP	BSA
2NC8b	RFLP	BSA	6NC2	RFLP	BSA, NILs
2ND1	RFLP	BSA	6NC3a	RFLP	BSA
2ND7b	RFLP	BSA	6NC12	RFLP	NILs
2NE10	RFLP	RM	6ND9	RFLP	NILs
3NB2	RFLP	BSA	7NA8	RFLP	BSA
3NB4	RFLP	BSA	0NF14b	RFLP[b]	BSA
3NC3	RFLP	BSA	OPA02	RAPD	BSA
3ND4b	RFLP	RM	OPA13a	RAPD	BSA
3NE11b	RFLP	BSA	OPA13b	RAPD	BSA
3NF2	RFLP	BSA, NILs	OPD05	RAPD	NILs
3NG3	RFLP	BSA	OPD08a	RAPD	BSA
3NH12	RFLP	BSA	OPD08b	RAPD	NILs
4NB3	RFLP	BSA	OPF14	RAPD	BSA
4NB6b	RFLP	BSA	OPF15	RAPD	NILs
4NB8	RFLP	BSA	OPG03a	RAPD	BSA
4NC1b	RFLP	BSA	OPG05	RAPD	BSA
4NC5a	RFLP	BSA	OPG13	RAPD	NILs
4ND1	RFLP	NILs	cRF1b	RFLP	BSA, NILs
4ND7b	RFLP	BSA, NILs	pCA15	RFLP	BSA
4NF4d	RFLP	NILs	pCOT39 rDNA	RFLP RFLP	NILs

[a] The markers had been selected as potentially linked to the *Rfp1* locus either by a bulked segregant analysis (BSA) performed with the KW population or by comparing a pair of 'Westar' NILs (Jean 1995). A few were selected during the random mapping (RM) of markers from the linkage map of the *B. napus* genome (Landry et al. 1991) in the KW population

[b] The RFLP locus 0NF14b was revealed when the amplified fragment known as the RAPD marker OPF14 was used as a probe (see Discussion)

high-stringency washes. The probes used in the present study (Table 2) had been either selected by targeted mapping as being potentially linked to the *Rfp1* locus (Jean 1995) or identified by a random survey of probes from the linkage map of the *B. napus* genome of Landry et al. (1991) (unpublished data). They were mostly anonymous cDNAs from a library of *B. napus* embryo cDNA clones. Most of the probes detected polymorphisms with more than one of four restriction enzymes tested. In such cases, that enzyme for which the clearest fragment differences were observed was used to follow the segregation of the marker in crosses.

A maize 17S cytoplasmic rRNA gene fragment (kindly provided by X. Q. Li, McGill University) was also used as probe. The cDNA inserts were amplified by the polymerase chain reaction (PCR), using vector-specific primers (B. S. Landry, unpublished) or excised by a *Pst*I digestion from plasmids purified using Magic Miniprep™ or Wizard Miniprep™ (Promega Corporation, Wis.). The cDNA inserts were further purified by electrophoresis and recovered from agarose with Geneclean™ (Bio 101, La Jolla, Calif.) or Sephaglas Band Prep™ (Pharmacia Biotech, N.J.). They were labeled with α-[^{32}P]dCTP using Multiprime™ (Amersham Int plc, UK) or T7 Quick Prime™ (Pharmacia Biotech, N.J.) and purified from unincorporated nucleotides by passage through Bio-Gel P-60 (Bio-Rad Laboratories, Calif.) in a PolyPrep column (Bio-Rad Laboratories, Calif.) or a Spin-X™ column (Costar, Mass.).

Genetic mapping

Co-segregation analyses were performed with MAPMAKER v. 2.01 (Lander et al. 1987, supplied by S. Tingey, DuPont) on a Macintosh LC III computer. The statistical thresholds were set at a minimum LOD score of 4.0 and a maximum recombination fraction of 0.30, unless specified otherwise. Putative double-crossover events were identified and re-checked for scoring errors. The Kosambi mapping function was used to convert the recombination frequency in centiMorgans. Loci are named after the probe or primer that revealed them, with lowercase letters (a, b …) designating separated loci displayed with the same probe or primer. Loci common with a previously published map of *B. napus* genome (Landry et al. 1991) are labelled identically.

Results

Genetics of fertility restoration

The sterility maintenance was generally incomplete in the KW and WW populations. Some pollen production

was observed in many sterile plants, especially in the WW population. This was probably related to the 'Westar' nuclear background (Fan et al. 1986). Because of the distinctive petal, sepal, and pistil morphology of *pol* CMS flowers, however, the differences between CMS and restored plants were evident, and in all cases the phenotype could be scored without ambiguity.

In the KW population (Table 1), 56 male-fertile and 55 male-sterile plants were observed (1 : 1 ratio, $Chi^2 = 0.000$). In the WW population, there were 30 male-fertile and 34 male-sterile plants (1 : 1 ratio, $Chi^2 = 0.141$). The fertility restoration of the *pol* CMS was thus confirmed to be controlled by a single dominant gene in these two populations segregating for the *Rfp1* restorer gene. In the KU population segregating for the *Rfp2* restorer gene for the *pol* CMS (Table 1), 13 male-fertile and seven male-sterile plants were found (1 : 1 ratio, $Chi^2 = 1.250$). This was also consistent with the premise that a single dominant gene controls fertility restoration in this population.

Genetic mapping of the *Rfp1* locus

To identify markers linked to the *Rfp1* gene, Jean (1995) screened the nearly isogenic lines (NILs) 'Westar' and 'Westar-Rf' with 208 probes and 140 primers. Fifteen RFLP and four RAPD loci (Table 2) were selected as being potentially linked to the *Rfp1* locus (Fig. 1). All these selected markers segregated in the expected 1 : 1 Mendelian ratio in the WW population, which was derived from a cross between the two 'Westar' NIL plants. Co-segregation analysis of these DNA markers with fertility revealed that six of the RFLP loci (markers cRF1b, 3NF2, 4ND7b, 5NB12, 5NF2, and 6NC2) and none of the RAPD markers were linked to *Rfp1* (Fig. 3).

A total of 242 probes and 140 primers were tested on DNA from the parents of the KW population (Jean 1995). The 143 probes and 35 primers that revealed polymorphisms expected to segregate in this BC_1 population were screened by the bulked segregant analysis, and 32 RFLP and seven RAPD loci (Table 2) were selected as markers potentially linked to the *Rfp1* locus (Jean 1995). All these selected markers segregated in the expected 1 : 1 Mendelian ratio in the KW population (Fig. 2). Co-segregation analysis of these DNA markers with fertility revealed that eight of the 32 RFLP loci (markers cRF1b, 3NF2, 4NB6b, 4ND7b, 5NE12b, 5NF4, 6NC2, and 0NF14b) and one of the seven RAPD loci (marker OPF14) were linked to *Rfp1* (Fig. 3).

We have therefore identified 11 distinct DNA markers linked to the *Rfp1* gene: five were present only in the KW population, two only in the WW population, and four in both populations (Fig. 3). The linkage group containing the *Rfp1* locus for the WW population is composed of seven markers and extends over 28.3 cM: the nine markers linked to *Rfp1* in the KW population span 44.7 cM (Fig. 3). No recombination was found between the *Rfp1* locus and the RFLP marker cRF1b in either population (Figs. 1 and 2). With a total of 175 individuals tested, this indicated a recombination frequency-lower than 0.006 ($< 1/175$). The nearest markers flanking *Rfp*/cRF1b were 5NE12b and 4ND7b in the KW population, at 5.4 and 10.8 cM, respectively, and 5NF2 and 4ND7b in the WW population, at 9.5 and 6.3 cM, respectively.

In the KW and WW crosses, the linkage groups containing the *Rfp1* locus had four DNA markers in common (markers cRF1b, 3NF2, 4ND7b, and 6NC2; Fig. 3). In both mapping populations, the order of these markers was identical, and the estimates of the genetic

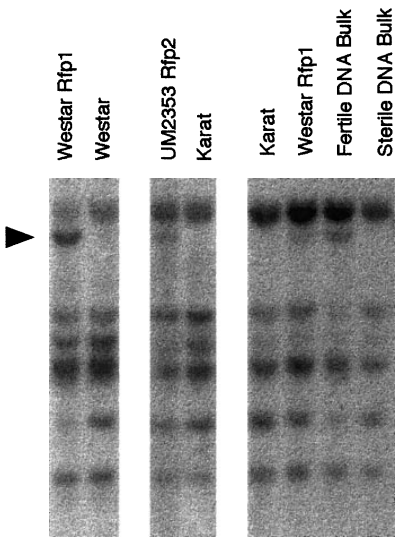

Fig. 1 RFLPs detected by the cRF1 probe on *Hin*dIII-digested DNA of *B. napus* parental lines. The *arrowhead* indicates the 4.3 kb *Hin*dIII fragment (cRF1b locus) perfectly linked to the *Rfp1* and *Rfp2* fertility restorer gene. Parental lines are indicated on *top*. The *first six lanes* are the parental lines of the three crosses. The *remaining two lanes* are two DNA bulks respectively made of DNA from fertile plants and sterile plants of the 'Karat' × 'Westar Rfp1' segregating population

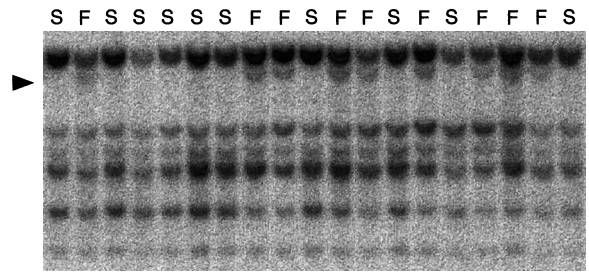

Fig. 2 Sample of the segregation of cRF1b in the backcross population derived from the cross 'Karat' × 'Westar Rfp1'. The *arrowhead* indicates the 4.3 kb *Hin*dIII fragment (cRF1b locus) perfectly linked to the *Rfp1* fertility restorer gene. The phenotype of each plant is indicated on top. *F* Fertile, *S* sterile

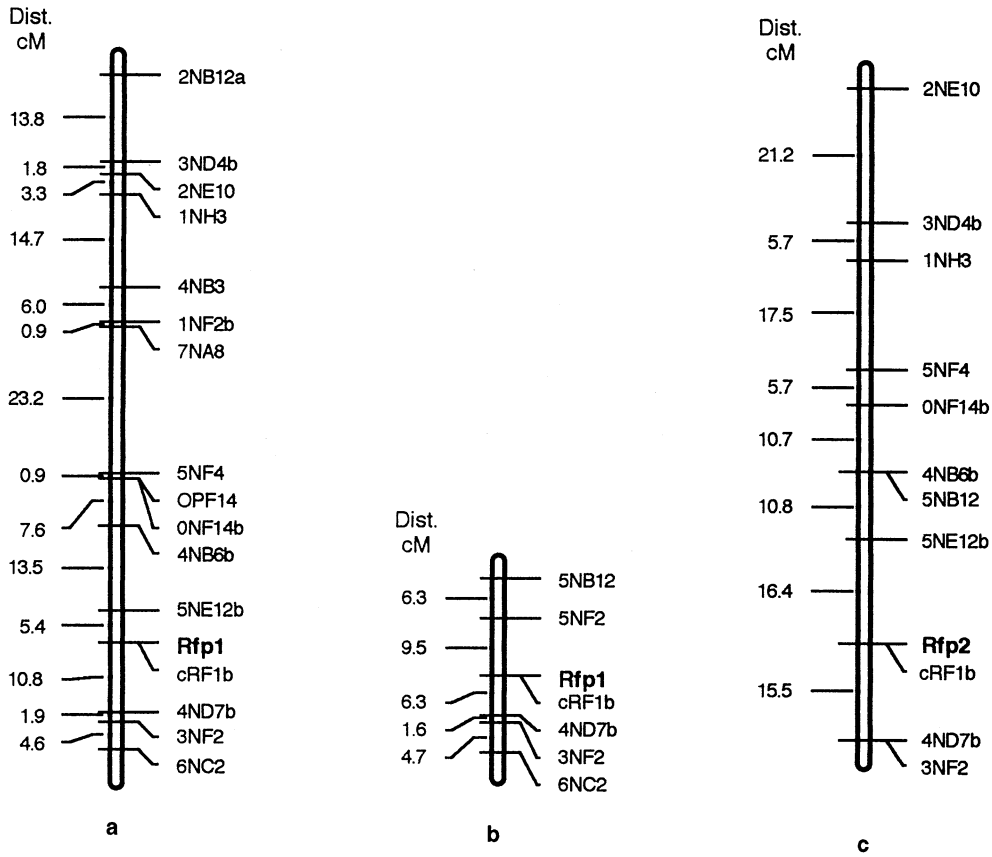

Fig. 3a–c Maps of the linkage group containing the restorer loci for the 'Polima' cytoplasmic male sterility of *Brassica napus* constructed from the backcross population derived from: **a** 'Karat' (*rfprfp;pol*) and 'Westar-Rf' (*Rfp1Rfp1; pol*) individuals, **b** 'Westar' (*rfprfp;nap*) and 'Westar-Rf' (*Rfp1Rfp1;pol*) individuals, **c** 'Karat' (*rfprfp; pol*) and 'UM2353' (*Rfp2Rfp2;pol*) indivduals. Distances (*left*) are in centi-Morgans (cM) (Kosambi mapping function). The names (*right*) represent RFLP markers, except OPF14, which is a RAPD marker. The *Rfp1* locus is indicated in *bold characters*

distances among were also similar. The relative order of the markers 4ND7b, 3NF2 and 6NC2 was confirmed by the observation of a single plant from the WW population that carried a deletion of the markers 3NF2 and 6NC2, but not of the marker 4ND7b (data not shown).

All the linked markers on one side of the *Rfp1* locus (markers 4NB6b, 5NB12, 5NE12b, 5NF2, 5NF4, OPF14, and its corresponding RFLP 0NF14b [see below]) were mapped only in one of the two populations (Fig. 3). The lack of common markers on this side of the *Rfp1* locus made it difficult to compare the two maps. However, the relationship between the marker cRF1b and the markers 4NB6b or 5NB12 suggested that the recombination frequency in this genomic area was probably similar in both the KW and WW populations. Indeed, the distance between the markers cRF1b and 5NB12 was about 15.8 cM in the WW population, which was similar to the distance of about 18.9 cM found in the KW population between the markers cRF1b and 4NB6b. If the relative order and distance of markers are conserved between the two maps, the markers 4NB6b and 5NB12 should therefore be quite near one another. In fact, no recombination was found between these markers in the KU population (see below). The relative order of RFLP markers in this region is therefore cRF1b-5NE12b-5NF2-(5NB12-4NB6b).

Transformation of the RAPD OPF14 marker into RFLP markers

RAPDs often originate from genomic regions containing repetitive DNA (Williams et al. 1990; Paran and Michelmore 1993) which cannot be sampled by conventional RFLP analysis using cDNA or low-copy genomic DNA probes. We were, however, able to easily transform the RAPD marker OPF14 into a RFLP marker linked to *Rfp1*. When the RAPD fragment (an intense band of about 1.5 kb) was extracted from the gel and used as a probe on *Hin*dIII-digested DNA samples from the KW population, it revealed two loci (designated as 0NF14a and 0NF14b): One of them (0NF14b) perfectly co-segregated with the RAPD marker OPF14 (Fig. 3), while the other was located on a different linkage group (data not shown). Since the RAPD marker almost co-migrated with a fainter, monomorphic fragment, this second locus could represent either a duplication of the OPF14 locus or hybridization to the other contaminating fragment.

Localization of the *Rfp1* locus on the linkage map of the *Brassica napus* genome

Co-segregation analysis performed on the KW population revealed that three of the RFLP markers selected

by bulked segregant analysis (1NF2b, 4NB3, and 7NA8) were not obviously linked to *Rfp1* but were, nevertheless, located on the same linkage group as the gene, at 50.6–57.6 cM from it (Fig. 3). These markers may represent a region of random homozygosity from the bulk, since the number of meiosis events involved in the generation of a BC_1 or F_2 population was too small to allow for the complete randomization of all loci in every chromosomal segment. Giovannoni et al. (1991) also reported the detection of a marker located 45 cM from the targeted locus using bulked segregant analysis. Further co-segregation analysis of this population with randomly selected markers from the linkage map of the *B. napus* genome (Landry et al. 1991) revealed four additional RFLP loci (markers 1NH3, 2NB12a, 2NE10, and 3ND4b) that map to the same linkage group as *Rfp1* (Fig. 3). Direct linkage was found between three of these markers (1NH3, 2NE10, and 3ND4b) and four markers linked to *Rfp1* (4NB6b, 5NF4, OPF14, and 0NF14b). The markers $1NH_3$, 2NB12a, 2NE10, and 3ND4b formed linkage group 18 in a previously published RFLP map of the *B. napus* genome (Landry et al. 1991). Since they have now been localized on the same linkage group as the *Rfp1* locus, this restorer gene is therefore also assigned to linkage group 18. The linkage group on which *Rfp1* is localized in the KW population therefore contains a total of 17 DNA markers and covers a total length of 109.5 cM (Fig. 3).

Relationship between restorer genes and nucleolar organizer regions (NOR)

Ribosomal RNA genes (rRNA genes or rDNA) are generally present as hundreds or thousands of tandemly repeated units of the 18S, 5.8S, and 26S genes and intergenic spacers and are localized at one or more loci in plant genomes. When expressed, the rRNA genes produce a nucleolus and secondary constrictions, termed nucleolar organizer regions (NOR), that can be visualized on some metaphase chromosomes. NOR have been used as visible markers for physical mapping studies, and some restorer genes have been located near a NOR locus (Tsunewaki 1974; Mukay and Tsunewaki 1979; Burns and Gerstel 1981; Tsunewaki 1982; Snape et al. 1985).

When a fragment of maize rDNA was used as a RFLP probe on *Eco*RV-digested DNA samples from the parents of the KW population, a polymorphism was visualized despite the overall pattern typical of repetitive DNA (data not shown). This RFLP marker segregated in a normal Mendelian ratio in the KW population but was unlinked to the *Rfp1* locus. Since there are six major rDNA loci in *B. napus* (Maluszynska and Heslop-Harrison 1993), the possibility that the *Rfp1* restorer gene of *B. napus* might also be located near a NOR locus cannot, however, be excluded.

Genetic mapping of the *Rfp2* locus

Thirteen markers (markers cRF1b, 1NH3, 2NB12a, 2NE10, 3ND4b, 3NF2, 4NB6b, 4ND7b, 5NB12, 5NE12b, 5NF4, 6NC2 and 0NF14b) from the linkage group containing the *Rfp1* locus were tested on the KU population segregating for the *Rfp2* restorer gene for the *pol* CMS (Fig. 1). Two of them (markers 2NB12a and 6NC2) were monomorphic and could therefore not be mapped. The segregation of the others did not differ significantly from a 1 : 1 Mendelian ratio and they were used for mapping studies.

The use of a small mapping population such as the KU population (20 individuals) affected the calculation of the LOD score used to assess the likelihood of linkage between markers, particularly for the linked markers that were relatively far away from one another. The LOD score is defined as the $\log_{10}$ of the ratio of the likelihood (probability) that the loci are linked, divided by the likelihood that they are not linked. In a small population, the probability that the observed data could have arisen by chance alone (unlinked loci) is necessarily high. The LOD score thus becomes very small even for markers that are truly linked.

When co-segregation analysis was performed with the KU population using our standard threshold (minimum LOD score of 4.0), only pairs of markers linked without recombination (markers 3NF2 and 4ND7b; marker cRF1b and the *Rfp2* locus) were scored as associated. Linkage between most of the markers found to be linked in the KW and WW populations was revealed when the minimum LOD score was lowered to 1.9. The pair of markers 1NH3 and 3ND4b, and the marker 2NE1O became included at LOD scores equal to 1.4 and 1.25, respectively. The length of these 'weaker' links was, however, still smaller than 30 recombination units (recombination fraction of 0.30), the threshold used to assume direct linkage between markers. Variations in the order or distance between these markers in the KU population can probably be attributed to inaccuracy in the estimation of the actual recombination frequencies resulting from the small size of this mapping population, and the organization of linkage group 18, as depicted in Fig. 3, was assumed to be correct. Linkage group 18 from the KU population thus contains 11 DNA markers and covers 103.5 cM.

The segregation of the 11 DNA markers from the linkage group known to contain the *Rfp1* locus was compared to that of the fertility restoration for the *pol* CMS induced by the *Rfp2* restorer gene from the cultivar 'UM2353' in the KU population. The *Rfp2* restorer locus was found to map to linkage group 18 (Fig. 3). No recombination was found between *Rfp2* and the marker cRF1b, the RFLP marker that perfectly co-segregated with *Rfp1* in the KW and WW populations. The nearest flanking markers for the *Rfp2* locus were the pair of markers 3NF2 and 4ND7b at 15.5 cM and the marker 5NE12b at 16.4 cM. The *Rfp1* and *Rfp2*

restorer genes of the *pol* CMS are therefore at least very tightly linked and likely reside at the same locus. The set of restriction fragments detected by the probe cRF1 in Westar-Rf and UM2353 were identical (Fig. 1), suggesting that the genomic regions surrounding *Rfp1* and *Rfp2* are similar at the molecular level.

Discussion

Fang and McVetty (1989) have reported data indicating that *Rfp1* and *Rfp2* are non-allelic, unlinked restorer genes. They first observed a 3:1 fertile:sterile segregation ratio ($Chi^2 = 1.33$) in a F_2-like population (100 plants) derived from a cross between two F_1 individuals, each heterozygous for one of the restorer genes. This was consistent with the hypothesis that the *Rfp1* and *Rfp2* genes are allelic as well as with the hypothesis postulating that they are non-allelic. However, a 15:1 fertile:sterile segregation ratio ($Chi^2 = 0.001$) was observed in five of 13 F_3 families (total: 694 plants) derived from the restored F_2 individuals, while the eight other F_3 families (total: 617 plants) displayed a 3:1 fertile:sterile segregation ratio ($Chi^2 = 0.004$). Such a result can only be consistent only with the hypothesis that the *Rfp1* and *Rfp2* genes are non-allelic. Analysis of the KU population, however, clearly showed that in our case the *pol* restorer *Rfp2* from the cultivar 'UM2353' mapped to linkage group 18 (Fig. 3). Furthermore, no recombination was found between *Rfp2* and marker cRF1b, a RFLP marker tightly linked to the *Rfp1* locus (Figs. 1 and 3).

Our work therefore provides strong evidence that the *Rfp1* and *Rfp2* genes map to the same locus or at least to very tightly linked loci. The finding that identical polymorphic fragments are associated with *Rfp1* and *Rfp2* indicates that the chromosomal regions surrounding the two genes are similar and suggests that they may, in fact, be the same gene. The basis for the discrepancy between our findings and those of Fang and McVetty (1989) is not clear. It is well-known that *pol* CMS can exhibit poor maintenance of sterility in certain genetic backgrounds and under high temperatures (Fan and Stefansson 1986; Fan et al. 1986), and conclusions based solely on the study of segregation ratios for phenotypes scored under field conditions might be misleading. Bias in the scoring might obscure the true segregation ratio and make the genetics of restoration appear more complex than it truly is. In support of this possibility, we found no evidence for the independent segregation of *Rfp1* and *Rfp2* in plant populations grown under controlled environmental conditions (X-Q Li, unpublished observations). Thus, unless data are obtained that clearly indicate otherwise, we suggest that *Rfp1* and *Rfp2* be considered to reside at the same nuclear locus, which we designate simply as *Rfp*.

The availability of a set of DNA markers linked to *Rfp* should prove useful for the development of new restorer lines for use in hybrid seed production. These markers should allow for rapid indirect selection for the restorer gene and permit an assessment of the amount of flanking DNA that accompanies the restorer during the introgression procedures used to develop such lines. The marker cRF1, which maps to less than one cM from the gene, should prove particularly useful in this regard.

The elucidation of the molecular identity of restorer genes is clearly beneficial to our understanding of CMS. The single restorer gene characterized at the molecular level, the *Rf2* restorer of *cms*-T maize, appears to specify a mitochondrial aldehyde dehydrogenase (Cui et al. 1996). This restorer is exceptional, however, in that it does not seem to affect mitochondrial gene expression (Dewey et al. 1987). *Rfp* has previously been shown to condition modifications of transcripts of the *pol* CMS-associated *orf224/atp6* mitochondrial gene region (Singh and Brown 1991; Singh et al. 1996) and thus, like most other restorers, appears to act by influencing the expression of mtDNA regions that may be involved in specifying CMS (Hanson 1991; Williams and Levings 1992; Bonen and Brown 1993). The isolation and characterization of *Rfp* therefore represent important fundamental goals. The availability of the isolated *Rfp* gene might have practical significance as well, in that it should allow the production of new restorer lines through plant transformation approaches. cRF1 is very tightly linked to *Rfp* and may prove useful for isolating the gene through a map-based cloning strategy. Brassica genomes may be particularly amenable to such approaches since the relationship between molecular and genetic distance appears to be relatively small, comparable to that of *Arabidopsis thaliana* (Lagercrantz et al. 1996).

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