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ORIGINAL PAPER

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Characterization of a radish introgression carrying the Ogura fertility restorer gene *Rfo* in rapeseed, using the *Arabidopsis* genome sequence and radish genetic mapping

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Abstract The radish *Rfo* gene restores male fertility in radish or rapeseed plants carrying Ogura cytoplasmic male-sterility. This system was first discovered in radish and was transferred to rapeseed for the production of F1 hybrid seeds. We aimed to identify the region of the *Arabidopsis* genome syntenic to the *Rfo* locus and to characterize the radish introgression in restored rapeseed. We used two methods: amplified consensus genetic markers (ACGMs) in restored rapeseed plants and construction of a precise genetic map around the *Rfo* gene in a segregating radish population. The use of ACGMs made it possible to detect radish orthologs of *Arabidopsis* genes in the restored rapeseed genome. We identified radish genes, linked to *Rfo* in rapeseed and whose orthologs in *Arabidopsis* are carried by chromosomes 1, 4 and 5. This indicates several breaks in colinearity between radish and *Arabidopsis* genomes in this region. We determined the positions of markers relative to each other and to the *Rfo* gene, using the progeny of a rapeseed plant with unstable meiotic transmission of the radish introgression. This enabled us to produce a schematic diagram of the radish introgression in rapeseed. Markers which could be mapped both on radish and restored rapeseed indicate that at least 50 cM of the radish genome is integrated in restored rapeseed. Using markers closely linked to the *Rfo* gene in rapeseed and radish, we identified a contig spanning six bacterial artificial chromosome (BAC) clones on *Arabidopsis* chromosome 1, which is likely to carry the orthologous *Rfo* gene.

Keywords Ogura cytoplasmic male-sterility · Restorer gene · Consensus genetic markers · Synteny · *Brassica napus*

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Introduction

The abundant genomic data available for *Arabidopsis thaliana* have already been successfully used in the identification of various genes in related species (Fourmann et al. 1998, 2001, 2002; Robert et al. 1998; Brunel et al. 1999; Lan et al. 2000; Sillito et al. 2000; Ku et al. 2001; Paterson et al. 2001). Non-anonymous markers can be used to compare the genomic organization, or synteny, of different species. We used the available genetic data for *A. thaliana* to develop non-anonymous markers for the radish sequences linked to the *Rfo* gene.

Efficient use of the Ogu-INRA cytoplasmic male-sterility (CMS) system (Pelletier et al. 1983, 1987) in hybrid rapeseed seed production depends on the generation of restorer lines with good agronomic properties. The breeding of restorer lines has therefore been a major objective for rapeseed breeders for several years. The *Rfo* restorer gene was introduced from radish into rapeseed

(Heyn 1976), together with a certain amount of linked radish genetic material (Pellan-Delourme and Renard 1988). The resulting intergeneric hybrids were improved by extensive breeding (Delourme et al. 1991, 1995). However, the restored rapeseed lines continue to display characters directly due to, or linked to, the radish DNA introgression. For example, some restored rapeseed lines display poor agronomic behavior and meiotic disturbance, leading to abnormal *Rfo* gene transmission. Random amplification of polymorphic DNA (RAPD) markers have been identified for the *Rfo* gene (Delourme et al. 1994). However, mapping of the *Rfo* gene region in rapeseed is hindered by the impaired meiotic recombination of the radish introgression with the homoeologous rapeseed chromosome (Delourme et al. 1998). A radish *Pgi-2* isozyme allele was found to be linked to the *Rfo* gene and can be used as a marker for the radish introgression (Delourme and Eber 1992). However, this marker may be lost in some improved lines with low glucosinolate content (Delourme et al. 1995).

The first aim of this study was to identify the region of the *Arabidopsis* genome syntenic to the *Rfo* locus. We used an amplified consensus genetic markers (ACGMs) approach (Brunel et al. 1999) to develop non-anonymous markers from the *Arabidopsis* genome sequence. We then carried out fine mapping of the *Rfo* gene in a segregating radish population, using AFLP markers followed by '*in silico* positioning'. By combining these two methods, we were able to identify a region on chromosome 1 of the *Arabidopsis* genome, covering six BACs, that is likely to contain the ortholog of the *Rfo* gene. Our data allowed a better characterization of the radish introgression in rapeseed, and we were able to generate a schematic diagram of it.

Materials and methods

Plant material and DNA preparation

The rapeseed plants used to develop the ACGMs for this study (Brunel et al. 1999) were the maintainer cultivar Samourai (SM) and two almost-isogenic derivatives (SR1 and SR2) carrying the *Rfo* gene on a radish introgression. The SR1 line is homozygous for *Rfo*. SR2 plants were obtained by back-crossing, with the SM line, a previously described derivative of the SR1 line that had lost some of the markers linked to *Rfo* (Delourme and Eber 1992; Delourme et al. 1995). SR2 plants are therefore heterozygous for *Rfo*.

In a hybrid breeding experiment, one plant was found to have male-sterile and fertile branches. The genetic stability of CMS in this plant was checked by PCR amplification of the *orf138* gene and the *Nco2.5* region (Bonhomme et al. 1991, 1992) in DNA extracted from several branches, some fertile and some sterile (data not shown). Based on the results obtained, this plant was considered chimeric for the *Rfo* gene. Fertile flowers were selfed and male-sterile flowers pollinated with a maintainer line; the resulting progenies were grown in the greenhouse. Total DNA was extracted individually from leaf material for each progeny before flowering, and the male fertility phenotype was checked upon flowering. We analyzed a total of 63 offspring from eight branches of the parental chimeric plant.

Two radish progenies segregating for the *Rfo* gene were produced for genetic mapping. Twelve F4 families were produced by selfing heterozygous-restored radish genotypes (D radish) originating from Asia. An F2 progeny of 20,000 seeds was obtained by selfing F1 hybrids between a European male-sterile radish line (7ms) and a radish genotype homozygous for *Rfo* (D81.8) selected from a D radish F3 family.

All radish plants were vernalized in a growth cabinet (8 weeks, 4°C, photoperiod: 12 h) and then grown to flowering in a greenhouse. Male fertility/sterility was assessed by eye.

Total DNA was prepared from rapeseed, *Arabidopsis* (Columbia ecotype) and from the 358 F2 and 359 F4 radish plants as described elsewhere (Doyle and Doyle 1990). DNA was extracted from a further 261 F2 plants by a simple sodium-metabisulfite method.

Development of consensus genetic markers

We developed ACGMs from *Arabidopsis* as described elsewhere (Brunel et al. 1999). PCR primers were designed with OLIGO v 6.0 for Macintosh (Rychlik and Rhoads 1989) and used for genomic DNA amplification. We amplified DNA from the three almost-isogenic lines—SM, SR1, and SR2—of *Brassica napus*, two bulks—BS and BF—of *Raphanus sativus*, and *Arabidopsis* as a control. The radish bulk DNAs were constituted by mixing equal amounts of DNA from 20 male-sterile (BS) and 20 fertile (BF) plants. PCR and sequencing were performed as previously described (Brunel et al. 1999).

We verified the amplification of orthologous genes by comparing the sequence data from *Brassica* and *Raphanus* with the *Arabidopsis* sequence, using BLAST (http://www.arabidopsis.org/Blast/). Multiple sequence alignments were generated with GCG (Wisconsin University, version 9.1, UNIX, September 1997) or CLUSTAL X (Thompson et al. 1994, 1997).

AFLP analysis, gel electrophoresis and staining

AFLP markers were analyzed as described by Vos et al. (1995), with the AFLP Analysis System I and AFLP Starter Primer Kits (Gibco BRL Life Technologies). Genomic DNA was digested with *Eco*RI and *Mse*I (Roche) or with *Pst*I and *Mse*I and was amplified with Eurobio *Taq* DNA polymerase, the 10× kit buffer and Sigma dNTPs. We heated 8 µl of each sample with a 1/5 vol. of loading buffer (98% formamide, 10 mM of EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue) for 4 min at 95°C to denature the DNA. We then subjected the resulting sample to electrophoresis in a denaturing 6% polyacrylamide gel (7 M of urea, 40% of acrylamide/bisacrylamide 19:1, 90 mM of Tris, 90 mM of boric acid, 2 mM of EDTA, 0.05% of TEMED and 0.26 µM of ammonium persulfate). Electrophoresis was performed at 65 W for 2.5 h. PCR products were visualized by silver nitrate staining, as follows. The acrylamide gel was incubated for 5 min in 10% ethanol, 3 min in 1% nitric acid, and then 15 min in 7.65 mM silver nitrate. Between incubations, the gel was carefully rinsed in distilled water. Bands were detected by incubation with 0.05% formaldehyde in 0.28 M sodium carbonate and the detection reaction was stopped by adding 10% acetic acid. The gel was then rinsed for 5 min in distilled water and air-dried at room temperature.

AFLP markers were designated according to the primer combination used and the molecular weight corresponding to the band.

Mapping

We used a bulk segregant analysis (BSA) strategy (Michelmore et al. 1991) to identify polymorphic markers linked to the *Rfo* gene. The F4 population was used to construct a first set of bulks. For each of the 12 F4 families, we pooled equivalent amounts of DNA from 12 fertile plants to generate one fertile bulk per F4 family and of DNA from 12 male-sterile plants to generate one sterile bulk per F4 family. We then pooled all the fertile bulks and all the sterile

bulks to generate two superbulks (fertile and sterile). These bulks were used to find the first polymorphic markers. We then used equivalent amounts of DNA from 12 fertile and 12 sterile genotypes to create two bulks (fertile and sterile) for the F2 population.

We mapped the polymorphic markers identified by BSA for 136 genotypes in the F4 and 358 genotypes in the F2 population. We then identified 20 F2 genotypes and 12 F4 genotypes displaying recombination between *Rfo* and its closest markers. These recombinant genotypes were used to map subsequently identified markers. We carried out genetic mapping independently for the F2 and F4 populations and then generated a combined map. We carried out multi-point linkage analysis of the marker loci with MAPMAKER/EXP version 3.0 (Lincoln et al. 1992). The threshold LOD score was 3.0 and the maximum distance between markers was 50 cM. The Kosambi function was used to obtain distances in centimorgans.

Cloning and sequencing

AFLP markers were cloned as follows. Bands were excised from the acrylamide gel, and the DNA they contained was isolated and re-suspended in ultrapure water. The DNA was then re-amplified by PCR, and run on a polyacrylamide gel to check the molecular sizes in the same conditions as described above. PCR products were cloned with the TA cloning kit (Invitrogen). Plasmid DNA was prepared with the Wizard *plus* SV miniprep DNA purification system (Promega). Insert sizes were checked by agarose-gel electrophoresis after digestion for 1 h at 37°C with *Eco*RI (Roche). We sequenced the inserts. Primers were designed with Oligo software (Rychlik et al. 1990) for selective amplification of the cloned marker. We used the Universal GenomeWalker kit (Clontech) to increase the size of the cloned markers by PCR walking. The final PCR products were cloned and sequenced as described above and new primers were designed to obtain specific PCR markers.

Sequenced AFLP markers and extended markers were aligned with the *Arabidopsis* genome sequence in databases, using BLAST.

Conditions for the development of specific PCR markers

To obtain specific markers, primers were designed from the sequences of extended AFLP markers and sequences generated from ACGMs markers by Oligo (Rychlik et al. 1990).

For markers derived from AFLP, the PCR conditions used were as follows: in a final vol. of 20 µl, 0.5 units of Eurobio Taq II, 2 mM of $MgCl_2$, 125 µM of each dNTP, 1.25 µM of each primer, 50 ng of total DNA sample subjected to heating at 94°C for 2.5 min followed by 30 cycles of 30 s at 94°C, 1 minute at annealing temperature and 2 min at 72°C, and a final extension step consisting of 5 min at 72°C. Annealing temperature was 50°C to 63°C, depending on the sequence considered.

For markers derived from ACGMs, PCR was routinely performed in a final volume of 50 μl, containing 40 ng of total DNA, 0.25 mM of each dNTP, 0.2 μM of each primer, and 1 unit of *Taq* polymerase from Eurobio, with the reaction buffer and $MgCl_2$ concentration recommended by the kit manufacturer. The amplification conditions were as follows: 4 min at 94°C, then 35 cycles (1 min at 94°C, 1 min at the annealing temperature, and 2 min at 72°C), and 4 min at 72°C. We subjected 8 μl of amplification products for each reaction to electrophoresis in a 1% or a 1.5% agarose gel, depending on the expected size of the PCR products.

We were able to obtain a co-dominant marker from E32M32.95 (see Results). A primer was designed from the sequence of the cloned marker. It was used for PCR with the M32 AFLP primer. PCR conditions were as described above for AFLP-derived markers except that the DNA samples were those used for AFLP genotyping and the annealing temperature was set at 50°C. Electrophoresis was performed in a non-denaturing 6% acrylamide gel for 5 h 30 min (280 V).

Sequences of primers

The sequences of primers developed in this study are available for academic research purposes only at the following address: budar@versailles.inra.fr

Results

Global survey of the *Arabidopsis* genome, using an ACGMs approach

Using the ACGMs approach developed by Brunel et al. (1999) we searched the entire *Arabidopsis* genome for regions in which radish orthologs were linked to *Rfo*.

We investigated a total of 131 ACGMs, corresponding to 106 different *Arabidopsis* genes. In some cases, several primer pairs had to be designed for the successful amplification of orthologs in rapeseed and radish. There may be several ACGMs (primer pair combinations) for a single target gene. For each BAC (or P1) clone carrying a target ACGMs sequence, we determined the approximate genetic distance from the origin by aligning physical and genetic maps on the online mapviewer of the TAIR webserver (http://www.arabidopsis.org/servlets/mapper/). According to these estimated positions, the mean physical distance (standard deviation: SD) between two target genes is 6.67 cM (SD=5.12), 4.43 cM (SD=4.07), 4.76 cM (SD=6.76), 5.84 cM (SD=5.61) and 5.57 cM (SD=7.03) for chromosomes 1, 2, 3, 4 and 5 respectively. This corresponds to a mean of 5.31 cM (SD=5.54) between two target genes over the entire *Arabidopsis* genome. We also estimated the mean physical distance between two target genes using their coordinates as given by AGI (http://www.arabidopsis.org/). According to these coordinates, the mean physical distance (SD) between two target genes is 1,683 kb (SD=1,057.4), 840 kb (SD=790.4), 1,130 kb (SD=1,711.2), 912 kb (SD=1,000) and 1114 kb (SD=1,930.9) for chromosomes 1, 2, 3, 4 and 5 respectively. This corresponds to a mean of 1,115 kb (SD=1,101.6) between two target genes over the entire *Arabidopsis* genome.

Radish and rapeseed orthologs were successfully amplified for 86 different target genes from the five *Arabidopsis* chromosomes (14, 19, 16, 17 and 20 target genes respectively on chromosomes 1, 2, 3, 4 and 5; data not shown). For five target genes, the PCR products obtained were not of a high-enough quality for validation by sequencing for both species. For 15 target genes, it was possible to amplify at least one orthologous gene and sequence it for rapeseed, but not for radish. In both these situations, we were unable to draw firm conclusions concerning the possible presence of the radish ortholog of the target gene in the restored rapeseed genotype.

Twelve ACGMs, corresponding to 11 target genes, revealed polymorphism between the restored and maintainer lines of rapeseed. Various types of polymorphism were identified (see legend of Table 1). We looked for markers giving an additional PCR product, corresponding

Table 1 Polymorphic ACGMs markers in rapeseed, identified in the global survey of the *Arabidopsis* genome, and their corresponding target genes. The locus name corresponds to the nomenclature of MIPS for the *Arabidopsis* target gene. The given accession number corresponds to the sequence used for developing ACGMs

A. thaliana chromosome	Locus name	Predicted function of encoded protein	BAC clone in *A. thaliana* contig	Accession number of reference sequence	Name of marker
1	AT1G60950	ferredoxin precursor	T7P1	AC018908	SG143 (c)
2	AT2G12400	unknown protein	F24C20	AC007112	MF2 (e, h)
2	AT2G14750	putative adenosine phosphosulfate kinase	F26C24	AC004705	MF1 (f, h)
3	AT3G10730	hypothetical protein	T7M13	AC011708	SG98 (f)
4	AT4G02780	ent-kaurene synthetase	T5J8	U11034	GA1 (d)
4	AT4G02780	ent-kaurene synthetase	T5J8	U11034	SG41 (d)
4	AT4G04470	peroxisomal protein	T26N6	NM_116685	SG131 (d)
5	AT5G24090	acidic endochitinase	MZF18	M34107	ATHCHIA (d)
5	AT5G42740	glucose-6-phosphate isomerase, cytosolic	MJB21	X69195	Pgi-2 D (d, g)
5	AT5G42740	glucose-6-phosphate isomerase, cytosolic	MJB21	X69195	Pgi-2 E (d, g)
5	AT5G43700	auxin-induced protein AUX2-11	MQD19	AB026651	AUX 2 (h)
5	*AT5G64040*	Photosystem I reaction centre subunit psaN precursor (PSI-N)	MBM17	AB019227	SG157 (d)

Name of marker: (c) Indicates a marker for which an additional PCR product in restored rapeseed does not correspond to any known radish sequence. (d) Indicates a marker for which an additional PCR product in restored rapeseed corresponds to the presence of the radish ortholog of the target gene. (e) Indicates a marker for which the additional PCR product in restored rapeseed corresponds to radish sequences not orthologous to the *Arabidopsis* target gene. (f) Indicates a marker for which an amplified fragment was absent from the SR1 restored rapeseed line but present in SM and SR2. (g) The Pgi-2D and Pgi-2E radish-specific bands were absent from SR2 plants, as expected from previous results (Delourme et al. 1995). (h) Consensus primers for amplification were developed by Marie Fourmann (Fourmann et al. 2002 and M. Fourmann, personal communication)

to the presence of the radish ortholog of the target gene. We identified seven such markers, corresponding to five different genes. These genes are dispersed over *Arabidopsis* chromosomes 4 and 5 (Table 1), and no single region was considered more likely than any other, at this point, to carry the ortholog of *Rfo*. A complete table of all tested ACGMs is available as electronic supplementary data.

Fine genetic mapping of the *Rfo* locus in radish, using a BSA strategy

In parallel, we constructed a fine genetic map of the *Rfo* gene in radish, using two mapping populations and a BSA strategy.

Both mapping populations displayed a ratio of male-fertile to male-sterile plants that did not differ significantly from 3:1. In the F2 families, 450 (72.9%) plants were male-fertile and 167 (27.1%) were male-sterile. In the F4 families, 263 (73.3%) plants were male-fertile and 96 (26.7%) were male-sterile. This confirmed the monogenic inheritance of male fertility restoration in this progeny.

We screened 850 *Eco*RI/*Mse*I and 480 *Pst*I/*Mse*I primer combinations on the bulks. This screening process identified 69 and 82 polymorphic markers, respectively. The polymorphic markers were first analyzed in the individual genotypes of the bulks. We then mapped two markers considered of particular interest (E36M55.110 and E33M48.160) in the F2 and F4 populations. This led to the identification of 20 F2 and 12 F4 genotypes displaying recombination around the *Rfo* gene, and markers were then analyzed for these recombinants only. Seventeen AFLP markers mapped to within 13.9 cM of the *Rfo* gene (Fig. 1). Ten of these markers mapped to within 3.4 cM of the gene, and four displayed no recombination and were considered completely linked to the *Rfo* gene (Fig. 1).

We cloned 15 of the AFLP markers closely linked to the *Rfo* gene (Table 2). We sequenced these markers and aligned their sequences with the sequence of the *Arabidopsis* genome. Five of these 15 markers were successfully extended by PCR walking (E36M65.120, E33M40.160, E86M76.195, E32M22.95 and E33M42.260). The extended sequences were aligned with the sequence in the *Arabidopsis* genomic database, which confirmed the results obtained with the original fragment (Table 2). Alignments of the cloned AFLP markers or of the extended markers with the *Arabidopsis* genome sequence identified a region of chromosome 1 and a region of chromosome 5 of the *Arabidopsis* genome. A single BAC (F24L7) from chromosome 2 was identified by the pVR8 marker (see Table 2).

Five BACs corresponding to two regions located close together on chromosome 1 were identified (Table 2): F1N19, F15H21, F22C12 and T12P18 in one region, and F25P12 in the other. Three additional, but more-distant BACs were identified on chromosome 1 with other markers: F20D22, F13M7 and F10F5. Three markers (pVR3, pVR6 and pVR7) also identified three adjacent BACs on chromosome 5: MYC6, MBK23 and K16L22, respectively.

Specific primers were designed from the extended sequences of four AFLP markers (E36M55.110, E33M40.160, E86M76.195 and E33M42.260). Specific

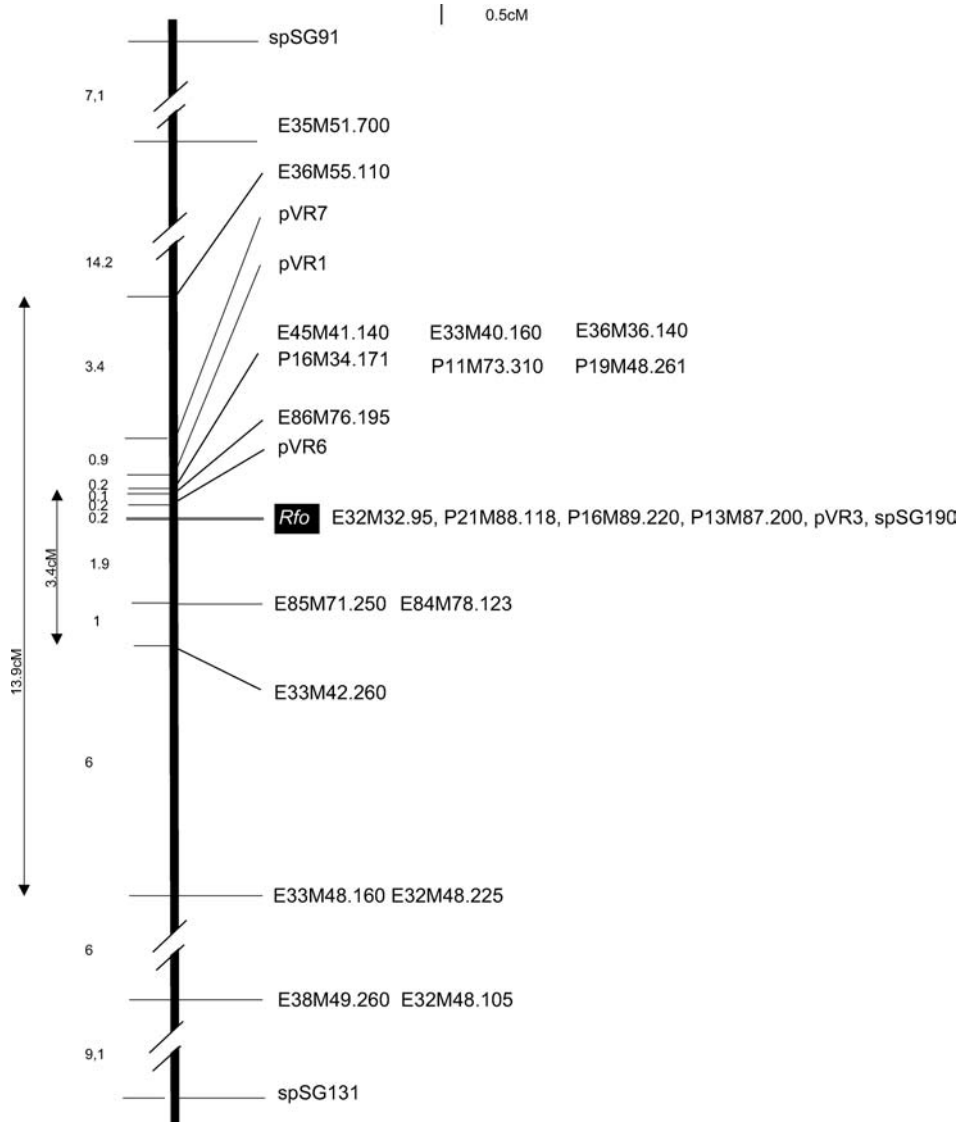

Fig. 1 Genetic map of the region containing the *Rfo* gene, produced by combining data for the F2 and F4 mapping populations, including that for AFLP (E_nM_n or P_nM_n) and specific PCR markers derived from AFLP (pVR1, pVR3, pVR6 and pVR7) or from ACGMs (spSG91, spSG131 and spSG190)

PCR markers pVR1, pVR6, pVR7 and pVR8 were derived from AFLP (see Table 2) and revealed either co-dominant (pVR1, pVR6 and pVR8) or dominant (pVR7) polymorphism between parents. The polymorphism identified between parents was also observed in the F2 and F4 generations, with the exception of the pVR8 marker, which was polymorphic only in the F2 population. Two specific primers were designed from the E32M32.95 AFLP fragment sequence, but neither revealed polymorphism between the parental lines of the F2 radish population. A co-dominant marker was identified by combining a specific primer pVR3UP with the AFLP primer M32.

The pVR1, pVR6 and pVR7 markers were mapped in 358 F2 plants and 359 F4 plants. These markers did not map to exactly the same locations as the corresponding original AFLP fragments (Fig. 1). This difference was accounted for by differences in the scoring data for some plants, the scoring of specific PCR markers being more reliable. We mapped pVR3 in 361 F2 plants and 288 F4 plants, and its complete linkage with *Rfo* was confirmed (Fig. 1).

This additional mapping of specific PCR markers confirmed that the markers closest to the *Rfo* gene in radish preferentially identified a region on chromosome 1 and a region on chromosome 5 (Table 2). We therefore focused on these two regions of the *Arabidopsis* genome for a more detailed analysis of the *Rfo* region of the rapeseed genome by the ACGM approach.

Focus on two regions of *Arabidopsis* chromosomes 1 and 5

The region of chromosome 1 considered spans a contig of approximately 2 cM between BAC clones T3P18 and F1N19. This region was selected based on the identification and sequencing of markers during mapping of the *Rfo* gene in radish (see Table 2). Fifteen ACGMs markers, corresponding to eight target genes, were tested, as

Table 2 Alignments of the sequences of the cloned or extended AFLP markers linked to the *Rfo* gene with sequences in the *Arabidopsis* genomic database. In the "Identities" column, the fraction gives the number of residues identical in our sequence and in the corresponding *Arabidopsis* sequence, over the entire length of the *Arabidopsis* sequence, as determined by the BLAST program. The number of regions is the number of different regions recognized in the *Arabidopsis* sequence. The last number in this column is the expectation value (E-value) given by BLAST

Original AFLP marker	Extended?	Name of the extended marker	cM from *Rfo*	Size of the sequenced fragment	*Arabidopsis thaliana*. Chromosome and gene numbers	Identities
E36M55.110	Yes	PVR1	5 (0.7**)	1397bp* (1500 bp)	Chromosome I, AT1G64480	282/307 (92%), 4 regions, 2×10^{-26}
E45M41.140	No	-	0.5	140bp	Not significant	
E33M40.160	Yes	PVR7	0.5 (1.6**)	1394bp* (600 bp)	Chromosome I, AT1G64320 Chromosome V, AT5G41780	161/182 (88%), 1 region, 4×10^{-44} 53/61 (86%), 1 region, 2×10^{-5}
P19M48.261	No	-	0.5	261bp	Chromosome I, AT1G56540	24/24 (100%), 1 region, 0.003
P16M34.171	No	-	0.5	171bp	Chromosome I, AT1G64550	37/41 (90%), 1 region, 6×10^{-6}
P11M73.310	No	-	0.5	310bp	Not significant	
E36M36.140	No		0.5	140bp	Not significant	
E86M76.195	Yes	PVR6	0.4 (0.2**)	1757bp* (800 bp)	Chromosome I, AT1G64000 Chromosome V, AT5G41570	262/296 (88%), 3 regions, 2×10^{-51} 136/148 (92%), 2 regions, 10^{-19}
E32M32.95	Yes	PVR3	0 (0**)	984bp	Chromosome I, AT1G63830 Chromosome V, AT5G41390	391/433 (90%), 6 regions, 2×10^{-38} 195/221 (88%), 3 regions, 6×10^{-26}
P16M89.220	No	-	0	220bp	Chromosome I, AT1G49870	112/174 (64%),1 region, 6.4×10^{-7}
P13M87.200	No	-	0	200bp	Not significant	
P21M88.118	No	-	0	118bp	Not significant	
E85M71.250	No		1.9	250bp	Chromosome I, AT1G04120	123/149 (82%), 1 region, 10^{-17}
E84M78.123	No		1.9	123bp	Not significant	
E33M42.260	Yes	PVR8	2.9	691bp (400 bp)	Chromosome II, AT2G32730 Chromosome I, AT1G04810	426/578 (74%), 2 regions, 2×10^{-99} 179/200 (89%), 3 regions, 4×10^{-17}

* Only the partial sequence was obtained despite sequencing from the ends of each fragment. The size in brackets is the approximate size of the specific marker

** Distance in brackets corresponds to the distance from the specific PCR marker to the *Rfo* gene (see Results)

Table 3 ACGMs corresponding to *Arabidopsis* sequences from selected regions on chromosomes 1 and 5. Legend as for Table 1

A. thaliana chromosome	Locus name	Predicted function of encoded protein	BAC (or P1) clone in *A. thaliana* contig	Accession number of reference sequence	Name of marker
1	AT1G62850	DS1-like putative protein	F16P17/F23N19	AC011000	SG129 (d)
1	AT1G63770	Similar to aminopeptidase N	F24D7	AC011622	SG190 (d)
1	AT1G63830	Unknown protein	T12P18	AC010852	SG177 (d)
1	AT1G63830	Unknown protein	T12P18	AC010852	SG178 (d)
1	AT1G63830	Unknown protein	T12P18	AC010852	SG179 (d)
5	AT5G29771	Ribosomal protein S1	F19I11	AQ010999	SG91 (d)
5	AT5G39740	Ribosomal protein L5-like	MIJ24	AB012243	SG144 (d)
5	AT5G40370	Glutaredoxin-like protein	MPO12	AB006702	SG164 (d, f)
5	AT5G41390	Putative protein	MYC6	AB006707	SG181 (d, f)

described above (see complete information in electronic supplementary data). The mean genetic distance between two target genes was 0.58 cM (SD=0.41). The mean physical distance between two target genes was 124 kb (SD=68.5). Rapeseed and radish orthologs were successfully amplified for all but one of the target genes. The exception was a resistance gene. A radish ortholog was successfully amplified and identified by sequencing, but the numerous amplification products generated from rapeseed resulted in too low a level of purification for efficient separation and subsequent identification by sequencing. Five ACGMs, corresponding to three different target genes, detected polymorphism between restored and maintainer lines of rapeseed. In all cases, this polymorphism corresponded to the presence of the radish gene, in addition to the rapeseed gene, in the restored rapeseed (Table 3).

The region of chromosome 5 considered, extends between markers ATCHIA and Pgi-2. Both markers correspond to genes for which orthologs are present in restored rapeseed, and a large portion of the genome (32 cM, 8.6 Mb) lying between these two markers has yet to be explored. Furthermore, this region spans BACs MYC6, MBK23 and K16L22, which display significant sequence similarity to the pVR3, pVR6 and pVR7 markers, respectively (Table 2), pVR3 being the most tightly linked to *Rfo* in radish. Unfortunately, this region corresponds to the centromeric region of chromosome 5, which made it difficult to develop ACGMs. Nevertheless, we tested 22 ACGMs, corresponding to ten target genes, in this region (see complete information in the electronic supplementary data). The mean genetic distance between two target genes was 2.67 cM (SD=4.2). The mean physical distance between two target genes was 791 kb

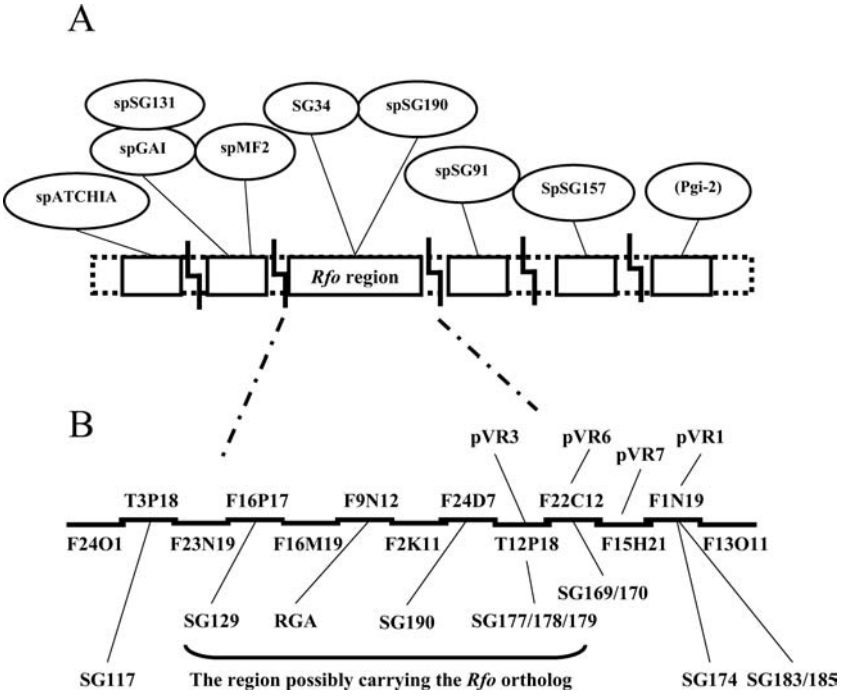

Fig. 2 (**A**) Arrangement of markers with respect to each other and to the *Rfo* gene, according to the results obtained for the unstable rapeseed progeny. The specific markers are named according to the ACGM from which they were developed. For example, the ATCHIA ACGM gave the specific PCR marker spATCHIA. SG34 is a specific PCR marker developed from RAPD9 (Delourme et al. 1998). spATCHIA, spSG91 and spSG157 are from various regions of chromosome 5, spGA1 and spSG131 are from chromosome 4, spSG190 is from chromosome 1, and spMF2 and SG34 have no clear ortholog in the *Arabidopsis* genome. The Pgi-2 isozyme was analysed on a subset of the progeny and is placed here according to the obtained results. *Boxes* represent "blocks" of markers that are retained or lost together, and *vertical lines* indicate breaks in colinearity with the *Arabidopsis* genome. (**B**) *Arabidopsis* contig map corresponding to chromosome 1. Extended AFLP markers (pVR) are indicated above the corresponding targeted BAC. The ACGMs tested in rapeseed (see Table 3, or Supplementary data for ACGMs not showing polymorphism) are indicated below the contig, those showing polymorphism in restored rapeseed are clustered in the horizontal bracket defining the *Arabidopsis* genomic region that may carry the *Rfo* ortholog. SG117 and SG169/SG170 are the markers determining the outer limits of the region of synteny between the *Arabidopsis* genome and the radish introgression

(SD=653.6). We successfully identified PCR products corresponding to rapeseed and radish orthologs by sequencing for nine target genes. Four polymorphic markers were identified. All of these markers made it possible to amplify an additional product in restored rapeseed, corresponding to the radish ortholog of the target gene. In addition, two of these markers also revealed the loss, in the SR1 line, of a PCR product corresponding to one rapeseed ortholog of the target gene (Table 3).

Use of an unstable rapeseed progeny for arranging markers on the radish introgression carrying the *Rfo* gene.

We used the unstable rapeseed progeny to determine the relative positions of the ACGMs markers described above, and produce a schematic diagram of the radish introgression in rapeseed.

We used ACGMs derived from three *Arabidopsis* target genes on chromosome 5 (ATCHIA, SG91, SG157), two on chromosome 4 (GA1 and SG31), one on chromosome 1 (SG190), and two markers with no identified ortholog in *Arabidopsis*: MF2 (Table 1) and a previously identified RAPD marker (Delourme et al. 1998). We developed radish-specific PCR markers, using the sequence alignments generated during the identification of ACGMs, and from the sequence of the cloned RAPD marker. These primers made specific amplification possible in the restored rapeseed plants, and were therefore used as dominant markers for the restoration locus. These dominant PCR markers were then tested in the 63 offspring of the unstable rapeseed progeny. We investigated the presence or absence of the characteristic amplification fragment for each of these specific primer pairs. For a comparison with previous reports, we also tested the Pgi-2 isozyme on a subset of the progeny to get an idea of its relative position. We assumed that markers lost together were more-likely to be linked than markers lost independently. Based on the results obtained, we drew a schematic diagram of the radish introgression around *Rfo* (Fig. 2A). We found that markers from the same *Arabidopsis* genomic region were not linked in the radish introgression. Two markers spSG91 and spSG131 located on each side of *Rfo* from the results on the unstable progeny were mapped in the radish segregating

population. They were confirmed to be on each side of *Rfo* at 26.3 cM and 24 cM, respectively (Fig. 1). The spSG190 and SG34 markers are perfectly linked to *Rfo* in this family. The spSG190 marker was mapped in the radish mapping population. No recombination between the *Rfo* gene and the spSG190 marker was found (Fig. 1), confirming that the radish ortholog of the *Arabidopsis* target gene is very tightly linked to *Rfo,* both in radish and in rapeseed. Fig. 2B shows the *Arabidopsis* contig in the corresponding region of chromosome 1 and the position of targets for the various ACGMs tested in this study. The positions of (extended) AFLP marker-orthologs identified in radish mapping studies are also indicated.

Our results suggest that the region carrying the *Rfo* gene is orthologous to the region comprising BACs F16P17, F16M19, F9N12, F2K11, F24D7 and T12P18 on *Arabidopsis* chromosome 1.

Discussion

The genomic data available for *Arabidopsis* are potentially very useful for the development of non-anonymous markers and the identification of candidate genes in other species, especially other members of the Brassiceae family, which includes important crop and vegetable species such as rapeseed, mustard, cabbage and radish (Brunel et al. 1999; Fourmann et al. 1998, 2001, 2002: Lan et al. 2000; Sillito et al. 2000; Paterson et al. 2001). One aim of this study was to identify the region of the *Arabidopsis* genome syntenic to the *Rfo* locus, to facilitate (1) the development of markers for use in the breeding of rapeseed restorer lines; and (2) the identification of the *Rfo* gene by identifying potential orthologs (candidate genes) in the *Arabidopsis* genome. It has been suggested that the radish introgression carrying the *Rfo* locus, which is present in restored rapeseed, is large, resulting in the replacement of about 50 cM of the rapeseed genome (Delourme et al. 1995, 1998; Pellan-Delourme and Renard 1988). Several studies on macro- and micro-synteny in plant genomes have been published in recent years (Devos et al. 1999; Bennetzen 2000). In cereal crops, such as maize, wheat, rice and millet, the large-scale colinearity of genomes has been preserved in large blocks, often comprising entire chromosomes or chromosome arms (Gale and Devos 1998), but fine-scale studies have reported small rearrangements of gene content, and of gene order and orientation, together with the disruption of colinearity (Keller and Feuillet 2000; Bennetzen and Ramakrishna 2002). In the Brassiceae, major rearrangements, including gene duplication, were reported following large-scale genome analyses (Lagercrantz and Lydiate 1996; Lagercrantz 1998). However, fine-scale studies have detected colinearity between *Arabidopsis* and *Brassica* crop-species genomes, around specific loci (Lagercrantz et al. 1996). Despite the apparently complex syntenic relationships of these species, the identification of collinear genomic regions is possible and is of potential value for genetic mapping and the identification of genes of agronomic importance (Lan et al. 2000; Fourmann et al. 2001, 2002; Paterson et al. 2001; Parkin et al. 2002).

In this study, we identified *Arabidopsis* genomic regions homologous to the *Rfo* locus region, by two different approaches.

The development of ACGMs made it possible to map radish orthologs of *Arabidopsis* genes unambiguously on the introgression carrying the *Rfo* gene in rapeseed. Thanks to intron divergence between species, it was possible in all cases to detect polymorphism between the radish alleles and the rapeseed alleles, at least by non-denaturing polyacrylamide-gel electrophoresis. Furthermore, direct sequencing of individual PCR products was used to verify that the orthologous gene was indeed amplified, and to identify radish and *Brassica* alleles unambiguously. Consequently, the absence of the radish ortholog in the PCR products from restored rapeseed clearly indicates that this radish gene is not carried by the radish-introgression in these lines. Conversely, the unequivoqual identification of a radish ortholog of an *Arabidopsis* gene in the genome of restored rapeseed lines provides a marker for the *Rfo* gene. In our initial global survey, we identified radish genes linked to *Rfo* in rapeseed that were orthologs of the *Arabidopsis* genes present on chromosomes 4 and 5, and showed that the apparent mosaic of regions present in the introgression consisted of limited genomic fragments, at least on this scale.

Genetic mapping in radish, combining AFLP techniques and BSA, led to the identification of four AFLP markers that were completely linked, and ten AFLP markers that were closely linked (within 3.4 cM) to the *Rfo* gene. The cloning and sequencing of AFLP markers, or extended markers, led to identification of the corresponding regions on *Arabidopsis* chromosomes 1 and 5. Three of these markers (pVR3, pVR7 and pVR6) were aligned with chromosomes 1 and 5, but the corresponding E-values were lower for chromosome 5 than for chromosome 1. These results are not surprising given the duplications observed in the genome of *Arabidopsis* (Blanc et al. 2000). It has been shown that duplications have occurred between *Arabidopsis* chromosomes (Kowalski et al. 1994; Blanc et al. 2000; Grant et al. 2000; Ku et al. 2000; Parkin et al. 2002), particularly between chromosome 5 and chromosome 1. This "*in silico* positioning" method has been successfully used in studies of the colinearity between *Arabidopsis* chromosome 5 and the corresponding regions of the *B. napus* genome (Parkin et al. 2002), and in the search for a candidate gene for a QTL conferring resistance to white rust in *Brassica rapa* species (Kole et al. 2002).

Combining these two set of results, and considering the genetic distances observed in radish, we focused on two *Arabidopsis* regions, on chromosomes 1 and 5, and tried to reduce the distance between the tested ACGMs. We identified four new genes (of the nine amplified and 11 tested) in the centromeric region of chromosome 5, and four new genes (of the seven amplified and eight tested)

in a 2-cM region of chromosome 1, for which orthologs were present on the radish introgression.

Unambiguous identification of a region of the *Arabidopsis* genome syntenic to the *Rfo* locus required determination of the arrangement of these markers with respect to each other and to *Rfo*. This information was provided by genetic mapping in radish for AFLP-derived markers. We used an unstable restored rapeseed line that spontaneously lost the *Rfo* locus and/or markers linked to the radish introgression, to determine the relative positions of the specific PCR markers (derived from ACGMs) around the *Rfo* gene. This made it possible to arrange all the tested markers in blocks according to their positions relative to the *Rfo* locus and to each other. As this analysis was performed on the progeny of a single plant, we cannot rule out the possibility that the relative positions of the markers established in this progeny is, at least in part, due to aspects of the radish introgression specific to this particular plant. Nevertheless, the positions of the three markers (spSG91, spSG190 and spSG131) that were common to the genetic map and to the map obtained from the unstable progeny were consistent. In addition, they indicate that more than 50 cM of the radish genome is present in the genome of restored rapeseed.

Our results, concerning both the characterisation of the radish introgression in restored rapeseed and the '*in silico* positioning' of markers around *Rfo* in radish, clearly identify a 2-cM region defined by *Arabidopsis* BACs F16P17, F16M19, F9N12, F2K11, F24D7 and T12P18. However, the colinearity between radish and *Arabidopsis* genomes in the *Rfo* region may cover a contig spanning these six BAC clones. Nevertheless, it is clear from our data that colinearity between the radish introgression carrying *Rfo* in rapeseed and the *Arabidopsis* genome is limited, because ACGMs corresponding to *Arabidopsis* target genes unlinked to the identified region have been identified in restored rapeseed. Interestingly, in some cases, we identified ACGMs displaying polymorphism due to the loss of a rapeseed-specific PCR product in homozygous restored rapeseed plants. In such cases, the rapeseed ortholog of the target gene may have been lost during introgression of the radish material into the rapeseed genome. Such a situation has already been observed and described for a *Pgi-2* allele (Delourme et al. 1998). This strongly suggests that the region of the rapeseed genome replaced by the radish introgression carries orthologs of the corresponding target genes. As these target genes are dispersed in the *Arabidopsis* genome, colinearity is unlikely to have been conserved between the *Arabidopsis* genome and the region of the rapeseed genome replaced by the radish introgression.

The identification of a region of the *Arabidopsis* genome syntenic to the radish *Rfo* locus opened up the possibility of looking for candidate genes for the *Rfo* ortholog in the *Arabidopsis* genome. Bentolila et al. (2002) and Koizuka et al. (2002) recently reported the identification of PPR protein-encoding genes such as the *Petunia Rf* gene, and the radish *Rfk1* gene, respectively. The region of the *Arabidopsis* genome syntenic to the *Rfo* locus contains several PPR protein-encoding genes, some of which are predicted to encode peptides that are imported into the mitochondria.The *Arabidopsis* ortholog of the *Rfo* gene may therefore be carried by this genomic region. However, we cannot rule out the possibility that the radish genome contains, at this locus, a gene for which there is no ortholog in *Arabidopsis*. In any case, fine physical mapping of the *Rfo* region in radish is required for the identification of this gene (Desloire et al. 2003).

This work demonstrates that the complete genome sequences of model plants constitute a rich and valuable source of readily developed markers for important traits in crop species.

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