Theor Appl Genet (2005) 111: 736–746
DOI 10.1007/s00122-005-2059-8

ORIGINAL PAPER

C. Primard-Brisset · J. P. Poupard · R. Horvais
F. Eber · G. Pelletier · M. Renard · R. Delourme

A new recombined double low restorer line for the *Ogu*-INRA cms in rapeseed (*Brassica napus* L.)

Received: 21 December 2004 / Accepted: 26 April 2005 / Published online: 18 June 2005

Abstract A major objective of breeders using the *Ogu*-INRA cytoplasmic male sterility (cms) system in rapeseed (*Brassica napus* L.) is to obtain double low restorer lines with a shorter introgression and a good agronomic value. The development of low glucosinolate content (low GC) restorer lines often occurs through the deletion of a part of the introgression. One of these lines has lost the radish *Pgi-2* allele expression, without recovering that of the rapeseed *Pgi-2* allele. This line shows a defect in the meiotic transmission of the restorer gene *Rfo* and a very poor agronomic value. We initiated a programme to force non-spontaneous recombination between this *Rfo*-carrying introgression and the rapeseed homologous chromosome from a low GC *B. napus* line. Gamma ray irradiation was used to induce chromosome breakage just prior meiosis aiming at just such a recombination. Low GC cms plants were crossed with the pollen of irradiated plants that were heterozygous for this introgression. The F_2 families were scored for their vigour, transmission rate of *Rfo* and female fertility. One family of plants, R2000, showed an improved behaviour for these three traits. This family presented a unique combination of molecular markers when compared to other rapeseed restorers analysed, which suggests that the recombination event allowed the recovery of *B. oleracea* genetic information that was originally replaced by the radish introgression in the original restorers. This resulted in a duplicated region (originating from radish and *B. oleracea*) on the chromosome carrying the introgression in the R2000 family.

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00122-005-2059-8

Communicated by H.C. Becker

C. Primard-Brisset · J. P. Poupard · G. Pelletier
SGAP INRA, 78026 Versailles Cedex, France

R. Horvais · F. Eber · M. Renard · R. Delourme (✉)
UMR INRA-Agrocampus Rennes, APBV,
BP 35327, 35653 Le Rheu Cedex, France
E-mail: Regine.Delourme@rennes.inra.fr
Tel.: +33-2-23485133
Fax: +33-2-23485120

Introduction

The breeding of restorer lines for the *Ogu*-INRA cytoplasmic male sterility (cms) system in rapeseed (*Brassica napus* L.) as a means of developing rapeseed hybrids has been a major objective during the past few years. Beginning with the *Ogura* rapeseed restored material obtained by Heyn (1976), extensive backcross and pedigree breeding has been necessary to both improve the female fertility of the restorer lines and decrease the glucosinolate content (low GC) (Delourme et al. 1991, 1995). However, the breeding of these restorer lines can still encounter some difficulties (introgression rearrangements, possible linkage with negative traits) due to the large size of the radish introgression. Random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers tightly linked to the *Rfo* gene have been identified, and recombination around the introgressed radish genome fragment in the *B. napus* restorer line has been shown to be very reduced (Delourme et al. 1994, 1998). This insertion has proved to be unstable on occasion, losing partial segments, thereby providing material for classifying molecular markers surrounding the *Rfo* gene (Giancola et al. 2003). Low GC restorer lines have often been developed through the deletion of a part of the radish introgression (Delourme et al. 1998, 1999). However, these events did not result from recombination with the orthologous chromosome of rapeseed as, until now, the loss of radish information was not necessarily compensated for by the recovery of the corresponding rapeseed information. One manner to overcome this problem is to clone the *Rfo* gene and produce transgenic restorer lines that are devoid of all surrounding radish genome and have not undergone the

loss of a rapeseed chromosome segment that the introgression may have caused. The recent successful cloning of the *Rfo* gene has led to the construction a fine molecular map of the *Rfo* region in the radish species itself (Brown et al. 2003; Desloire et al. 2003; Giancola et al. 2003; Imai et al. 2003; Koizuka et al. 2003).

Another way to obtain improved restorer lines—without the use of transgenic lines—is to attempt to force recombination between the radish insertion and the homoeologous *B. napus* chromosome in the hope of getting a shorter radish insertion. Ionising irradiation is known to induce double-strand-breaks (DSBs). Repair can occur via homologous recombination—although in plants this is quite inefficient—or, preferentially, via aberrant end rejoining leading to rearrangements that often include deletions, inversions or insertions (Shirley et al. 1992; Gorbunova and Levy 1999). The expected event could either be the result of a homoeologous recombination or be the result of DSB repair.

Two approaches can be used to obtain this event. The first one is somatic hybridisation, which has been proven to facilitate the integration of donor chromosome fragments into a receptor genome when the donor has been exposed to ionising radiation (X-ray or γ-rays) or to UV light. Forsberg et al. (1998a, 1998b) showed that the higher the exposure dose is, the higher the degree of asymmetric hybrids obtained by the sorting out of DNA from the donor protoplasts. Sakai et al. (1996) successfully transferred the restorer *Rf* gene from *Raphanus sativus* into a cms-*B.napus* cybrid line by protoplast asymmetrical fusion and produced a complete male-sterile/restored fertility system using X-ray irradiation.

The second approach is sexual cross with donor pollen issued from an irradiated plant prior to its pollen formation. Muhanna et al. (1991a, b) studied the cytological effects of gamma irradiation on pollen mother cells (PMCs) from plants raised from irradiated tobacco seeds (0–1,000 Gy) or from excised inflorescences (3,000 Gy). They observed fragmentation and stickiness of the chromatin, which subsequently promoted the formation of chiasmata even between non-homologous chromosomes. Kaneko et al. (1992) observed that gamma irradiation of dry seeds of an alien chromosome addition line of radish with a single kale chromosome enhanced the proportion of PMCs with one trivalent, which increased the expectation of translocations in the following generations.

The investigation reported here is based on this second approach in which the irradiation of an heterozygous restored rapeseed plant carrying the *Rfo* introgressed radish segment associated to a normal rapeseed chromosome would generate chromosomal lesions. During subsequent meiotic events, natural repair mechanisms would then hopefully induce recombination between the orthologous chromosome segments, and these would be transmissible through rearranged pollen grains. As our objective was to obtain an improved low GC restorer *B. napus* line, our breeding programme was initiated from a low GC spring restorer selected line (R211) which carries a deleted radish insertion; for example, having lost the radish *Pgi-2* allele (as in Delourme and Eber 1992). This R211 line was also chosen because it has a very poor agronomic value. Three traits are among its most deleterious characters: (1) the fertile:sterile ratio in F_2 progenies derived from this material is lower than expected; (2) its low female fertility is expressed by a poor seed set by selfing; (3) the whole homozygous plant exhibits a very poor vigour. Consequently, it was possible to base selection in the F_2 generation derived from irradiated material on phenotypic observations of these three chosen traits.

One family, R2000, showed the expected positive expression of the analysed characters and was selected and characterised by molecular markers defined around the restorer gene. This R2000 family has a new combination of these markers, indicating that a recombination event took place in the irradiated heterozygous plant.

Materials and methods

Plant material

To obtain the required heterozygous restored genotype R211*Dk in a low GC rapeseed background, we crossed the low GC spring rapeseed restorer line R211 (lacking the radish *Pgi-2* allele and also the original *Brassica oleracea* one) to a low GC spring French line, Drakkar. The spring low GC cms Wesroona (Australian origin) and cms Pactol lines were used as female parent for testcrosses. Controls in molecular analysis were: *Ogura*-INRA restorer lines derived from the French winter cultivar Samouraï carrying the complete (RRH1) or incomplete (R113) introgression, an Asiatic *Ogura* (*Ogu*) restorer radish line (D81), rapeseed maintainer lines (Samourai, Pactol and Drakkar), a European maintainer (Rad7) and cms *Ogu* (7ms) radish lines. The *Brassica* diploid species *B. oleracea*, (a cauliflower cultivar) and *B. rapa* cv. Asko as well as the wild radish (*Raphanus raphanistrum*) were also included in the analyses.

Irradiation treatment

Either seeds or inflorescences from heterozygous R211*Dk restored plants were irradiated with γ-rays from a panoramic Co60 source in a controlled area. In a given time of exposure, ranges of radiation doses could be tested simultaneously on several sets of material arranged around the source at precisely recorded positions, as the dose flow is a function of the distance to the Co60 source (controlled by the Fricke dosimetry test). Seeds (25–100 per assay) were exposed to doses ranging from 150 Gy to 2,500 Gy during either 1 h [short irradiation (SIR)] or 16 h [long irradiation (LIR)], under three moisture conditions: (1) either soaked in water; (2) after 1 h of imbibition; (3) dry. Whole flowering plants

in their pots were irradiated (six per assay). Each one was arranged horizontally around the source with the first flower crown at the chosen dose flow position. Distances of the axillary nods receiving decreasing dose flow were recorded. Flowers were exposed to radiation doses ranging first from 250 Gy to 2,000 Gy, then from 65 Gy to 90 Gy during 1 h (LIR) or 6 min (SIR). An addition two sets of 35 plants received 65 Gy during 1 h.

Testcrosses and F_2 production

The R211*Dk F_1 plants issued from irradiated dry seeds were selfed to produce M2 and M3 progeny and crossed to cms Wesroona plants to produce F_1' progeny (Fig. 1). Irradiated R211*Dk F_1 flowering plants were transferred to an insect-proof greenhouse following removal of flower buds larger than 2 mm (off the meiosis stage) to produce the F_1' progeny on the cms Wesroona line. Hand pollinations were carried out keeping records of the flower rank (pooled by ten) and the inflorescence axes rank used. Pollen viability was estimated by the Alexander dye coloration technique (Alexander 1969), and by the ability to produce offsprings.

The F_2 families were produced from the fertile derived F_1' plants and were precisely sown in field assays along with M2/M3 families and non-irradiated controls. Only families having produced 100 seeds or more were retained for field analysis.

Phenotypic selection and production of homozygous lines

Two visual criteria were scored over a 2-year period (2000–2001) in field assays on 965 F_2 offspring (860 from buds and 105 from seeds), 218 M2/M3 families (158 from buds and 60 from seeds) derived from irradiated plants and 54 controls: (1) goodness of fit of the ratio of fertile:sterile plants in the F_2 segregation was tested by estimating the confidence interval for the proportion of sterile plants ($\alpha = 5\%$); (2) female fertility (pod aspect and seed set) was recorded on a scale of 1 (high) to 7 (low).

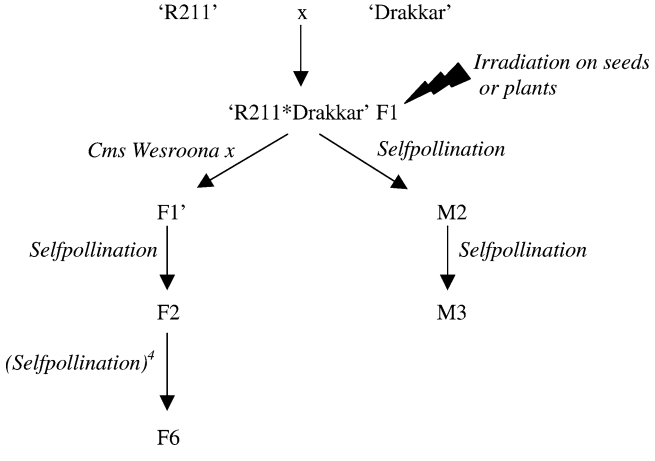

Fig. 1 Pedigree of the plant materials studied

Advanced selfed generations were obtained from the selected F_2 families either in the field or the greenhouse, allowing identification of homozygous *Rfo*/*Rfo* lines (F_4–F_6) for further analyses (Fig. 1). The number of seeds per pod was assessed on these homozygous F_4 plants and on heterozygous plants issued from testcrosses of homozygous F_4 plants onto the Pactol cms line. Three samples of 50 pods were taken per progeny. Glucosinolate content was estimated by near infrared reflectance spectrometry (NIRS) on the above three samples of 50 pods.

Molecular analysis

DNA for the PCR analyses was extracted from a piece of leaf or flower bud in an Eppendorf tube using either a quick preparation with isopropanol precipitation (derived from Edwards et al. 1991) or a cleaner DNA extraction (G. Gendrot, personal communication) as follows. For each assay, 400 µl of buffer [200 m *M* Tris-HCl pH 7.5, 250 m *M* EDTA, 25 m *M* NaCl, 0.5% sodium dodecyl sulfate (SDS)] was added to the tube, and the tube then vortexed for 5 s and centrifuged for 7 min. A 350-µl aliquot of the supernatant was then treated with 350 µl phenol:chloroform:isoamylic alcohol, (25:24:1), centrifuged 15 min and precipitated with 95% ethanol plus 10% 3 *M* NaCl. The supernatant was rinsed with 70% ethanol, resuspended in 100 µl TE (10:1) plus 10 µg/ml RNAse and quantified.

Isozyme analysis was performed as in Delourme and Eber (1992). We choose molecular markers from the radish introgression and from the *B. napus* genome that were positioned relatively to *Rfo* either on a schematic diagram of the introgression obtained from unstable restored rapeseed progeny or on the radish and rapeseed genetic maps (Fig. 2). This map and the markers were derived from bulk segregant analysis (Delourme et al. 1994, 1998, 1999; P. Barret, unpublished data; Lombard and Delourme 2001) and from colinearity studies between the radish introgression and *Arabidopsis* (Giancola et al. 2003, unpublished data; Desloire et al. 2003). The *Pgi-2* gene was positioned on the radish map from isozyme analysis on a F_2 radish population. Three sequence characterised amplified region (SCAR) markers, ScA14, ScH03 and SG34 (Table 1), were derived, respectively, from the RAPD markers A14.800, H03.1350 and J01.1000 (RAPD2, RAPD6 and RAPD9 in Delourme et al. 1998). For these SCARs, the PCR conditions used were as follows: in a final volume of 20 µl, 1 U Eurobio *Taq*II, 0.8 m *M* $MgCl_2$, 150 µ*M* of each dNTP, 0.4 µ*M* of each primer, 10 ng of total DNA sample was subjected to heating at 94°C for 2.5 min followed by 30 cycles of 30 s at 94°C, 1 min at 55°C and 2 min at 72 C, and a final extension step consisting of 5 min at 72°C.

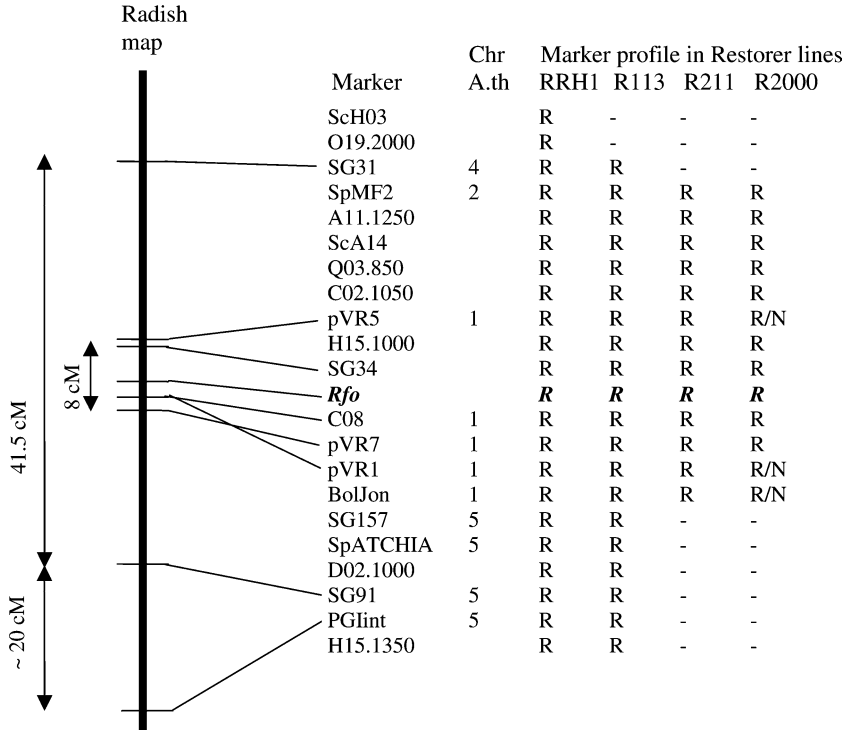

Fig. 2 Schematic diagram of the introgression obtained on the different restorers (RRH1, R113, R211 and R2000). When known, the position of the markers on the genetic radish map is indicated. Otherwise, their position on the introgression was deduced from restorer plants with rearranged introgression (Giancola et al. 2003). *R* Presence of a radish fragment, – absence of the radish fragment, *R*/*N* presence of a radish fragments and of a *B. napus* fragment that was originally absent from the restorers

Four additional PCR markers (Table 1) were defined to characterise the R2000 family in comparison to the known rapeseed restorers R211, RRH1 and R113, using the *Arabidopsis-Brassica* micro-synteny and genomic sequences available in the *Brassica* Data base (UK-CropNet DB) to choose specific primers (as in Fourmann 1998). Two markers were orthologous to the *Arabidopsis* phosphoglucose isomerase (PGI) gene (locus AT5G42740.1) on the MJB21 genomic clone on chromosome V. The PCR upper primer of PGIol was chosen to be specific to the *B. oleracea* genome. It overlaps a small 9-bp deletion present in the *B. oleracea Pgi-2* sequence (EM: AF258277, 94–102 bp) relative to the *B. rapa* one (EM: AF258278) (F. Budar, personal communication). Two markers (CP418L and BolJon) were derived from SG129, which was found to be close to *Rfo* by Giancola et al. (2003). The DNA amplifications were performed in a final volume of 25 µl, with using the buffer kit recommended by the kit manufacturer plus $MgCl_2$ for 1 U Eurobio *Taq*II, 0.25 m *M* of each dNTP, 0.5 µ*M* of two relevant primers and about 20 ng of total DNA sample. Samples were subjected to heating at 94°C for 3 min, followed by 30 cycles of 45 s at 94°C, 50 s at 55°C, 1 min 30 at 72°C, followed by 7 min at 72°C. These were generally separated on 1.2% agarose gels. The PCR products were validated by sequencing (GENOMEXPRESS). *blast* searched were carried out in the National Center for Biotechnology Information (NCBI) and UK Crop Net *Brassica* data bases, and alignments were made to deduce and compare reliable consensus sequences using MULTIALIN software (INRA, Toulouse).

Table 1 PCR primers for the three SCAR markers and the four markers defined in the present study

Marker name	Primer name	Sequence (5′ → 3′)
ScA14	ScA14 U	TCT GTG CTG GTG GGA TGA AAG
	ScA14 L	TCT GTG CTG GCT ACT TAT TCA TAC AG
ScH03	ScH03 U	AGA CGT CCA CCA TAC C
	ScH03 L	AGA CGT CCA CTC CTA GTT C
SG34	SG34 U	TAT ATT GTA CCT TTG CCT CTT C
	SG34 L	CTT TTC TTT TAG TTT TTG GTT T
PGIint	PGIint U	CAG CAC TAA TCT TGC GGT ATG
	PGIint L	CAA TAA CCC TAA AAG CAC CTG
PGIol	PGIol U	TCA TTT GAT TGT TGC GCC TG
	PGIol L	TGT ACA TCA GAC CCG GTA GAA AA
CP418L	CP418 U	AAT TTC TCC ATC ACA AGG ACC
	SG129U	GTT CAT TGA CAT CGG AAA CTT CT
BolJon	BolJon U	GAT CCG ATT CTT CTC CTG TTG
	BolJon L	GCC TAC TCC TCA AAT CAC TCT

Results

Choice of the sublethal irradiation dose

Radiation doses were first tested on seeds. Germination occurred at all doses, but plantlet survival was dependant upon the moisture content of the seed at the time of irradiation: a 350-Gy dose was lethal when the seeds were soaked, a 700-Gy dose was lethal when the seeds were moistened (1-h imbibition) and a 2,500-Gy dose was lethal when the seeds were dry. We subsequently studied F_2 progenies issued from moistened seeds irradiated with 500 Gy (LIR) and dry seeds irradiated with 2,100 Gy (LIR and SIR), allowing 70% survival.

Flowers of whole irradiated plants appeared very sensitive to the treatment as they were all burned in the first assay (250–2,000 Gy in 1 h or 6 min). However, after the irradiated plants had been cultured for 1 month of culture, some axillary buds started to develop. The higher the γ-ray dose received by the plant, the lower the surviving buds were initiated on the stem. This allowed us to estimate a sublethal dose of about 70 Gy, which was subsequently verified in a second experiment (range of 65, 90 and 110 Gy). Only the lowest dose, 65 Gy, applied in either 1 h (LIR) or 6 min (SIR) permitted the survival of the first inflorescence and pollen maturation in flower buds less than 2 mm in size. The 65-Gy dose was chosen for further experiments.

Irradiation effect on phenotype

The phenotype of the irradiated flowering plants was unchanged immediately following the irradiation treatment, but their growth pattern was disturbed, as was that of plants issuing from seed subjected to a high dose of gamma irradiation.

The dose flow applied (the energy of a 6-min treatment is tenfold higher than that applied in 1 h) affected differentially the fertility of whole irradiated plants. First, the gamete viability was reduced. In control flowers in the greenhouse, 97% of the pollen grains showed stainability (indicating viability). In flowers scored on the first four or five inflorescences emerging after a LIR treatment, pollen stainability was estimated to be between 40% and 60%; this dropped to 30–50% for SIR plants. Second, seed set was reduced accordingly in crosses with cms Wesroona plants, (about 20 seeds per pod in LIR and seven in SIR). Female fertility was also affected in selfing; LIR pods showed a low number of seeds and SIR ones were always almost empty.

Selection of R2000 family

Based on results from earlier investigations, we expected the percentage of fertile plants in segregating F_2 progenies derived from R211 to be lower than 75% since the proportion of homozygous restored plants was found to be lower than 25%. In addition, in such progenies, the homozygous restored plants had a very poor seed set. In the field, they had short curved pods with few seeds and a less erect stem, which contrasted with the normal looking heterozygous fertile plants. The screening was thus based on these two criteria based on the hypothesis that an improved family would have a 1:3 sterile:fertile ratio and homogeneity of female fertility among the fertile offsprings. The sterile:fertile ratio was first calculated for the treated F_2 and M2/M3 families and for the control F_2 familes sown in the field assays (Table 2). A great range of variation was observed within each type of family, and the mean ratio was very similar (41–43%) between the three types of families. The percentages of families fitting the 1:3 (sterile:fertile ratio) were equal to 28–32% and were not significantly different between the three types of families ($\chi^2 = 0.48$; $P = 0.49$)

Second, the overall aspect of the inflorescences was observed in the families fitting the expected 1:3 sterile:fertile ratio. Most of these exhibited heterogeneous phenotypes among the fertile segregating plants, and these were assumed to have kept the defective R211 parental line trait. Only two treated families showed a homogeneous good female fertility. These were screened among families derived from irradiated flowering plants; none were sorted among families derived from treated seeds but only a small number of families were analysed. One selected family was a M2 (R2695, from the LIR treatment), while the other was a F_2 family (R2000, from the SIR treatment). The latter issued from the irradiation of the first ten flowers of the first inflorescence (10% of the tested F_2 progenies) and was unique among the 21 F_2 progenies issued from the same SIR plant.

The PGI isoenzyme analysis was performed on these two families. R2695 showed the typical segregation obtained from a heterozygous restored plant having lost the *Pgi-2* radish allele—i.e. the same pattern as the one obtained from R211. On the other hand, R2000 progeny

Table 2 Results of sterile:fertile plant segregation in the control and treated families

	Number of families	Total number of plants	Percentage of sterile plants		Number of families fitting a 1:3 ratio
			Mean	Range	
Control F_2	54	4,736	42	23–67	15
Treated F_2	965	62,840	43	13–90	310
Treated M2/M3	218	13,084	41	5–76	71

Table 3 Observed segregations across the successive generations in self-pollinations and testcrosses of R2000

Generation	Number of fertile plants	Number of sterile plants	Total number of plants	Percentage of fertile plants	χ^2 (P) for 3:1 ratio[a]
Self-pollinations					
F_3	304	111	415	73.2	0.63 (0.42)
F_4	315	121	436	72.2	1.69 (0.19)
F_5	774	384	1158	66.8	40.91 (< 0.0001)
Testcrosses					
From F_4	83	86	169	49.5	0.09 (0.76)
From R211	70	98	168	41.6	5.0 (0.025)

[a] χ^2 for homogeneity between $F_3/F_4/F_5$ (in self-pollinations) = 8.12 ($P = 0.004$); χ^2 for homogeneity between F_3/F_4 (in self-pollinations) = 3.31 ($P = 0.07$); χ^2 for homogeneity between F_4/R211 (in testcrosses) = 66.5 ($P = 5.10^{-16}$)

Table 4 Number of seeds per pod assessed on the best R2000 F_5 families and on R211 in self-pollinations (SP) and in testcrosses (TC) for cms Pactol and rapeseed controls

Genotype	SP	TC
Drakkar	29.3	
Pactol	23.1	
R211	11.2	25.5
R2000	26.5 (24.0–31.1)	27.0 (24.0–28.7)

expressed the typical rapeseed pattern with the *Pgi-2* allele from the *B. oleracea* genome, which had been lost in R211, indicating the originality of this family. We then focused on this R2000 family.

In the following generations of the R2000 family, fertile:sterile segregation was assessed in the heterozygous families (Table 3). The fertile:sterile ratio fitted the 3:1 ratio in the self-pollinations from the F_3 and F_4 generations. The number of homozygous restored plants among the fertile plants was checked in the self-pollinations of F_3 plants and fitted the expected ratio (14 *Rfo*/*Rfo*:42 *Rfo*/*rfo*; $\chi^2 = 0.14$; $P = 0.71$). More advanced generations may reveal some residual abnormalities, such as a slightly deficit of fertile plants in the segregating F_5 population. However, we could verify an improvement in the R2000 line relative to the testcrosses obtained from F_4 families and R211 (Table 3), which gave significantly different ratios ($\chi^2 = 66.5$; $P < 0.0001$). In the F_5 generation, female fertility was assessed on screened homozygous progenies relative to that of R211 during self-pollination and in testcrosses on male-sterile Pactol (Table 4). The number of seeds per pod was significantly higher in the R2000 families than in R211 (Fig. 3), and differences in the number of seeds per pod between homozygous (self-pollination) and heterozygous (testcross) plants no longer existed. Glucosinolate content was assessed in F_5 families and in self-pollination for F_6 families. Some variation in glucosinolate content could still be observed among the R2000 families (Table 5).

Table 5 Glucosinolate content assessed on the best R2000 F_5 (Spring 2002) and F_6 (Spring 2003) families and on R211 in self-pollinations (SP) and testcrosses (TC) for cms Pactol and rapeseed controls

Spring 2002		TC	Spring 2003	
Genotype	SP		Genotype	SP
Drakkar	11.7		Drakkar	17.3
Pactol	6.9		Pactol	6.6
R211	9.9	8.2	R211	13.4
R2000	14.9 (10.3–21.4)	10.9 (7.9–15.0)	R2000	17.8 (14.5–25.3)

Meiosis analysis

Meiosis of R2000 plants was compared to that of R211 plants. In two R211 plants, we observed 0.21 I(univalent) + 18.77 II (bivalent) + 0.07 IV (quadrivalent) (83.4% of cells with19 II); in 22 R2000 plants, we observed

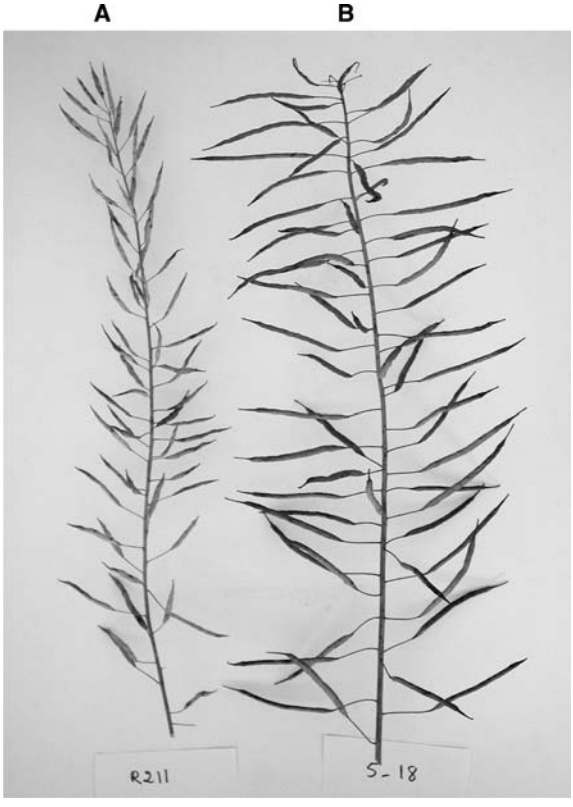

Fig. 3 Comparison between mature inflorescences of R2000 (**B**) and its progenitor R211 (**A**)

0.20 I + 18.87 II + 0.02 IV (88.6% of cells with 19 II). One plant showed an irregularity with $2n = 40$. Following the first cross of R2000 with a winter cultivar (20 plants), we observed a few more multivalents: 0.21 I + 18.71 II + 0.02 III + 0.09 IV (81.75% of cells with 19 II). Although no direct controls were made at the same moment, it appeared that meiosis was still slightly affected in this R2000 progeny.

Analysis of markers in the radish introgression

Molecular markers evenly spaced on the radish introgression were chosen to compare R2000 to other restorer lines (Fig. 2). The minimum size of the introgression in RRH1 was estimated at 60 cM, excluding markers that are thought to be outside this interval (ScH03, O19.2000 and H15.1350); R113 had retained all of the radish markers but ScH03 and O19.2000. In comparison to R113, R211 had lost markers on both side of the introgression (SpSG131, SpSG157, SpSG91, SpATCHIA, *Pgi-2* and the two RAPD markers, D02.1000 and H15.1350), but we had not enough markers to estimate precisely the size of the remaining radish introgression. At this point, no difference was apparent with respect to the screened markers between R2000 and its parental line R211.

Markers in the *PGI-2* gene

As the isozyme analysis indicated a modified *Pgi-2* pattern in the R2000 family, two PCR markers derived from PGI sequences were tested on the winter/spring rapeseed maintainers/restorers and on *B. oleracea* and *B. rapa* species, and the genomic origin of the sequence of the different fragments were compared. The results are summarised in Table 6.

PGIol amplified nothing in *Arabidopsis* nor in the tested radish, but it did amplify one band, homologous to the *B. oleracea* sequence in the spring cultivar Drakkar and in the F_1 (R211*Dk). The parental line R211 did not show any PGIol band. However, PGIolU primer specificity was not observed in the tested cultivars Samouraï and Darmor neither in the *B. rapa* species cv. Asko. The winter restored RRH1 and R113 lines, which lacked the *B. oleracea Pgi-2* isozyme allele, still showed one PGIol band that was found to be closely homologous to the *B. rapa* cv. Asko one. One G/T substitution was visible on a concentrated acrylamide gel upon restriction polymorphism following digestion by the *Mse*I enzyme (T/TAA) of the *B. oleracea* and *B. rapa* PGIol fragments. The homozygous R2000 PGIol/ *Mse*I profile appeared to be identical to those of *B. oleracea* (Primard-Brisset et al. 2003). A co-dominant marker, PGIint, was designed to amplify a PCR product in the various genomes tested (Fig. 4). By choosing primers in a conserved area between the *Arabidopsis* and *B. napus Pgi-2* sequences, only one PGIint band was obtained in each diploid species. The *B. oleracea* and *B. rapa* bands were of the same size (870 bp) on the agarose gel but could be distinguished from their sequences. Both were amplified in the rapeseed winter maintainer lines but not in the spring lines Drakkar and Wesroona in which only one clear PGIint DNA sequence was obtained, homologous to that of *B. oleracea*. As expected, R211 did not show any band. The winter restored lines RRH1 and R113, displayed the European radish band and a *Brassica* PGIint band. This latter band showed a sequence highly homologous to that of *B. rapa*, which was closer to the *B. rapa* cv. Asko sequence than to the Brassica DB *B. rapa* one.

The R2000 plants didn't show the PGIint radish band as did R211, but they did show the *Brassica* band that was homologous to the *B. oleracea* sequence, thereby confirming the hypothesis rising from isozyme analysis that the plants of the R2000 family have recovered the *Pgi-2* allele from *B. oleracea* (Fig. 4, sequence 1).

Markers closer to *Rfo* gene

The SG129 marker was located close to the *Rfo* gene on the segment of the radish insertion orthologous to the *Arabidopsis* chromosome 1, precisely at the overlapping ends of the AC011000 clone (BAC F16P17) and of its

Table 6 Summary of PGI and BolJon markers[a]

Marker	Band size (bp)	Maintainers		Restorers				*Brassica oleracea*	*B. rapa* cv. Asko
		Winter	Spring	Winter		Spring			
				RRH1	R113	R211	R2000		
PGIol	248	+ [A]	+ [C]	+ [A]	+ [A]	–	+ [C]	+ [C]	+ [A]
PGIint	950	–	–	+ [R]	+ [R]	–	–	–	–
	870	+ [A& C]	+ [C]	+ [A]	+ [A]	–	+ [C]	+ [C]	+ [A]
CP418L	650	+ [C]	+ [C]	–	–	–	+ [C]	+ [C]	–
BolJon	950	+ [C]	+ [C]	–	–	–	+ [C]	+ [C]	–
	870	+ [A]	+ [A]	+ [A]	+ [A]	+ [A]	+ [A]	–	+ [A]
	630	–	–	+ [R]	+ [R]	+ [R]	+ [R]	–	–

[a] [A], [C] and [R] indicate that the amplified fragment had a *B. rapa* cv Asko-type, a *B. oleracea* or a *R. sativus* sequence, respectively; [A&C] indicates that a *B. rapa* fragment and a *B. oleracea* fragment are co-amplified. Winter and spring only were included in the design of the tested genotypes

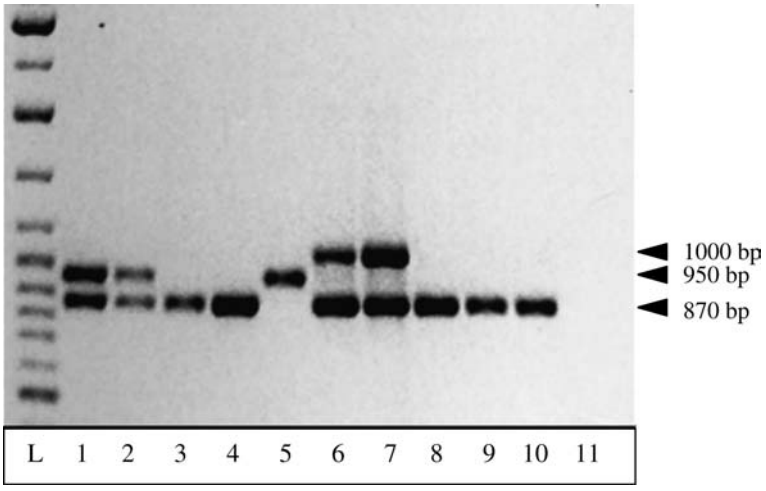

Fig. 4 Electrophoresis agarose gel showing PGIint marker analysis on the tested genotypes: the PGIint marker in diploid species *Brassica oleracea* (*3*), *B. rapa* (*4*), *R. sativus* line 7 (*5*), *B. napus* × wild *R. raphanistrum* hybrids (*6*, *7*), rapeseed maintainer Drakkar (*8*), Wesroona (*9*) and restored rapeseed R113 (*1*), RRH1 (*2*), R211 (*11*) and R2000 (*10*). *L* 100-bp ladder

adjacent contig AC07190 clone. The AC011000 and AC07190 clones are located about 300 bp distant from the *Rfo* gene on the *Arabidopsis* map. The SG129 co-amplified two distinct bands of about 900 bp and 850 bp in *B. napus*. Only the 900-bp band was amplified in all of the restored rapeseed lines but R2000. In *Brassica*DB, the 900-bp band sequence blasted partly to two *B. oleracea* clones [EMBH: 448336 (764 bp, orthologous to the beginning of AC011000 clone) and EMBH: 53971 (644 bp, orthologous to the AC07190 clone end)].

A genome-specific CP418 PCR marker was designed to exploit the 5-bp polymorphism found between the two *B. napus* SG129 bands for the CP418 lower primer. The CP418L primer has no homology with the orthologous area in *Arabidopsis*. The SG129U primer amplified one PCR band (650 bp) in *B. napus* maintainers, whose sequence was strictly homologous to the *B. oleracea* EM: BH448336 sequence (sequence 2). In the restored lines RRH1, R113 and R211, there was no CP418 band, indicating that the *B . oleracea* orthologue of the target gene could have been deleted following the radish insertion. On the contrary, R2000 showed the *B. oleracea* CP418 band (Table 6).

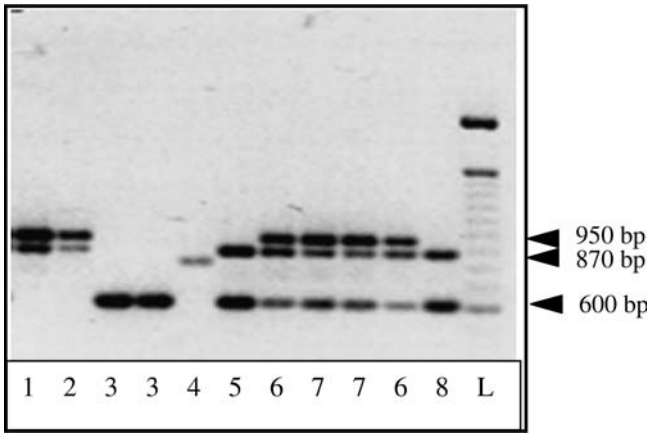

Fig. 5 Electrophoresis agarose gel showing BolJon marker analysis on the tested genotypes: the Boljon marker in Samourai (*1*), cms Pactol (*2*) *R. sativus* line 7 (*3*), *Arabidopsis thaliana* (*4*), restored rapeseed RRH1 (*5*) and R211 (*8*), F_1 hybrid R211*Dk (*6*) and R2000 (*7*). *L* 100-bp ladder

We also designed a co-dominant PCR marker, BolJon, by choosing primers in conserved areas between *Arabidopsis* and the orthologous *B. oleracea* clones, EM:BH53971 (for the U primer) and EM:BH448336 (for the L primer). We verified that it allowed amplification of a specific PCR profile in the different genotypes compared (Fig. 5). In *Arabidopsis*, we obtained a 815-bp BolJon band that was homologous to the overlapping segments of the *Arabidopsis* contigs. In radish, the restored D81 BolJon band was slightly smaller than its 600-bp counterpart of the Rad7 European line (not shown). In *Brassica* diploid species, the BolJon marker showed distinct bands of 950 bp in *B. oleracea* and 870 bp in *B. rapa* cv. Asko. Both were obtained in *B. napus* and exhibited a high homology, except for a long insertion of 75 bp in the *B. rapa* BolJon orthologue of the *B. napus* sequence (sequence 3).

In the restorer lines RRH1, R113 and R211, the BolJon marker showed the 600-bp radish band plus only the 870-bp *B. rapa* cv. Asko-type band (Fig. 5). In contrast, R2000 showed the 600-bp radish band plus the two *B. napus* BolJon bands (Figs. 2, 5).

In order to confirm that R2000 recovered some *B. oleracea* genetic information, markers of the *B. napus* linkage group LG15, in which the radish introgression has occurred, were screened on the parental lines, i.e. R211, Drakkar and Wesroona. Five markers (four RAPDs, AE19.700, AJ06.1100, O20.1380, M13.1420, and one specific PCR marker, IID12.1), which are located close to the position of the introgression or in the replaced region (Delourme et al. 1998; Lombard and Delourme 2001), were present in Drakkar and absent in R211 and Wesroona. These markers spanned a 42-cM segment in *B. napus* and were found to be present in R2000 as in Drakkar. Two other markers, pVR1 and pVR5 (Fig. 2), exhibited a co-dominant pattern between the *B. napus* maintainer and restorer lines. The two bands were shown to be present in R2000 for these two markers.

Localisation on the *B. napus* genetic map

We checked whether or not the radish introgression in R2000 has taken place in the same linkage group as it was in the original restorers. Two markers, AE19.700 and AJ06.1100 from LG15, were mapped relatively to *Rfo* in a BC_1 segregating progeny derived from R2000 crossed to the Pactol cms line. These two markers were found to be linked with each other ($d = 13$ cM) and linked to the radish introgression. The distance between AE19.700 and *Rfo* was 4.2 cM.

Discussion

In this investigation, we selected a novel recombined low GC restorer line with good female fertility following gamma irradiation treatment of the first flowers of an heterozygous restored plant with a deleted radish introgression.

We choose to test progenies derived from either seeds or flowering plants irradiated with a sublethal γ-ray dose. In seeds, the treatment will have a delayed effect and, after meiosis, will give rise to plant sectors for any induced aberrations and hopefully to several F_1 families carrying the expected event. We most likely did not test enough F_2 progenies from treated seeds to recover any event for the analysed traits. When the PMCs are the target of the irradiation treatment on flowering plants just before meiosis, independent events will be created. These will be transmitted to individual F_1 plants after a testcross on cms plants and can be screened for among all of the F_2 progenies that are subsequently produced. In this manner, we selected the R2000 F_2 plants, which were obtained from the first crown of irradiated flowers (SIR, 65 Gy in 6 min).

As Sears (1993) pointed out, the frequency of recovery of desirable rearrangements is low. Consequently, a favourable selection must be performed. In our investigation, it is the choice of the poor line, R211, which determined the ability to, first, select in field for a rare event and, second, to characterise the R2000 family. Both of these were possible through the combination of two criteria: the sterile:fertile ratio, which enabled a first round of selection among the treated F_2 and M2/M3 families in comparison to the control ones, and the female fertility. The percentage of families fitting the 1:3 sterile:fertile ratio was not significantly different in the treated families than in the control ones, but the range of this ratio was greater among the treated F_2 families (8–96% fertile), perhaps due to the irradiation effect. The recovery of a normal sterile:fertile ratio, which is associated with a good homogeneity for female fertility among the fertile F_2 offsprings, allowed us to retain two families. Only the R2000 family showed a good overall vigour and a modified PGI isozyme pattern compared to R211 and was further characterised.

Molecular characterisation of the homozygous R2000 revealed that it presents a unique combination of markers when compared with the restorer lines of rapeseed analysed.

Interestingly, the two areas revealed by the PCR markers, BolJon and PGI, were different with respect to homology among the tested rapeseed genotypes. In either spring or winter lines, the *B. rapa* BolJon sequences were highly homologous and very close to that of *B. rapa* cv. Asko. The *B. oleracea* BolJon and CP418 marker were found to be *B. oleracea*-specific in all backgrounds. This was not the case with the PGI markers, as the *B. oleracea* PGI specificity found in the spring rapeseed lines was not recovered in the winter background nor in the diploid species *B. rapa* cv. Asko. Moreover, Samourai *B. rapa* PGI sequences were more homologous to *B. rapa* cv. Asko sequences than to the corresponding ones in the UKCropNet DB (EM: AF258276). It would be interesting to investigate other markers and other lines of different origins, as genetic diversity studies performed on oilseed *B. napus* germplasm have always shown a clear separation between winter and spring cultivars (Diers and Osborn 1994; Lee et al. 1996; Lombard et al. 2000).

Our marker analysis led to the conclusion that R2000 had recovered some *B. oleracea* sequences that were originally replaced by the radish introgression in all of the homozygous restored rapeseed lines. The PGI isozyme and DNA sequence analyses showed that R2000 had no radish PGI band, as in its deleted parental line R211, but it had gained one *Brassica Pgi-2* allele that was homologous to the *B. oleracea* one. R2000 had also gained the *B. oleracea* area closer to the *Rfo* gene, as designed by the BolJon sequences. The pVR1 and pVR5 markers present in the Drakkar region that correspond to the one that was originally lost were also shown to be present in R2000. For these three latter markers, the radish homoeologous sequences were still amplified in R2000. Moreover, mapping of the introgression in R2000 showed that it was located on the same linkage group as it was in the original restorers. Our hypothesis is that a recombination event took place between the introgressed chromosome and the corresponding *B. oleracea* rapeseed chromosome in the PMC that gave rise to R2000 plants. This event brought back at least some of the missing genetic information lost in the deleted R211 and resulted in a duplicated region, one from the radish and one from *B. oleracea* rapeseed genome. If chromosome breaks were induced by ionisation in R2000, repair took place within *B. oleracea* chromosomes, which were indeed homologous but impaired by the presence of the radish introgression on one of them. However, as we screened only one family, it is difficult to connect this event directly to the γ-radiation effect in a PMC and to know if the higher SIR energy of the gamma rays hitting the flower buds in a shorter time might have been determinant for the expected event recovery.

More markers are required to investigate whether the already shortened R211 insertion has been modified or not by the rearrangement, the size of the duplicated region and the precise structure of the new introgressed

chromosome. *Arabidopsis* genome structure have been studied in detail (Blanc et al. 2000), and comparative physical/genetic maps of *Arabidopsis* and *Brassica* have been established, assigning *Arabidopsis* marker loci on the *B. oleracea*, *B. napus* or other *Brassica* AFLP-, RFLP-, EST- or SSR-based linkage groups. Through the comparison of these recently acquired data (Babula et al. 2003; Li et al. 2003; Lowe et al. 2004; Lukens et al. 2003; Parkin et al. 2003), we found that one linkage group in *B. oleracea* and in *B. napus* (O9 in *B. oleracea* and N19 in *B. napus* in the Brassica UKcropNet DB) seems to present the same organisation of the stretches of collinear markers used here in the introgressed LG15. These comparative mapping data will be used to design new markers on the region of interest for further characterisation purposes.

The new arrangement in R2000 enabled the female fertility to be improved. This improvement can be related to the recovery of some of the *B. oleracea* chromosome segment that was originally replaced by the radish introgression and that was missing in the deleted parental line R211. The progeny of homozygous *Rfo*/*Rfo* plants derived from R2000 are completely male-fertile and stable. The transmission of the *Rfo* gene in successive progeny in testcrosses or in self-pollination from heterozygous plants was greatly improved, although some irregularities were still observed. More backcross generations are needed to precisely determine the transmission and the stability of the restored material derived from R2000.

This work describes the development of a new low GC restorer material. A relatively wide range of variation in glucosinolate content was observed among the R2000 families. This could be explained by the fact that no selection was applied for glucosinolate content during the pedigree breeding of R2000. Low GC families were nonetheless identified, and this trait will be easily introduced in the best low GC winter oilseed rape lines through backcross breeding. It will then be possible to assess the agronomic value of such material in winter genetic background.

Acknowledgements We would like to thank Mrs. Belloni and Mrs. Pique for having provided the opportunity to realise the Co60 irradiation treatments in the 'Laboratoire de Physico-Chimie des rayonnements' Orsay Cedex-France, S. Marhadour for her technical help, A.M. Chèvre for critical reading of the manuscript, V. Favaudon and C. Raquin for their valuable discussions.

References

Alexander MP (1969) Differential staining of aborted and non-aborted pollen. Stain Technol 44:117–122

Babula D, Kaczmarek M, Barakat A, Delseny M, Quiros CF, Sadowski J (2003) Chromosomal mapping of *Brassica oleracea* based on ESTs from *Arabidopsis thaliana*: complexity of the comparative map. Mol Genet Genomic 268:656–665

Blanc G, Barakat A, Guyot R, Cooke R, Delseny M (2000) Extensive duplication and reshuffling in the *Arabidopsis* genome. Plant Cell 12:1093–1102

Brown GG, Formanova N, Jin H, Wargachuk R, Dendy C, Patil, P, Laforest M, Zhang J, Cheung WY, Landry BS (2003) The radish *Rfo* restorer gene of Ogura cytoplasmic male sterility encodes a protein with multiple pentatricopeptide repeats. Plant J 35:262–272

Delourme R, Eber F (1992) Linkage between an isozyme marker and a restorer gene in radish cytoplasmic male sterility of rapeseed (*Brassica napus* L.). Theor Appl Genet 85:222–228

Delourme R, Eber F, Renard M (1991) Radish cytoplasmic male sterility in rapeseed: breeding restorer lines with a good female fertility. In: Proc 8th Int Rapeseed Congr. University Extension Press, University of Saskatchewan, Saskatoon, Canada, pp 1506–1510

Delourme R, Bouchereau A, Hubert N, Renard M, Landry BS (1994) Identification of RAPD markers linked to a fertility restorer gene for the Ogura radish cytoplasmic male sterility of rapeseed (*Brassica napus* L.) Theor Appl Genet 88:741–748

Delourme R, Eber F, Renard M (1995) Breeding double low restorer lines in radish cytoplasmic male sterility of rapeseed (*Brassica napus* L.). In: Proc 9th Int Rapeseed Congr, vol 1. Cambridge, UK, pp 6–8

Delourme R, Foisset N, Horvais R, Barret P, Champagne G, Cheung WY, Landry BS, Renard M (1998) Characterisation of the radish introgression carrying the Rfo restorer gene for the Ogu-INRA cytoplasmic male sterility in rapeseed (*Brassica napus* L.). Theor Appl Genet 97:129–134

Delourme R, Horvais R, Vallée P, Renard M (1999) Double low restored F_1 hybrids can be produced with the Ogu-INRA CMS in rapeseed. In: Proc 10th Int Rapeseed Congr. Canberra, pp 26–29

Desloire S, Gherbi H, Laloui W, Marhadour S, Clouet V, Cattolico L, Falentin C, Giancola S, Renard M, Budar F, Small I, Caboche M, Delourme R, Bendahmane A (2003) Identification of the fertility restoration locus, *Rfo*, in radish, as a member of the pentatricopeptide-repeat protein family. EMBO Rep 4:588–593

Diers BW, Osborn TC (1994) Genetic diversity of oilseed *Brassica napus* germplasm based on restriction fragment length polymorphisms. Theor Appl Genet 88:662–668

Edwards K, Johnstone C, Thompson C (1991) A simple rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19-6:1349

Forsberg J, Lagercrantz U, Glimelius K (1998a) Comparison of UV light, X-ray and restriction enzyme treatment as tools in production of asymmetric somatic hybrids between *Brassica napus* and *Arabidopsis thaliana*. Theor Appl Genet 96:1178–1185

Forsberg J, Dixelius C, Lagercrantz U, Glimelius K (1998b) UV dose dependent DNA elimination in asymmetric somatic hybrids between *Brassica napus* and *Arabidopsis thaliana*. Plant Sci 131:65–76

Fourmann M (1998) Cartographie de gènes de fonction connue chez le colza. Exploitation de données acquises sur le génome modèle *Arabidopsis thaliana*. Thèse Docteur es Sciences de l'Université Paris XI, Orsay

Giancola S, Marhadour S, Desloire S, Clouet V, Falentin-Guyomarc'h H, Laloui W, Falentin C, Pelletier G, Renard M, Bendahmane A, Delourme R, Budar F (2003) Characterisation of a radish introgression carrying the Ogura fertility restorer gene *Rfo* in rapeseed, using the *Arabidopsis* genome sequence and radish genetic mapping. Theor Appl Genet 107:1442–1451

Gorbunova V, Levy AA (1999) How plant make ends and meet: DNA double-strand break repair. Trends Plant Sci 4:263–269

Heyn FW (1976) Transfer of restorer genes from *Raphanus* to cytoplasmic male sterile *Brassica napus*. Cruciferae Newsl1:15–16

Imai R, Koisuka N, Fujimoto H, Hayakawa T, Sakai T, Imamura J (2003) Delimitation of the fertility restorer locus *Rfk1* to a 43-kb contig in Kosena radish (*Raphanus sativus* L.). Mol Gen Genomics 269:388–394

Kaneko Y, Namai H, Matsuzawa Y, Sarashima M (1992) Induction of translocation in radish by g-ray Irradiation of seeds in the chromosome addition lines of radish with a single kale chromosome. Jpn J Breed 42:383–396

Koizuka N, Imai R, Fujimoto H, Hayakawa T, Kimura Y, Kahno-Murase JK, Sakai T, Kawasaki S, Imamura J (2003) Genetic characterisation of a pentetricopeptide repeat protein gene, orf687, that restores fertility in the cytoplasmic male-sterile Kosena radish. Plant J 34:407–415

Lee D, Reeves JC, Cooke RJ (1996) DNA profiling and plant variety registration: 2. Restriction fragment length polymorphism in varieties in oilseed rape. Plant Var Seeds 9:181–190

Li G, Gao M, Yang B, Quiros CF (2003) Gene for gene alignment between the *Brassica* and *Arabidopsis* genomes by direct transcriptome mapping. Theor Appl Genet 107:168–180

Lombard V, Delourme R (2001) A consensus linkage map for rapeseed (*Brassica napus* L.): construction and integration of three individual maps from DH populations. Theor Appl Genet 103:491–507

Lombard V, Baril CP, Dubreuil P, Blouet F, Zhang D (2000) Genetic relationships and fingerprinting of rapeseed cultivars by AFLP: consequences for varietal registration. Crop Sci 40:1417–1425

Lowe AJ, Moule C, Trick M, Edwards KJ (2004) Efficient large-scale development of microsatellites for marker and mapping applications in *Brassica* crop species. Theor Appl Genet 108:1103–1112

Lukens L, Zou F, Lydiate D, Parkin I, Osborn T (2003) Comparison of a *Brassica oleracea* genetic map with the genome of *Arabidopsis thaliana*. Genetics 164:359–372

Muhanna S, Souvre A, Albertini L (1991a) Karyological and cytological effects of gamma rays on pollen ontogenesis and viability and on tapetum of *Nicotiana tabacum* L var. *xanthi*. Dulieu Cytol 56:17–25

Muhanna S, Souvre A, Albertini L (1991b) Effect of gamma rays on nuclear DNA and cellular RNA in meiocytes, microspores and tapetum of *Nicotiana tabacum L.* var. *xanthi*. Dulieu Cytol 56:107–115

Parkin IAP, Sharpe AG, Lydiate DJ (2003) Patterns of genome duplication within the *Brassica napus* genome. Genome 146:291–303

Primard-Brisset C, Poupard JP, Horvais R, Budar F, Pelletier G, Renard M, Delourme R (2003) A new recombined double low restorer line for the Ogu-INRA cms in rapeseed. In: Proc 11th Int Rapeseed Congr, vol 1. KVL Copenhagen Denmark. BP7, pp 300–302

Sakai T, Liu HJ, Iwabushi M, Kohno-Murase, Imamura J (1996) Introduction of a gene from fertility restored radish (*Raphanus sativus*) into *Brassica napus* by fusion of X-irradiated protoplasts from a radish restorer line and iodoaceoamide-treated protoplasts from a cytoplasmic male-sterile cybrid of *B. napus*. Theor Appl Genet 93:373–379

Sears ER (1993) Use of radiation to transfer alien chromosome segments to wheat. Crop Sci 33:897–901

Shirley BW, Hanley S, Goodman HM (1992) Effects of ionising radiation on a plant genome: analysis of two *Arabidopsis transparent testa* mutations. Plant Cell 4:333–347