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Characterisation of the radish introgression carrying the *Rfo* restorer gene for the *Ogu*-INRA cytoplasmic male sterility in rapeseed (*Brassica napus* L.)

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Abstract Bulked segregant analysis and comparative mapping were applied to identify molecular markers linked to the *Rfo* restorer gene used for the *Ogu*-INRA cytoplasmic male-sterility system in rapeseed. These markers were then used to localise the radish introgression on the *B. napus* genetic map constructed from the cross 'Darmor.*bzh*' x 'Yudal'. The introgression mapped on the DY15 linkage group. From the comparison of this latter group to the linkage group constructed on a F_2 progeny segregating for the radish introgression, it was concluded that the introgression had occurred through homoeologous recombination, that it was not distal and that it had replaced a *B. napus* region of around 50 cM. A QTL involved in aliphatic seed glucosinolate content was located on the DY15 linkage group at a position corresponding to one end of the introgression. The DNA markers identified in this study are being used in map-based cloning of the *Rfo* gene and in marker-assisted selection.

Key words *Brassica napus* · *Raphanus sativus* · Restorer gene · Introgression · RFLP · RAPD · Genetic mapping

Introduction

Breeding restorer lines for the *Ogu*-INRA Cytoplasmic Male Sterility (CMS) (Pelletier et al. 1983, 1987) in rapeseed (*Brassica napus* L.) has been a major objective during the past few years. A restorer gene *Rfo* was introgressed from radish (*Raphanus sativus* L.) into rapeseed (Heyn 1976) through intergeneric hybridisation. Extensive backcross and pedigree breeding (Delourme et al. 1991) were necessary to improve the low female fertility of the restorer lines, which was attributed to a long piece of radish genetic information remaining around the *Rfo* gene or elsewhere in the genome (Pellan-Delourme and Renard 1988). This breeding resulted in restorer lines ($2n = 38$) in which female fertility was equal to that of rapeseed maintainer lines and meiotic behaviour was greatly stabilised, thus leading to a more regular transmission of the restorer gene through backcross or self pollination (Delourme et al. 1995). However, the use of these restorer lines in double-low F_1 hybrid breeding has been slow because of a tight linkage between this introgression and high glucosinolate content (Delourme et al. 1995).

Some radish DNA still remains around the introgressed *Rfo* gene. A radish isozyme allele at the *Pgi-2* locus was found to be tightly linked to the *Rfo* gene (Delourme and Eber 1992). Random Amplified Polymorphic DNA (RAPD) markers were then identified, the polymorphic DNA fragments being associated either with the restorer allele or with the sterility maintainer allele (Delourme et al. 1994). From a comparison of the *Pgi-2* alleles found in the two progenitors (*B. rapa* L., AA and *B. oleracea* L., CC) of rapeseed (Chèvre et al. 1995) to the ones of rapeseed, it can be assumed that the introgression has taken place in the C genome of rapeseed. Moreover, the introgression might have occurred through homoeologous recombination since the introgressed *Pgi-2* allele of radish has replaced the *Pgi-2C* allele of rapeseed (Delourme and Eber 1992).

In the study presented here, the region around the *Rfo* gene was further saturated with molecular markers using bulked segregant analysis (BSA) (Michelmore et al. 1991) and comparative mapping in order to characterise more precisely the radish

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introgression. Its localisation on the *B. napus* genetic map built from the cross 'Darmor.*bzh*' x 'Yudal' (Foisset et al. 1996) was achieved, and co-localisation with QTL (quantitative trait loci) involved in the variation of total glucosinolate content was studied.

Materials and methods

Plant materials

The origin of the restored *B. napus* lines carrying the *Rfo* radish restorer gene has been described previously (Pellan-Delourme and Renard 1988). 'R40', an homozygous single-low rapeseed restorer line was derived from the family improved for female fertility (Delourme et al. 1991) through pedigree breeding (F6). 'R40' was crossed to 'Yudal', one of the parents of the cross used to construct the *B. napus* genetic map (Foisset et al. 1996), and a F_2 population of 220 plants was produced. The male fertility/sterility of these F_2 plants was scored and homozygous restored *Rfo*/*Rfo* plants were screened among the male-fertile ones using the linkage of the *Rfo* gene with the *Pgi-2* radish allele as described in Delourme and Eber (1992). Ten homozygous restored (*Rfo*/*Rfo*) and ten male-sterile (*rfo*/*rfo*) plants were chosen to construct the 'R40' x 'Yudal' (RY) bulks for RAPD analyses. For RFLP studies, two pairs of bulks were constructed according to the male-fertile/male-sterile phenotype of the plants.

The *B. napus* genetic map (Foisset et al. 1996) used to localise the radish introgression was elaborated from a doubled-haploid (DH) progeny (152 lines) derived from the cross 'Darmor.*bzh*' x 'Yudal'. The map comprised 254 markers assembled in 19 linkage groups, *i.e.* 1765 cM. When the linkage group carrying the radish introgression was identified, bulks were constructed to expand this group by pooling the DNA of plants carrying the 'Darmor.*bzh*' (D) allele or 'Yudal' (Y) allele for the markers at the targeted region.

Plant genomic DNA was isolated as described in Doyle and Doyle (1990).

RAPD analyses

The procedure for RAPD analyses was as described in Foisset et al. (1996). RAPD primers were purchased from Operon Technologies and the *Taq* DNA polymerase from Eurobio. RAPD primers are designated by an 'RAPD' prefix, followed by a number. RAPD loci are then specified by adding a letter if two markers are obtained from a same primer.

Southern blot hybridization

Probes coded BN and DLM correspond to anonymous clones from embryo and seedling cDNA libraries of *B. napus*. DNA samples were digested with *Bam*HI, *Eco*RI, *Eco*RV and *Hind*III (5 U/μg DNA). Digested DNAs (3 μg per lane) were electrophoresed on 0.8% TAE agarose gels and transferred onto Hybond N^+ membranes (Amersham) by the alkaline transfer procedure. Probes were radioactively labelled with [^{32}P] using the T7 Quick Prime™ kit (Pharmacia). Hybridisations and autoradiography were as described in Landry et al. (1991).

Linkage analyses

Segregation analyses on the 'R40' x 'Yudal' progeny were made on the population of 220 F_2 plants for RAPD markers and on 99 F_2 plants for restriction fragment length polymorphism (RFLP) markers. Codominant markers were first analysed. Dominant markers were then added taking into account the coupling/repulsion phase. Mapping of the markers on the *B. napus* genetic map was done on the 152 DH lines. Multi-point linkage analyses of the marker loci were performed with MAPMAKER/EXP version 3.0 (Lincoln et al. 1992). Linkage groups were established with a threshold LOD score of 6.0 and a maximum recombination fraction of 0.4. Centimorgan distances were obtained using the Kosambi function.

Results

Localisation of the radish introgression on the *B. napus* genetic map

Twenty-six RAPD primers representing loci evenly spaced on the 19 linkage groups of the *B. napus* genetic map were analysed on the *Rfo*/*Rfo* and *rfo*/*rfo* bulks. A total of 58 mapped loci could be scored (Table 1). Nine markers corresponding to loci whose dominant allele originated from 'Darmor.*bzh*' were absent in both bulks, indicating that these markers were not present in 'R40'. All the other markers but one (OPJ07.2960) were monomorphic on the two bulks. This locus was located on the DY15 linkage group. The DY15 linkage group was only 23.5 cM long and comprised two RFLP (3ND4a and 2NB12a) and two additional RAPD (OPP11.715 and OPF15.700) markers. One of these RAPD markers (OPF15.700) was among the nine markers that were absent in both bulks (Table 1); the other one (OPP11.715) was tested later on and was also found to be absent in both bulks. The two RFLP markers were found to be completely linked to the introgression.

While the 26 primers of the *B. napus* map were tested, 4 new RAPD markers (RAPD1, RAPD2, RAPD3, RAPD4) were found polymorphic between the *Rfo*/*Rfo* and *rfo*/*rfo* bulks and totally linked to the *Rfo* gene.

Identification of new markers

RAPD markers

A total of 295 primers were analysed for polymorphism between the *Rfo*/*Rfo* and *rfo*/*rfo* bulks made from the

Table 1 Polymorphism obtained with the 26 selected primers of the *B. napus* genetic map on the bulks made on the 'R40' x 'Yudal' F_2 progeny

Origin of the markers	Bulk *Rfo*/*Rfo*	Bulk *rfo*/*rfo*	Number of markers
Darmor.*bzh*	−	−	9
Darmor.*bzh*	+	+	19
Codominant	Yudal	Yudal	2
Yudal	+	+	26
Yudal	−	−	1
Yudal	−	+	1

Table 2 Number of markers obtained with bulked segregant analysis on the 'R40' (R) x 'Yudal' (Y) F_2 progeny and on the 'Darmor.*bzh*' (D) x 'Yudal' (Y) DH progeny

Bulks used	Number of primers	*Rfo*/*Rfo-rfo*/*rfo* bulks		DY15 bulks	
		Number of polymorphic markers	Number of markers on RY linkage group	Number of polymorphic markers	Number of markers on DY15 linkage group
Rfo/*Rfo-rfo*/*rfo*					
Bulks	36	10	5		
Both bulks	221	20	8	37	5
DY15 Bulks	38			3	2

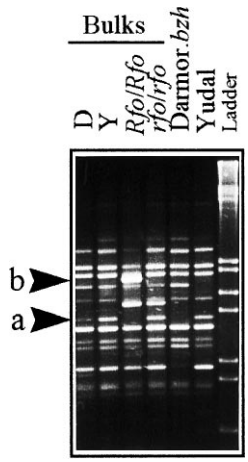

Fig. 1 Amplification profiles obtained with the RAPD13 primer on the D and Y bulks (*lanes* 1 and 2), on the *Rfo*/*Rfo* and *rfo*/*rfo* bulks (*lanes 3* and *4*) and on the two parents of the *B. napus* genetic map (*lanes 5* and *6*). Two polymorphic fragments (RAPD13a and RAPD13b) are revealed

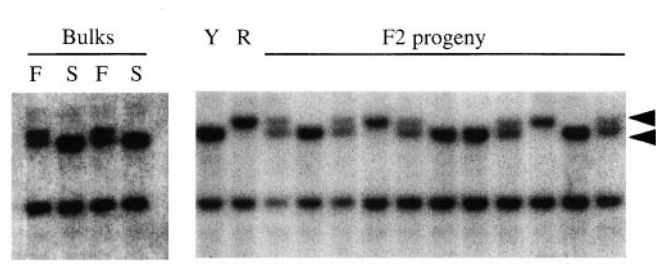

Fig. 2 Hybridisation patterns obtained with the probe DLM316 on the two pairs of male-fertile (*F*) and male-sterile (*S*) bulks, on 'Yudal' (*Y*) and 'R40' (*R*), the two parents of the F_2 progeny and on 11 F_2 individuals

'R40' x 'Yudal' F_2 progeny and/or on the D and Y bulks made from the 'Darmor.*bzh*' x 'Yudal' DH progeny using the DY15 markers (Table 2). This generated 30 and 40 amplified products capable of distinguishing the *Rfo*/*Rfo* and *rfo*/*rfo* bulks and the D and Y bulks, respectively. This resulted in 13 new markers linked to the *Rfo* gene and 7 new markers on the DY15 linkage group. Two of them (RAPD13a and RAPD16) were polymorphic between the two pairs of bulks (Fig. 1). Two markers (RAPD18 and RAPD19), identified when screening the *Rfo*/*Rfo* and *rfo*/*rfo* bulks only, were also polymorphic on 'Darmor.*bzh*' x 'Yudal' progeny and mapped on the DY15 linkage group. Reciprocally, one marker (RAPD5), identified when screening the D and Y bulks only, was polymorphic on 'R40' x 'Yudal' progeny and found to be linked to the *Rfo* gene.

RFLP markers

The two parental lines 'R40' and 'Yudal' were screened for polymorphism with 325 cDNA probes using four restriction enzymes. A total of 205 probes revealed polymorphism with at least one restriction enzyme. Due to the high level of polymorphism observed between the parents, further screening of this collection of 205 probes with the two pairs of bulks made from the phenotypes (male-fertile – male-sterile) of F_2 plants was made with only one restriction enzyme. From these, 23 candidate probes detected polymorphism between the 'male-fertile' and 'male-sterile' bulks. Linkage analyses showed that four of them were perfectly linked to the *Rfo* gene (Fig. 2).

By comparative mapping of in-house genetic maps constructed from different *Brassica* mapping populations (DNA LandMarks), we selected a collection of 31 RFLP probes. Some of these probes had been missed in the previous screening with BSA either because only one restriction enzyme was used or because the results were ambiguous or missing. A few of them are from a new seedling cDNA library that has never been screened before, neither on the bulks nor on the parents. These probes were used in segregation analyses on the 'R40' x 'Yudal' F_2 progeny. Ten RFLP markers were found to be linked to the *Rfo* gene.

Comparison of the linkage groups

Linkage analyses were performed on the two segregating populations, and two linkage groups were built: the RY linkage group on the 'R40' x 'Yudal' F_2 progeny

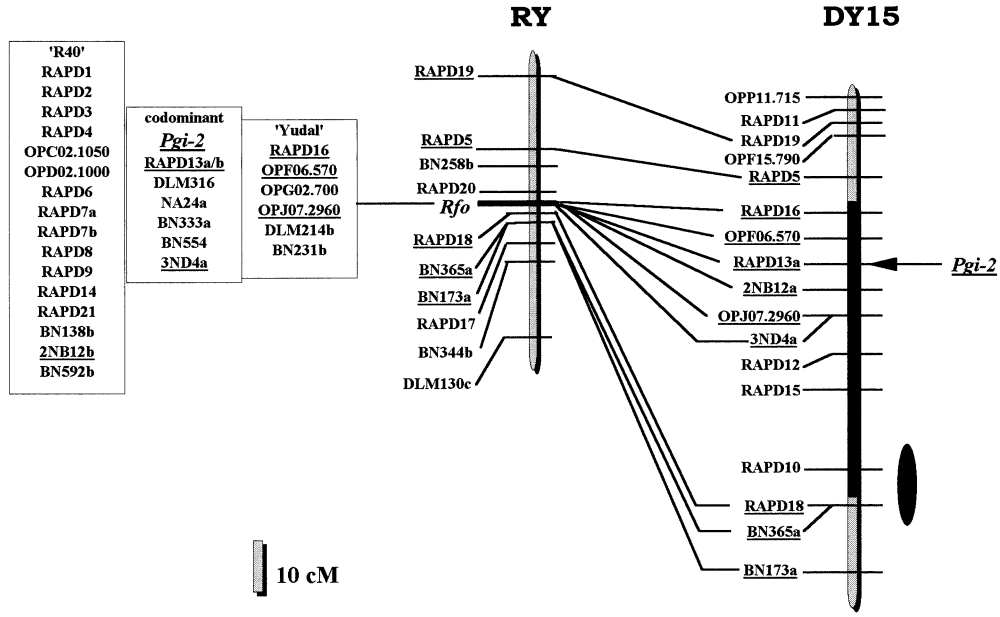

Fig. 3 Comparison of the RY linkage group from the 'R40' x 'Yudal' F_2 progeny to the DY15 linkage group from 'Darmor.*bzh*' x 'Yudal' DH progeny. *Underlined* markers are common to the two linkage groups. *Framed* markers are completely cosegregating with the *Rfo* gene. The *black box* on the DY15 linkage group represents the size of the *B. napus* segment that was replaced by the radish introgression. The *black oval* on the *side* of the DY15 linkage group indicates the position of the QTL involved in total aliphatic seed glucosinolate content

and the DY15 linkage group on 'Damor.*bzh*' x 'Yudal' DH progeny (Fig. 3). The markers provided by the primers OPC02, OPD02, OPG02 and OPF06 were identified in Delourme et al. (1994). The locus *Pgi-2C* did not segregate in 'Darmor.*bzh*' x 'Yudal' DH progeny. Its position was estimated from the analysis of common segregating markers in another *B. napus* cross (unpublished data).The comparison of the two linkage groups RY and DY15 was possible because of 11 common segregating markers. It indicated that the introgression was not distal since markers of rapeseed incompletely linked to it were found at each side. The two ends of the introgression were positioned from the recombination frequency with the flanking markers. It corresponds to a region covering approximately 50 cM on the DY15 linkage group (Fig. 3).

In all, 30 markers completely cosegregated with the *Rfo* gene (Fig. 1), of which 17 are dominant markers whose dominant alleles are associated with the radish fertility restorer allele present in 'R40', 6 are codominant markers and 7 are dominant markers whose dominant alleles are associated with the sterility maintainer allele of 'Yudal' (Fig. 3). Most of the RAPD markers are dominant markers. However, the RAPD primer RAPD13 revealed one DNA fragment (RAPD13a) in 'Yudal' and one DNA fragment (RAPD13b) in 'R40' which are completely linked to *rfo* and *Rfo* alleles, respectively. RAPD13b was not present in 'Darmor.*bzh*'. RFLP markers are either dominant or codominant markers. All the markers located outside of the introgression on the RY linkage group are dominant markers, the dominant allele being mostly associated with the sterility maintainer allele of 'Yudal' except for RAPD17, RAPD20 and BN173a whose dominant allele is carried by 'R40'.

Discussion

Breeding rapeseed restorer lines with a good agronomic value for the *Ogu*-INRA CMS has been difficult due to the fact that the restorer gene *Rfo* was introduced into rapeseed through an intergeneric cross. During the transfer of the *Rfo* gene from the radish genome to the *B. napus* genome, radish DNA adjacent to the restorer gene was introgressed. For the size of this radish segment to be estimated, precise mapping of the introgression on the *B. napus* genetic map (Foisset et al. 1996) and its characterization were carried out.

Characterisation of the introgression

The use of bulked segregant analysis and of selected mapped RAPD markers was a very efficient means to rapidly identify the *B. napus* linkage group carrying the radish introgression. It was also very efficient to add new RAPD and RFLP markers in the introgression area and on the corresponding *B. napus* linkage group. Comparative mapping also proved to be very useful for adding new markers in a targeted region. The quite

high number of markers found in the introgression could be explained by the high degree of polymorphism between radish and rapeseed genomes and/or by the large size of this introgression. A large introgression had been hypothesised previously from the disturbed meiotic behaviour and the poor male transmission of the restorer gene in some segregating progenies (Delourme et al. 1995). Our present study confirmed this large size since the radish introgression replaced a *B. napus* region covering around 50 cM. However, from these results, we cannot know if the *B. napus* segment has been replaced by a radish segment of equivalent size.

Common segregating markers on RY and DY15 linkage groups which were incompletely linked to the introgression showed that this introgression was not distal. The fact that codominant RFLP markers were observed is consistent with the replacement of a *B. napus* segment by a corresponding homoeologous radish one, which had already been hypothesised from the *Pgi-2* patterns (Delourme and Eber 1992). Some RFLP markers were scored as dominant. In these cases, it is possible that the fragment corresponding to the other allele was masked by a fragment from another locus. This hypothesis is supported by the observation that these dominant markers are also homoeologous ones: probes (such as 2NB12) giving a dominant allele in 'R40' mapped loci on the DY15 linkage group (Fig. 3), and probes giving a dominant allele in rapeseed 'Yudal' mapped loci in the region surrounding the restorer gene in a radish segregating population (unpublished data). These results indicate that the introgression has occurred by homoeologous chromosome pairing between radish and rapeseed. This is consistent with the known homoeology between the R genome of radish and the A and C genomes of rapeseed; even if allosyndetic pairing between the chromosomes of A, C and R genomes are rare, they have been reported by several authors (McNaughton 1973; Harberd and McArthur 1980; Dolstra 1982; Lelivelt et al. 1993). A number of other introgressions have been found to have occurred by chromosomal exchanges in homoeologous regions. This was the case in rapeseed for a *B juncea* introgression carrying a *Leptosphaeria maculans* resistance gene (Barret et al. 1998) as well as in other species such as peanuts (Garcia et al. 1994), wheat (Autrique et al. 1995; Bonhomme et al. 1995) or rice (Jena et al. 1992).

Linkage with glucosinolate content

The DH progeny from the cross 'Darmor.*bzh*' x 'Yudal' have been used to identify genomic regions involved in seed glucosinolate content in rapeseed (Foisset et al. 1995). A QTL involved in total seed glucosinolate content was located on linkage group DY15 (unpublished data) at a position corresponding to one end of the radish introgression (Fig. 3). Two hypotheses could be drawn from this observation. (1) The locus controlling seed glucosinolate content is located on the rapeseed genome very close to the introgression; the rapeseed line originally used for the transfer of the restorer gene was single-low. The difficulties in breeding low glucosinolate restorer lines would therefore result from the extremely low recombination rate in this area. The genetic distance between BN365a and BN173a is lower on the RY linkage group than on the DY15 linkage group. (2) The locus controlling seed glucosinolate content is located in the radish introgression; examination of glucosinolate profiles of radish seeds showed that the dominant aliphatic glucosinolate was glucoraphenin (4-methylsulphinylbut-3-enyl-GS), which contains an unsaturated sulphinyl R group (Sang et al. 1984). This glucosinolate is not found in seeds of rapeseed restorer lines, whereas it is present in seeds of *Raphanobrassica* (hybrid between radish and rapeseed) in addition to the glucosinolates typically found in rapeseed (data not shown). Therefore, a gene responsible for the presence of glucoraphenin is not present in the introgression, but it is possible that the introgression carries a radish gene catalysing another step of the biosynthetic pathway which would be common to the rapeseed glucosinolate biosynthetic pathway. This cannot be excluded because of the homoeology of the introgressed radish region with the replaced rapeseed region. Until now, we could not distinguish between these two hypotheses. In both hypotheses, the gene present in this region acts on all individual aliphatic glucosinolates and on total seed glucosinolate content of the restorer lines.

The development of low-glucosinolate restorer lines needs a recombination event between the restorer gene and the gene involved in seed glucosinolate content. Until now in our breeding programmes, the modifications of the introgression that have been observed have occurred through deletion. This has been shown in some lines that have lost the radish *Pgi-2* allele but have not recovered the original rapeseed *Pgi-2* allele (Delourme and Eber 1992). Different low-glucosinolate rapeseed restorer lines have been selected since 1992. These lines still carry or have lost the radish *Pgi-2* allele. These different lines are being screened with the DNA markers identified in this study to characterise the rearrangements that have occurred in the introgression. Meanwhile, the markers are also being mapped in a radish population in which *Rfo* is segregating. The clustering of the cosegregating markers with the *Rfo* gene in the 'R40' x 'Yudal' progeny is likely to result from the suppression of the recombination around the introgressed radish fragment rather than from genuine close physical proximity to the *Rfo* gene. The fine mapping of the markers in a pure radish cross and the results of the characterisation of the different restorer lines selected will allow us to order the markers relative to the *Rfo* gene and use them in map-based cloning and marker-assisted selection.

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