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Identification of RAPD markers linked to a fertility restorer gene for the *Ogura* radish cytoplasmic male sterility of rapeseed (*Brassica napus* L.)

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Abstract Bulked segregant analysis was employed to identify random amplified polymorphic DNA (RAPD) markers linked to the restorer gene (*Rfo*) used in the *Ogura* radish cytoplasmic male sterility of rapeseed. A total of 138 arbitrary 10-mer oligonucleotide primers were screened on the DNA of three pairs of bulks, each bulk corresponding to homozygous restored and male sterile plants of three segregating populations. Six primers produced repeatable polymorphisms between paired bulks. DNA from individual plants of each bulk was then used as a template for amplification with these six primers. DNA polymorphisms generated by four of these primers were found to be completely linked to the restorer gene with the polymorphic DNA fragments being associated either with the fertility restorer allele or with the sterility maintainer allele. Pairwise cross-hybridization demonstrated that the four polymorphic DNA fragments did not share any homology. Southern hybridization of labelled RAPD fragments on digested genomic DNA from the same three pairs of bulks revealed fragments specific to either the male sterile bulks or to the restored bulks and a few fragments common to all bulks, indicating that the amplified sequences are low copy. The four RAPD fragments that were completely linked to the restorer locus have been cloned and sequenced to develop sequence characterized amplified regions (SCARs). This will facilitate the construction of restorer lines used in breeding programs and is the first step towards map-based cloning of the fertility restorer allele.

Key words *Brassica napus* · *Raphanus sativus* *Ogura* cytoplasmic male-sterility restorer gene Bulked segregant analysis RAPD markers

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Introduction

The cytoplasmic male sterility (cms) originally found in radish (*Raphanus sativus* L.) by Ogura (1968) has been transferred to *Brassica oleracea* and *Brassica napus* by interspecific crosses (Bannerot et al. 1974). Male sterile *B. napus* cybrids were then produced through protoplast fusion (Pelletier et al. 1983) to generate male sterile lines without any defects (Pelletier et al. 1987). Fertility restorer alleles were introduced from radish into rapeseed (Heyn 1976) through intergeneric crosses between a CMS line of rapeseed and a *Raphanobrassica* (*R. sativus* × *B. napus* amphidiploid, and fully restored plants with one dominant restorer allele (*Rfo*) were selected on the best cybrid cytoplasms (Pelletier et al. 1987). However, the introduction of the restorer allele was accompanied by a large decrease in seed set (Pellan-Delourme and Renard 1988). It was assumed that restored plants had retained too much radish genetic information around the restorer allele or elsewhere in the genome. During the last few years, improvement in restorer material has been achieved and restorer lines with a good female fertility have been obtained (Delourme et al. 1991). This success was possibly due to the elimination of more radish genetic information. However, isozyme studies performed on the improved restorer lines revealed the presence of a radish *Pgi-2* allele tightly linked (0.25 cM) to the radish restorer allele (Delourme and Eber 1992) Therefore, some radish DNA still remains around the restorer gene, and this may explain why the transmission of the restorer gene through the pollen is still a little lower than expected in some progeny (unpublished data). In order to estimate the length of the *Pgi-2-Rfo* segment, more genetic markers in this area are needed. Furthermore, saturation of the radish segment with additional markers will permit the construction of the physical map of this chro-

mosomal area and ultimately lead to the cloning of the restorer allele.

Recently, random amplified polymorphic DNA (RAPD) markers have been developed (Wiliams et al. 1990; Welsh and McClelland 1990). RAPDs are generated by the amplification of genomic DNA using a single primer of arbitrary nucleotide sequence to drive the amplification reaction. Several research groups are now using RAPD to construct genetic maps. The most useful application of RAPD markers is, however, to quickly generate markers within a genomic region of interest using near-isogenic lines (NILs) (Klein-Lankhorst et al. 1991; Martin et al. 1991: Paran et al. 1991; Penner et al. 1993). We call this approach "targeted genetic mapping". However, several generations of backcrosses are required to create NILs and several regions of the donor genome can be co-introgressed into the NIL. An alternative method called bulked segregant analysis has been proposed by Michelmore et al. (1991). It aims at replacing the NILs by two bulked DNA samples collected from individuals identical for alleles at a specific locus in a single population, each bulk being homozygous for one or the other allele of the gene of interest. The advantage of this technology is that markers are targeted to a smaller region within the genome and the likelihood of identifying false positive markers is small (Michelmore et al. 1991). Bulked segregant analysis has been successfully applied by Pineda et al. (1993) and Barua et al. (1993) to detect markers tightly linked to a gene conferring resistance to a potato cyst nematode and to *Rhynchosporium* in barley. Pooling strategy has also been used to target RAPD markers to a chromosome (Reiter et al. 1992) or to specific regions of chromosome (Giovannoni et al. 1991) using existing mapping populations.

Here we report on the identification of RAPD markers lying within the *Pgi-2-Rfo* radish chromosome segment using the bulked segregant analysis approach.

Materials and methods

Plant materials

All plants used in this study were selected on cybrid 58 cytoplasm, one of the *Ogu* cybrids produced by Pelletier et al. (1983). The detailed pedigree of the restored *B. napus* lines carrying the radish fertility restorer gene has been previously described (Pellan-Delourme and Renard 1988). Two F_2 populations (R63F2 and R72F2) and a segregating doubled haploid population produced by in vitro microspore culture (RB3HD) of a heterozygous plant at the restorer locus were used. These progenies were screened for the presence of the restorer gene using its tight linkage with the *Pgi-2* isozyme marker (0.25 cM) as previously described (Delourme and Eber 1992). Although the RB3HD population had lost the *Pgi-2* allele of radish, phosphoglucose isomerase (PGI) could, nevertheless, be used to screen homozygous restored and male sterile plants (Delourme and Eber 1992). Consequently, homozygous restored plants and male sterile plants could be identified and the plants which would constitute the bulks could be chosen before flowering. Their male fertility/sterility was then checked at the flowering stage. DNA from each individual was extracted using the method described by Dellaporta et al. (1983) with an additional phenol-chloroform extraction. DNA concentrations were determined by fluorescence in the presence of bisbenzimide (Hoechst dye 33258, Kodak) using a fluorescence spectrophotometer.

RAPD analysis

For each sample, DNA concentration was adjusted to 12.5 ng/µl with ddH20. DNA aliquots (20 µl) of homozygous fertile individuals and homozygous sterile individuals were combined in two separate pools for bulked segregant analysis. Between 13 and 19 individuals were used in each bulk and six DNA bulks were constructed each corresponding to the homozygous fertile and sterile plants of the three segregating populations (R63F2, R72F2 and RB3HD). RAPD markers were tested for their ability to detect polymorphisms using DNA from the bulks as templates and 138 arbitrary 10-bp-long oligonucleotides as primers (Operon Technologies, kits A to G). Solutions for polymerase chain reactions were as described in Deragon and Landry (1992). Approximately 25 ng DNA was used as template in a final volume of 25 µl. The DNA amplifications were performed in a Hybaid™ thermal reactor programmed as follows: 30 s at 94 °C (ramping 1.0) and 10 min at 42 °C (ramping 2.0) for one cycle; 1 s at 50 °C (ramping 5.0), 30 s at 72 °C (ramping 1.0), 56 s at 94 °C (ramping 1.0) and 30 s at 42 °C (ramping 2.0) for 45 cycles; 5 min at 72°C (ramping 1.0) for one cycle. Amplification products were resolved by electrophoresis at 5 V/cm for 2 h in a 1.4% agarose gel. Primers detecting polymorphisms between the paired bulks were then tested on all individuals constituting the bulks.

Southern blot analysis

The same DNA bulks as those used for RAPD analysis were constructed. Aliquots (10 µg DNA) of each individual were bulked together. DNA samples of the six bulks were digested with the following restriction endonucleases: *Bam*HI, *Eco*RI and *Eco*RV. Digested DNAs (5 µg/lane) were electrophoresed in 0.8% TAE agarose gels and transferred onto Hybond N^+ membranes (Amersham) by capillary action (Southern 1975). Amplification products whose polymorphisms were confirmed on the individuals of the bulks were isolated from the agarose with the Sephaglas Band Prep kit (Pharmacia) and labelled with ^{32}P by random priming with the T7 Quick Prime kit (Pharmacia). Prehybridization, hybridization, washing, exposure and stripping of the membranes were as described in Landry et al. (1991). Polymorphic amplification products were cloned in the PCR II vector using the TA cloning system (Invitrogen Corp) by the direct ligation of PCR products into the vector. Cloned inserts were then isolated by *Eco*RI digestion for further analysis. They were radiolabelled and used to probe Southern blots of digested genomic DNA and PCR products amplified with the corresponding primers using as template DNA from 8 restored plants and 8 male sterile plants of the three populations.

Results

Identification of RAPD markers linked to the restorer gene

DNA from the three pairs of bulks was used as a template in PCR reactions with each of 138 arbitrary 10-mer oligonucleotide as primers. Of these 30 (22%) did not give any amplification product and were not analyzed further. From the remaining 108 primers, an average of five amplified DNA fragments were scored for each. Six primers produced repeatable polymorphisms between paired bulks constructed from the two F_2 and the DH populations (Table 1); the sequence of these primers is indicated in Table 2. The size of the polymorphic DNA amplification fragments for each RAPD primer is indicated in subscript and

Table 1 RAPD markers identified between bulks differing for male fertility restoration (*R* restored plants, *r* male-sterile plants)

Marker	R63F2		R72F2		RB3HD	
	R	r	R	r	R	r
$OPB06_{650}$	–[a]	+	–	+	–	+
$OPC02_{1150}$	+	–	+	–	+	–
$OPC02_{1050}$	+	–	+	–	+	–
$OPD02_{1000}$	+	–	+	–	–	–
$OPD13_{670}$	–	+	–	+	–	+
$OPF06_{1200}$	–	+	–	+	–	+
$OPG02_{1350}$	–	+	–	–	–	–
$OPG02_{700}$	–	+	–	+	–	+
$OPG02_{600}$	+	–	+	–	+	–

[a] ±: Presence/absence of the DNA fragment

Table 3 Linkage relationships between the restorer gene and the RAPD markers identified by bulked segregant analysis (*R* restored plants, *r* male-sterile plants)

Marker	R63F2		R72F2		RB3HD	
	R	r	R	r	R	r
$OPB06_{650}$	18–[a]	17+/1–	19–	12+	13–/6+	14+
$OPC02_{1150}$	18+	19–	19+	10–	18+	14–
$OPC02_{1050}$	18+	19–	19+	10–	18+	14–
$OPD02_{1000}$	18+	19–	19+	12–	18–	14–
$OPD13_{670}$	17–	19+	19–	12+	16–/3+	12+/1–
$OPF06_{1200}$	18–	19+	18–	12+	15–	13+
$OPG02_{1350}$	17–	19+	19–	13–	17–	14–
$OPG02_{700}$	17–	19+	19–	13+	17–	14+
$OPG02_{600}$	17+	19–	19+	13–	17+	14–

[a]: ±: Presence/absence of the marker

Table 2 Nucleotide sequence of primers detecting polymorphism between pairs of bulks

Primer	Sequence
OPB06	TGCTCTGCCC
OPC02	GTGAGGCGTC
OPD02	GGACCCAACC
OPD13	GGGGTGACGA
OPF06	GGGAATTCGG
OPG02	GGCACTGAGG

used to identify the different clones. Three primers OPB06, OPD13 and OPF06 each generated one polymorphic DNA fragment (respectively $OPB06_{650}$, $OPD13_{670}$ and $OPF06_{1200}$) between the pairs of bulks constructed from the segregating populations, and the polymorphic DNA band was associated in each case with the sterility maintainer allele. One primer OPD02 generated one polymorphic DNA fragment ($OPD02_{1000}$) only between two of the pairs of bulked DNA (Table 1); the DNA band was associated with the fertility restorer allele. The primer OPC02 generated two polymorphic DNA fragments ($OPC02_{1150}$ and $OPC02_{1050}$) between the pairs of bulked DNA. In each, the RAPD allele was associated with the fertility restorer allele. The primer OPG02 generated three polymorphic DNA fragments ($OPG02_{600}$, $OPG02_{700}$ and $OPG02_{1350}$). Two of these ($OPG02_{600}$ and $OPG02_{700}$) were polymorphic in all pairs of bulks. $OPG02_{1350}$ was polymorphic only in one pair of bulks. For the markers $OPG02_{700}$ and $OPG02_{1350}$, the DNA band was associated with the sterility maintainer allele while for the marker $OPG02_{600}$, it was associated with the fertility restorer allele.

DNA from individual plants of each bulk was then used as a template for amplification with the 6 primers detecting polymorphisms between paired bulks for segregation and linkage analysis. The results are shown in Table 3. The RAPD alleles generated by primers OPC02, OPD02, OPG02, and OPF06 were found to be completely linked with the presence/absence of the restorer gene (Fig. 1). The RAPD allele generated by the primer OPD02 was found in both F_2 populations but not in the DH population. For primers OPB06 and OPD13, some recombinants were observed between the markers and the restorer gene.

Cloning of RAPD fragments linked to the restorer locus

Only RAPD markers perfectly linked to the restorer locus were further characterized. They were: $OPC02_{1150}$, $OPD02_{1000}$, $OPF06_{1200}$ and $OPG02_{700}$. Each fragment was cross hybridized, and no homology was detected between these sequences (Fig. 2). Three of them ($OPC02_{1150}$, $OPF06_{1200}$, $OPG02_{700}$) were then isolated from the gel, radiolabelled, and hybridized to filters containing DNA from the six bulks, each of which had been individually digested with *Bam*HI, *Eco*RI and *Eco*RV. Hybridization results with $OPC02_{1150}$ revealed the presence of several fragments common to restored and male sterile bulks and one fragment specific to the three restored bulked DNA digested with *Bam*HI. Hybridization with $OPF06_{1200}$ revealed two extra fragments in the sterile bulks on two restriction digests (*Bam*HI, *Eco*RI). Hybridization with $OPG02_{700}$ revealed two fragments common to all bulks on the three restriction digests and one fragment specific to the three male sterile bulks. Southern hybridization of the $OPG02_{700}$ probe on genomic DNA from the six bulks digested with *Eco*RI are presented in Fig. 3.

We then proceeded to clone each of the four RAPD fragments that were perfectly linked to the *Rfo* locus. The DNA bands were isolated from the gel and used as templates for re-amplification using the corresponding RAPD primers. The correct size of the re-amplified DNA was confirmed by gel electrophoresis; a single DNA band corresponding to the original fragment size was obtained in each case. The remaining re-amplified DNA was used for cloning in the PCR II vector. The size and homogeneity of the cloned inserts were tested by digesting the recombinant plasmid of the positive clones with *Eco*RI. There are two *Eco*RI sites on the PCR II vector, each flanking the inserted DNA fragment, and *Eco*RI digestion revealed heterogeneity with two of the four cloned DNA fragments. Three different re-

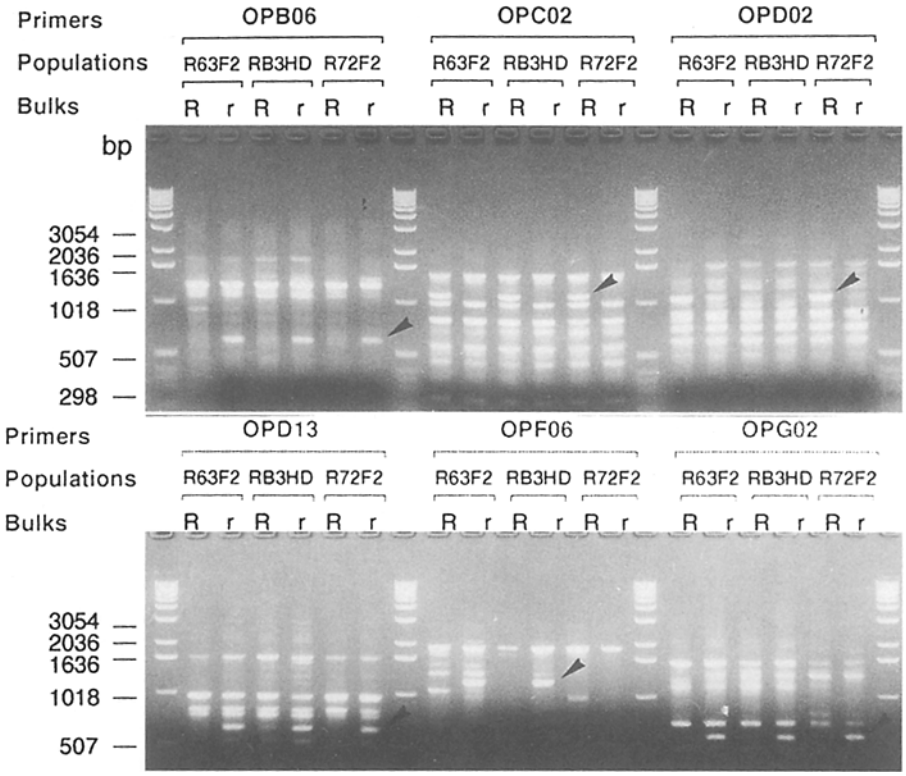
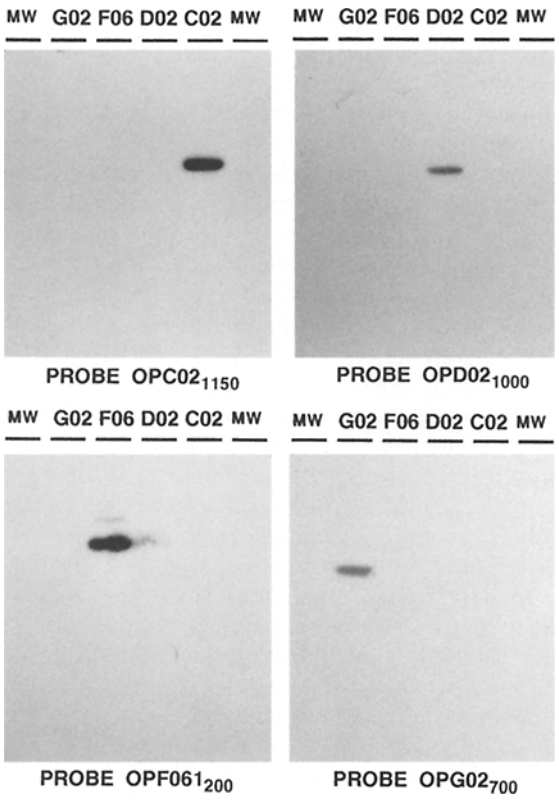


Fig. 1 DNA amplification patterns obtained with RAPD primers OPB06, OPC02, OPD02, OPD13, OPF06, and OPG02 on DNA bulks made of restored (*R*) and sterile (*r*) plants from three segregating populations (R63F2, RB3HD, and R72F2). The polymorphic DNA fragments are indicated by *arrows*. The size of molecular weight markers is indicated at *left*



combinant clones of OPD02$_{1000}$ were obtained. Inserts were characterized by zero, one or two internal *Eco*RI sites (labelled OPD02a$_{1000}$, OPD02b$_{1000}$ and OPD02c$_{1000}$). Similarly, three different clones were obtained from OPF06$_{1200}$; inserts with zero, one or three internal *Eco*RI sites (OPF06a$_{1200}$, OPF06b$_{1200}$ and OPF06c$_{1200}$). However, a single homogeneous insert of the expected size without any internal *Eco*RI restriction site was found in all recombinant clones derived from OPC02$_{1150}$ and OPG02$_{700}$.

To confirm their identity, inserts from all of the different clones were used individually as probes on Southern blots of genomic DNA amplified with the four original primers OPC02, OPD02, OPF06 and OPG02, of both restored and male sterile plants.

As expected, perfect co-segregation with the restorer allele was obtained when the single *Eco*RI insert of the OPC02$_{1150}$ clone was used as the radiolabelled probe (Fig. 4A). Three hybridizing bands all specific to the restored

Fig. 2 Results of pairwise cross-hybridization of the four RAPD fragments OPG02 (*G02*), OPF06 (*F06*), OPD02 (*D02*), and OPC02 (*C02*) that were linked to the *Rfo* locus. Probes were OPC02$_{1150}$, OPD02$_{1000}$, OPF06$_{1200}$, and OPG02$_{700}$. No cross-homology was detected

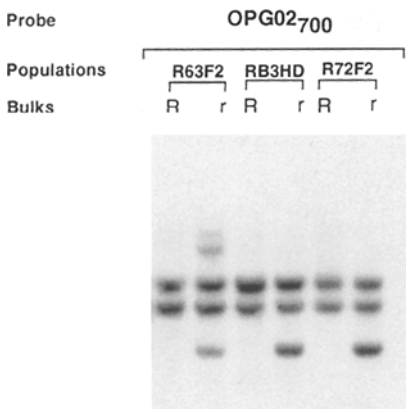


Fig. 3 Southern hybridization of *Eco*RI digested genomic DNA from bulks of restored (*R*) and sterile (*r*) plants from three segregating populations (R63F2, RB3HD, and R72F2). The probe was $OPG02_{700}$. Two DNA bands were common to all bulks and one additional DNA band was present in the sterile bulks. In population R63F2, two additional fragments of higher molecular weight were detected

plants were detected, one of which corresponded to the original polymorphic RAPD marker linked to the restorer allele. No detectable hybridization signal was detected on the amplified DNA of male sterile plants.

Of the three different clones derived form $OPD02_{1000}$, only the insert which contained one internal EcoRI restriction site ($OPD02b_{1000}$) hybridized to the RAPD allele specifically linked to the restorer allele (Fig. 4B). Two hybridizing bands, both specific to the restored plants, were detected, the largest one corresponding to the original polymorphic RAPD marker linked to the restorer allele. No detectable hybridization signal was detected on the amplified DNA of male sterile plants.

The three types of recombinant clones derived from $OPF06_{1200}$ were also analyzed. Cloned inserts with zero and one internal *Eco*RI restriction site, i.e. $OPF06a_{1200}$ and $OPF06b_{1200}$ failed to reveal any polymorphic hybridizing DNA band between the male fertile and male sterile plants. The insert of $OPF06c_{1200}$ which carried three internal *Eco*RI restriction sites, revealed three hybridizing DNA bands common to male fertile and male sterile plants and one DNA specific to the male sterile plants. Each of the four *Eco*RI fragments of $OPF06c_{1200}$ was then individually tested as the probe. The 200-bp *Eco*RI fragment gave a single hybridizing DNA fragment specific to the sterility maintainer allele, and its size corresponded to that of the original RAPD marker (Fig. 4C). This 200-bp fragment was then subcloned for further analysis and named $OPF06_{200}$.

The insert of $OPG02_{700}$ hybridized only to DNA amplified from male sterile plants. Three DNA bands were detected, one corresponding to a RAPD marker monomorphic between the male sterile and the male-fertile, while the remaining two DNA bands corresponded to the original RAPD associated to the sterility maintainer allele (Fig. 4D).

Discussion

To be useful for F_1 hybrid production, a CMS system requires strong fertility restorer and sterility maintainer alleles. Recently, the *Ogura* CMS system for F_1 hybrid seed production in *B. napus* was improved so as to have such alleles. The fertility restorer allele was introgressed from radish into rapeseed. During this transfer from the radish genome to the *B. napus* genome, the fertility restorer allele may have carried adjacent radish DNA. A *Pgi-2* radish allozyme has been found to be tightly linked (0.25 cM) to the fertility restorer allele in *B. napus* (Delourme and Eber 1992). In our study we identified six additional dominant RAPD markers, two of which were linked to the fertility restorer allele and the other four to the sterility maintainer allele. These results illustrate the efficiency of bulked segregant analysis coupled with RAPD markers. One of the reasons for this efficiency is the ability of individual primers to detect multiple loci. The Southern blot analyses performed in this study illustrate some other properties of RAPD markers. Hybridization with labelled RAPD fragments on digested genomic DNA from the bulks indicated that these amplified fragments corresponded to low- or middle-repetitive sequences. Similar results had already been obtained in *Brassica* (This 1992) as well as in soybean (Williams et al. 1990) and *Arabidopsis thaliana* (Reiter et al. 1992). Southern hybridizations with the same amplified fragment as probes on RAPD profiles showed that additional bands were hybridized in addition to the RAPD marker fragment. These could be fragments of different sizes resulting from the amplification of the same sequence with internal repeats corresponding to the RAPD primer; they could also be repeated or duplicated sequences, at different loci, that had undergone size modifications through insertion or deletion. However, the additional bands hybridized only on RAPD profiles of either the restored or the male sterile plants (depending on the allelic side of the marker) although RAPD fragments of the same size were observed on all the plants. This rather supports the first hypothesis of a sequence with internal repeats.

In this study, we analyzed in detail only four RAPD markers (two on each allelic side) that showed no recombination with the restorer locus. One of these RAPD markers ($OPC02_{1150}$) hybridized to filters of PCR products obtained with the corresponding primer using DNA from *R. sativus* plants and from different *B. napus* varieties as the template. Hybridization was only observed on PCR products of the radish plants (Barret and Delourme, results not shown). This confirms the hypothesis that radish DNA has been retained around the restorer allele. It is possible that recombination could be suppressed due to a lack of homology; physical distance between markers may be large even if no recombination has been detected. The precise positioning of the four RAPD markers around the restorer locus will therefore require segregation analysis in a pure *R. sativus* cross. However, there are indications from segregation data that the remaining radish DNA around the re-

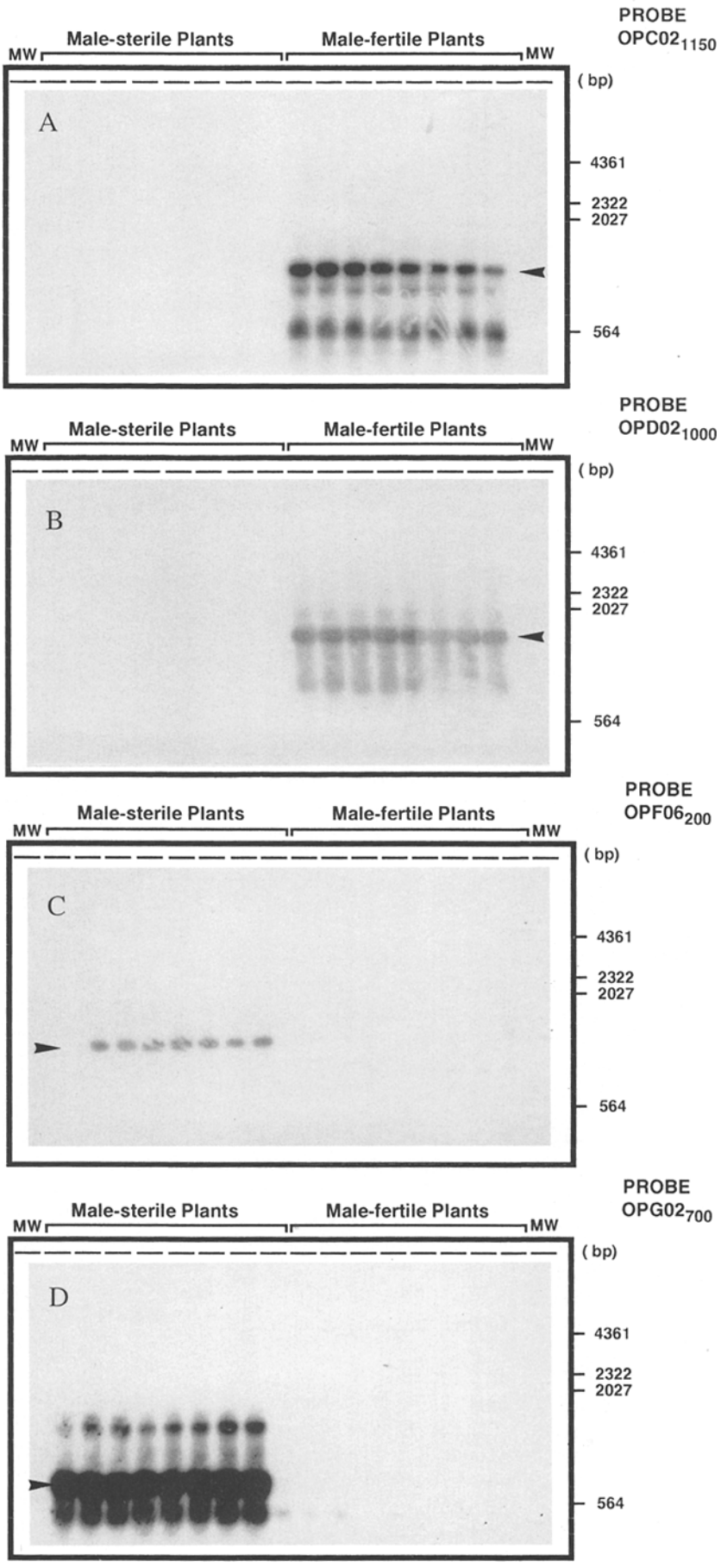


Fig. 4A–D Southern blots of amplified DNA from 8 sterile and 8 fertile plants and probed with the four cloned RAPD fragments perfectly linked to the *Rfo* locus.
A Probe OPC02$_{1150}$ hybridized to three major DNA bands that were amplified by OPC02 only in fertile plants.
B Probe OPD02$_{1000}$ hybridized to one major DNA band that was amplified by OPD02 only in fertile plants. **C** Probe OPF06$_{200}$ (a subclone of OPF06$_{1200}$; see text) hybridized to one major DNA band that was amplified by OPF06 only in sterile plants. DNA from the first sterile plant did not amplify at all which resulted in no hybridization.
D Probe OPG02$_{700}$ hybridized to three major DNA bands that were amplified by OPG02 only in sterile plants although a very faint band of 564 bp could be seen in all fertile plants after longer exposure. The hybridizing DNA fragment corresponding to the size of the original RAPD fragments is indicated for each probe by an *arrow*. DNA sizes are indicated at *right*

storer locus is smaller in the doubled haploid population RB3HD. This doubled haploid population is derived from a plant that was heterozygous at the restorer locus but had lost the radish *Pgi-2* allele. In this population, the radish-specific DNA fragment $OPD02_{1000}$ has also been lost, while the radish-specific DNA fragment $OPC02_{1150}$ is still present in this population. This indicates that $OPC02_{1150}$ is either located between the restorer allele and the *Pgi-2* allele at a distance less than 0.25 cM from the restorer locus or that $OPC02_{1150}$ is located on the other side of the restorer allele. $OPD02_{1000}$ is most likely located on the same side as *Pgi-2* and tightly linked to it since more recombinants were observed in the RB3HD population.

Homozygous plants which have lost the *Pgi-2* radish allele have not recovered the *Pgi-2* allele of rapeseed that was originally replaced by the radish allele. Thus, these plants lack a *B. napus* DNA segment and this seems to have a negative effect on some of their characters such as vigor, female fertility, or transmission of the restorer allele through the pollen (Delourme, unpublished results). In order to remove as much radish DNA as possible while being sure to recover the whole *B. napus* genome, we have to look for recombination around the restorer locus starting from plants that still possess the *Pgi-2* radish allele. For this purpose, we need codominant markers. Transformation of dominant RAPD markers into codominant markers can be attempted by developing sequence characterized amplified regions (SCAR) as PCR-based markers (Michelmore et al. 1991). The ends of the RAPD fragments are sequenced to design pairs of longer and highly specific primers that will allow only the amplification of the marker loci. In this way codominant SCAR markers were successfully derived from RAPD fragments in lettuce (Paran and Michelmore 1993). The four RAPD marker fragments identified in our study are at present being sequenced to develop SCAR primers and also to determine if they have homology to any published DNA sequence (Bouchereau et al. in preparation). One of the advantages of SCAR markers is the facility by which they can be followed in different crosses. They may enable us to localize the *Rfo* locus on the developed *B. napus* genetic maps and provide information on the length of the introgressed radish chromosome segment. The application of these SCAR markers combined to recently developed seedling leaf disk DNA microextraction procedures (Deragon and Landry 1992; Cheung et al. 1993) promise to facilitate breeding of this agronomically important gene.

Since no recombination between the restorer locus and the four RAPD markers has been detected using 100 plants from the three populations, we are also attempting to construct the physical map of this chromosome area using pulsed field gel electrophoresis technology. This is the first step towards cloning the fertility restorer allele. For these experiments, the four RAPD fragments that have been cloned in this study will be used as landmark for precise estimation of the physical distance between them. Our ultimate goal is the isolation of the *Rfo* coding DNA sequence through map-based cloning. This approach, originally developed for the isolation of human genes, has recently been successfully applied in plants (Giraudat et al. 1992; Arondel et al. 1992). If the *Rfo* gene becomes available, it should be possible, by applying transgenic technology, to produce new restorer lines without the disadvantge of linkage drag. In addition, as no restorer genes have been isolated for any CMS system in any species, the isolation and characterization of this gene would constitute a true breakthrough at the fundamental level. The characterization of this gene should lead to a better understanding of the molecular processes governing pollen production and may lead to the development of alternate methods of pollination control.

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