Theor Appl Genet (1992) 85:222–228

Linkage between an isozyme marker and a restorer gene in radish cytoplasmic male sterility of rapeseed (*Brassica napus* L.)

R. Delourme and F. Eber

INRA, Station d'Amélioration des Plantes, BP 29, F-35650 Le Rheu, France

Received January 5, 1992; Accepted March 10, 1992
Communicated by H. F. Linskens

Summary. Co-segregation studies of isozyme markers and male fertility restoration showed that a restorer gene from radish was introduced into rapeseed along with an isozyme marker (*Pgi-2*). The radish chromosome segment carrying these genes was introgressed into rapeseed through homoeologous recombination, substituting for some of the rapeseed alleles. By crossing heterozygous restored plants to male-sterile lines and to maintainers, tight linkage was found between the restorer gene and the marker. The recombination fraction was estimated at $0.25 \pm 0.02\%$. Although few restored plants lacked the radish isozyme marker, it was still possible to distinguish male-fertile from male-sterile plants by their PGI-2 patterns. Furthermore, homozygous and heterozygous restored plants could be separated by specific PGI-2 phenotypes. Thus, the *Pgi-2* marker is now currently used in restorer breeding programs.

Key words: *Brassica napus* – *Raphanus sativus* – Cytoplasmic male sterility – Restorer – Isozyme

Introduction

An effective system of pollination control is required for the production of rapeseed (*Brassica napus* L.) F1 hybrids on a commercial scale. Male-sterile hybrids have been obtained through protoplast fusion between a male-fertile rapeseed line and a cytoplasmic male-sterile (CMS) line of rapeseed (with Ogura radish male sterility-inducing cytoplasm; Pelletier et al. 1983). No restorer was found in *B. napus* (Rousselle 1982) but restorer genes were introduced from radish into rapeseed (Heyn 1976) through intergeneric crosses between a CMS line of rapeseed and *Raphanobrassica* (*Raphanus sativus* × *B. napus* amphidiploid, $2n = 56$). Restored *B. napus* plants were selected from the progeny of this cross (Pellan-Delourme 1986). Fully restored plants with one dominant restorer gene were obtained for the best CMS cybrids (Pelletier et al. 1987).

Male fertility restoration in these plants was accompanied by a decrease of female fertility (Pellan-Delourme and Renard 1988). Therefore, it is likely that the restored rapeseed plants have retained other radish genetic information in addition to the restorer gene.

Progenies segregating for the radish restorer gene were used to study possible association between this gene and isozyme markers. This should provide information on the genetic structure of the restored plants and would be a useful tool in a breeding programme using this cytoplasmic male sterility.

Materials and methods

Plant material

The origin of the *B. napus* restored lines was described in Pellan-Delourme and Renard (1988). Breeding activities with this material included self pollinations, backcrosses to double low rapeseed lines and test crosses to male-sterile cybrid lines. Restored plants carrying a single dominant restorer gene were selected on cybrid 27 and 58 cytoplasms.

The following *B. napus* progenies were used for the co-segregation studies (Fig. 1). The numbers in square brackets in Fig. 1 refer to progeny numbers: Progeny A: heterozygous restored plants (Rr) were crossed to a male-sterile (rr) line to produce F1s. Some F1s [1] were selfed to produce an F2 [2] and then test crossed to a male-sterile line (TC [3]). Other F1s were selfed (F2 [4], [5]) and crossed to a maintainer (rr) line (BC1 [6]).

Progeny B: heterozygous restored plants (Rr) were crossed to a maintainer line to give an F1 [7] and then backcrossed three

Correspondence to: R. Delourme

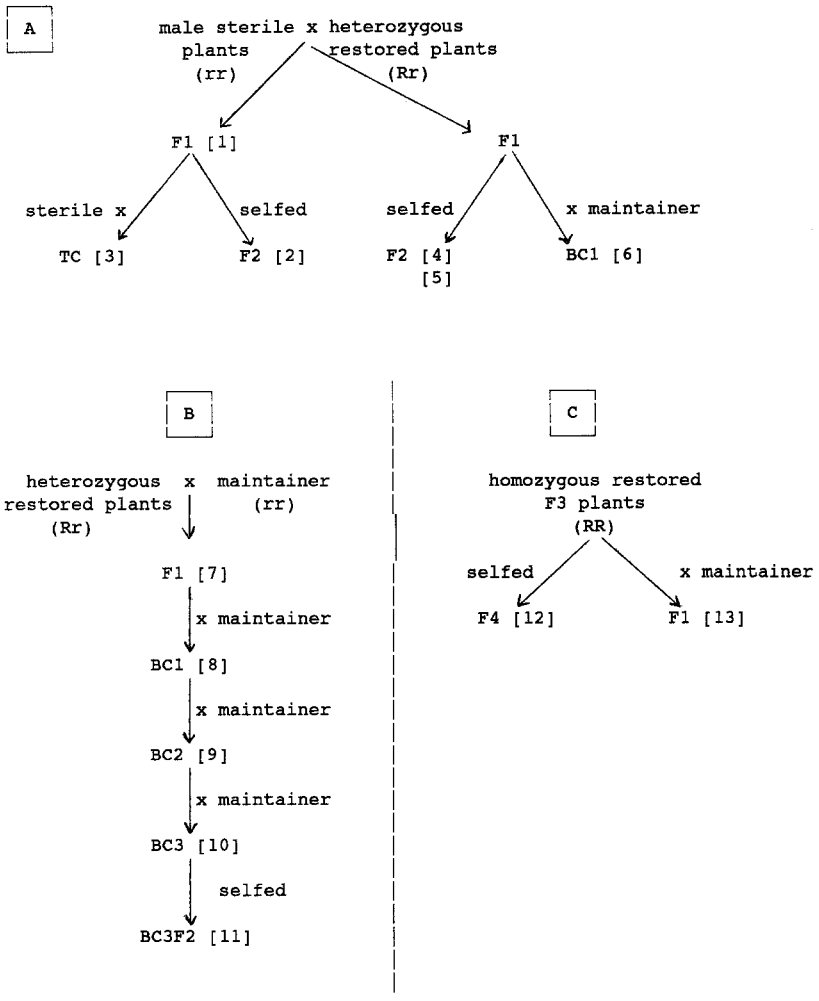


Fig. 2. Genealogy of the restored material

times to the same maintainer line to produce successively BC1 [8], BC2 [9], and BC3 [10]. Finally, the third backcross was selfed (BC3F2 [11]).

Progeny C: homozygous plants (RR) from a F3 progeny were selfed (F4 [12] and crossed to a maintainer line (F1 [13]).

Isozyme studies

Isozyme studies were performed on all progenies. In addition, a few radish varieties (Remus, Resal, Iris, Pegletta) were sampled. In the analysis of the first progeny [1], nine isozyme systems were studied using starch-gel electrophoresis. Young leaves were crushed in a Tris-HCl 0.1 M buffer (pH 7.5) containing 1% w/v glutathione. Gel/electrode buffers for starch gels were those given by Shields et al. (1983). Malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), 6-phosphogluconate dehydrogenase (6PGD) were separated on a G buffer system (pH 6.1), glutamate oxaloacetate transaminase (GOT), triose phosphate isomerase (TPI) on a C buffer system (pH 8.3), and aconitase (ACO), phosphoglucomutase (PGM) on an E buffer system (pH 7.0). Phosphoglucoisomerase (PGI) was studied on the three preceding buffer systems and on B buffer (pH 5.7). Staining procedures were as reported by Vallejos (1983) except for LAP and ACO which followed the protocols given by Arus and Orton (1983) and Wendel and Stuber (1984) respectively. Only PGI was analysed in the rest of the progenies.

Results

Search for markers

In progeny [1], five isozyme systems (ACO, GOT, MDH, PGI, 6PGD) were segregating but only PGI showed different patterns distinguishing male-fertile restored plants and male-sterile plants. The restored plants had two additional bands, absent in the male-steriles, in the PGI-2 region of activity (Fig. 2). This was explained by the presence of a *Pgi-2* radish allele interacting with *Pgi-2* alleles 1 and 2 of rapeseed in the restored plants. Because of its amphidiploid nature, two *Pgi-2* genes express in rapeseed: one from *B. oleracea* and the other from *B. campestris*. PGI is a dimeric enzyme. Thus, an additional allele results in two more bands: a dimer encoded by the radish allele and an interallelic dimer encoded by this allele and an allele of rapeseed. The heterodimer of the radish allele with the other allele of rapeseed superposed to one of rapeseed isozymes. The *Pgi-2* radish allele was observed in radish varieties included in the electrophoretic analysis (Fig. 3).

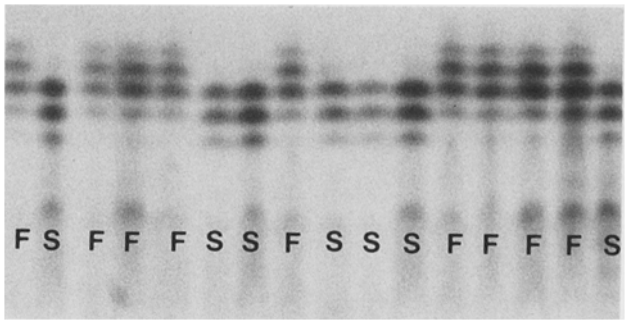


Fig. 2. A progeny segregating for male fertility restoration and presence/absence of the *Pgi-2* radish allele. *F*, male-fertile; S, male-sterile

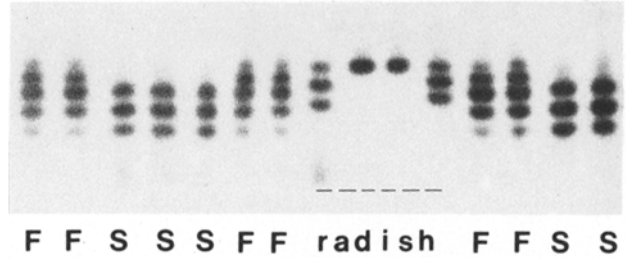


Fig. 3. *Pgi-2* patterns of male-sterile and restored plants and comparison with radish varieties. *F*, male-fertile; S, male-sterile

Linkage studies

Linkage studies between the restorer gene and the *Pgi-2* radish isozyme marker were carried out in the progenies described in Fig. 1. Table 1 shows the segregation data for male-fertility restoration and the presence or absence of the isozyme marker for the different progenies. The presence of the restorer gene was correlated with the *Pgi-2* radish allele except in very few plants. A few male-fertile plants did not possess the marker. These included:

- One plant in the F2 [2] of a heterozygous restored plant.
- Three F2 plants [4].
- One plant in the cross [8] between a heterozygous restored plant and a maintainer line.
- One plant in the cross [13] between a homozygous restored plant and a maintainer line.

No male-sterile plant had the radish isozyme marker.

Only the crosses between heterozygous plants and maintainer or male-sterile lines could be taken into account when estimating the recombination rate between the genes. In F2 [2] a deviant segregation ratio for male-fertile and male-sterile plants was observed. In the other crosses, male-sterile plants were not studied.

Thus, the recombination fraction between the two genes was assessed on 401 plants and was estimated to be $0.25 \pm 0.02\%$.

Table 1. Segregation data for male fertility and *Pgi-2* radish allele presence (PGI+) or absence (PGI−) in different progenies

Progeny	Type of crosses	Number of plants		
		Fertile PGI+	Fertile PGI−	Sterile PGI−
Progeny A				
F1 [1]	Sterile × heterozygous[a]	45	0	36
F2 [2]	Heterozygous × maintainer	111	1	2
TC [3]	Sterile × heterozygous	44	0	72
F2 [4]	Heterozygous × maintainer	0	3	0
F2 [5]	Heterozygous × maintainer	66	0	–[b]
BC [6]	Heterozygous × maintainer	64	0	–
Progeny B				
F1 [7]	Heterozygous × maintainer	36	0	23
BC1 [8]		72	1	72
BC2 [9]		38	0	–
Progeny C				
F4 [12]	Homozygous[a] × maintainer	57	0	0
F1 [13]	Homozygous × maintainer	78	1	0

[a] Heterozygous or homozygous for the restorer gene
[b] No sterile plant was studied

Isozyme studies on plants with the marker

Two heterozygous restored plants (plant I and plant II) from progeny [1] were self pollinated and test crossed to a male-sterile line (Cybrid 27 × 'RP1'). Their genotypes and gametes were deduced from their progeny. The different patterns observed and the numbers of plants within each class are given in Table 2.

Plants I and II were selected from progeny where the recurrent parent was the rapeseed variety 'Drakkar'. They carried *Pgi-2* alleles 1 and 2 of 'Drakkar' and the *Pgi-2* radish allele (Table 2). They were both test crossed to the male-sterile cybrid line 'RP1' which carried the rapeseed allele 3 and a null allele at *Pgi-2* loci but their progenies did not show the same patterns.

Plant I produced two types of gametes (r,1; 1,2) and each gamete could carry two alleles: one of each homoeologous *Pgi-2* gene from *B. oleracea* and *B. campestris*. The radish allele replaced allele 2 of rapeseed in some of the gametes. As expected two patterns could be observed in the test cross in equal proportions ($\chi^2 = 0.05$, $P = 0.82$). No sterile plant was found in the self pollination, so one of the three expected patterns was not observed.

Plant II produced four types of gametes (r,1; r,n; 1,2; 2,n). Therefore, it is likely that the *Pgi-2* radish allele replaced allele 2 of rapeseed on one of the parental chromosomes and a null allele was present on the homologue. All the expected patterns were observed in the progenies except for the self pollination (Table 2) where the *Pgi-2* homozygotes (n,n; 2,2) were absent, perhaps due to the

Table 2. *Pgi-2* genotypes of two restored plants (plant I and plant II) and of their progeny in test crosses to the male-sterile line 'RP1' (TC) and in self pollination

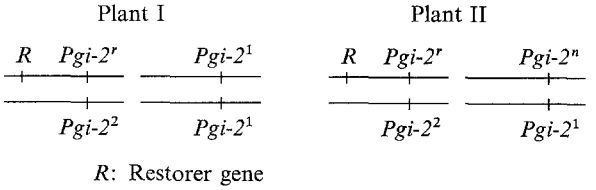
	Drak-kar	RP1	Plant I	TC			Self pollination					
Patterns	1 2	3	r 1 2	r 1 3	1 2 3		r 1	r 1 2	1 2			
Genotypes[a]	11 22	nn 33	11 r2	1n r3	1n 23		11 rr	11 r2	11 22			
Theoretical number				15	15		7.5	15	7.5			
Observed number				13	17		9	21	0			

	Drak-kar	RP1	Plant II	TC				Self pollination						
Patterns	1 2	3	r 1 2	r 3	r 1 3	1 2 3	2 3	r	r 1	r	r 1	1	2	
Genotypes	11 22	nn 33	1n r2	nn r3	1n r3	1n 23	nn 23	nn rr	n1 rr + 11 rr	nn r2	ln r2 + 11 r2	11 22 + 1n 22	nn 22	
Theoretical number				7.5	7.5	7.5	7.5	1.8	5.4	3.6	10.9	5.4	1.8	
Observed number				5	9	8	8	2	7	5	14	1	0	

[a] 1, 2, 3, n, *Pgi-2* alleles of rapeseed; r, *Pgi-2* allele of radish

small size of this progeny. However, the segregation was not significantly different from the expected ratio ($\chi^2 = 7.31$, $P = 0.20$).

Thus, the following two genotypes could be proposed for the restored plants; each pair of homoeologous chromosomes (from *B. oleracea* and *B. campestris*) being depicted:



R: Restorer gene

Four other progenies (self pollinations and backcrosses) were also analysed. The same patterns, genotypes and segregation ratios were observed (data not shown).

Homozygous restored lines obtained from the selfing of plants which had the same genotypes as plant I or plant II were also studied. They possessed the *Pgi-2* radish allele and allele 1 of rapeseed. This confirms that the *Pgi-2* allele of radish has replaced allele 2 of rapeseed.

Isozyme studies of plants without the marker

The progeny of one restored plant which lacked the radish isozyme marker was analysed. This plant appeared in the cross between a heterozygous restored plant and a maintainer line (BC1 [8], Fig. 1). The results presented here were obtained from BC3 [10] and BC3F2 [11] progenies.

It was possible to differentiate male-fertile from male-sterile plants by their PGI-2 phenotypes. The male-sterile plants showed a three-banded pattern and the male-fertile ones showed either a three-banded or a one-banded pattern (Fig. 4, Table 3). The difference between the two three-banded patterns was based on the intensity of the most cathodal band. The separation made on isozyme

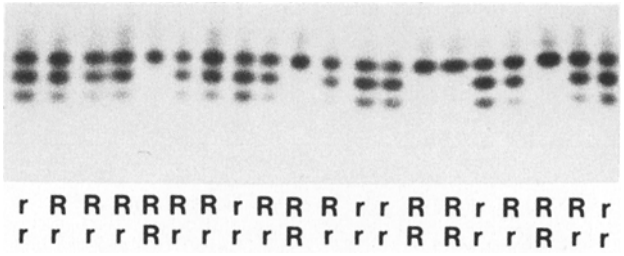


Fig. 4. *Pgi-2* patterns of male-sterile and restored plants lacking the radish isozyme marker. *rr*, male-sterile plants; *RR*, homozygous restored plants; *Rr*, heterozygous restored plants

Table 3. Segregation in progenies of restored plants lacking the radish isozyme marker

Progeny	BC3 [10]		BC3F2 [11]		
Patterns	—	—[1][a]	—	—	—
	—	—	—	—	
	—	—2	—	—	
Number of plants	12	18	138	206	80
Number of plants noted for male fertility	12	18	81	73	30
Male fertility[b]	12S	18F	78S:3F	70F:3S	30F

[a] 1, 2, *Pgi-2* alleles of rapeseed
[b] S, sterile; F, fertile

Table 4. Genotypes of a mother restored plant lacking the *Pgi-2* radish allele and of its progeny

Mother plant						
Pattern	—[1][a]					
	—					
	—2					
Genotype	1	R[b]				
	+	+				
	+	+				
	1	2				
Progeny						
Patterns	—		—		—	
	—		—			
	—		—			
Genotypes	1	2	1	R	1	R
	+	+	+	+	+	+
	+	+	+	+	+	+
	1	2	1	2	1	R
Male fertility	Sterile		Fertile heterozygous		Fertile homozygous	

[a] 1, 2, *Pgi-2* alleles of rapeseed
[b] R, restorer gene

phenotypes was in agreement with the male fertility/male sterility of the plants except for six cases (Table 3).

The genotypes of the mother plant and its progeny were deduced from the isozyme phenotypes (Table 4). The production of two types of gametes could explain the segregations obtained for *Pgi-2* and the male-fertility restoration genes. Thus, male-fertile and male-sterile plants could be quite easily distinguished by their PGI-2 patterns and it was possible to differentiate homozygous restored plants from heterozygous ones even when the *Pgi-2* radish allele had been eliminated.

Discussion

In the present study, the restorer gene introduced from radish into rapeseed was found to be associated with a *Pgi-2* isozyme allele of radish.

Observation of metaphase-I pairing in heterozygous restored plants showed that most of the pollen mother cells had 19 bivalents (data not shown). Thus, the introgression of the restorer gene could not be the result of a substitution. The *Pgi-2* radish allele was co-segregating with the *Pgi-2* alleles of rapeseed. The plants which were homozygous for the restorer gene have lost one *Pgi-2* allele of rapeseed. The most likely hypothesis is that a radish chromosome segment had, by recombination, replaced its homoeologue carrying the *Pgi-2* gene.

Similar results have been obtained by Yu (1991) through the transfer of resistance to the root knot nematode (*Meloidogyne naasi*) from *Aegilops variabilis* into *Triticum aestivum*. The chromosome segment introgressed into wheat carried the resistance gene and an esterase gene (*Est-5*) from *Ae. variabilis* which replaced its homoeologous segment carrying one *Est-5* gene of wheat. Another example has been reported for the transfer of resistance to eyespot (*Pseudocercosporella herpotrichoides*) from *Aegilops ventricosa* to wheat (Gale et al. 1984; McMillin et al. 1986).

Recombination between radish and rapeseed genomes could occur since possible allosyndetic pairing between these two species has been observed (Dolstra 1982; Rousselle 1983). The study of the meiotic behaviour of restorer material in the earlier generations showed that there were meiotic irregularities: up to three multivalents per pollen mother cell were observed (Pellan-Delourme and Renard 1988). These multivalents implied that radish chromosome segments had been retained. Since meiotic pairing was greatly regularised (data not shown), it can be assumed that, during the successive backcrosses, new recombinations could have eliminated most of the remaining radish chromosome segments, thus reducing their amount. This is supported by the fact that female fertility improved as the number of backcrosses increased (Delourme et al. 1991).

When searching for isozyme markers, only *Pgi-2* from radish was present in the restored plants and was closely linked to the restorer gene. In any case, repeated backcrosses could quite easily remove radish chromosome segments which were unlinked to the restorer gene. By contrast, elimination of linked segments would be more difficult due to 'linkage drag' (Zeven et al. (1983). Young and Tanksley (1989) measured the size of introgressed segments flanking the *Tm-2* locus in several cultivars of tomato using a high density map of restriction fragment length polymorphism markers (the *Tm-2* locus was introduced from *Lycopersicon peruvianum* and conferred resistance to tobacco mosaic virus). The smallest introgressed segment was estimated to be 4 cM in length, while the longest was over 51 cM. Moreover, Young and Tanksley (1989) showed that, through additional backcrosses, plants containing desirable recombination near the resistance gene were rarely selected and, as a result, the backcross breeding method was largely ineffective in reducing the size of linked DNA around the *Tm-2* locus. By contrast, the use of a marker closely located near the target gene during the backcrosses quickly reduced the length of the introgressed segment.

Actually, in the present study, recombination between the restorer gene and the *Pgi-2* gene occurred in very few plants. However, the recombination rate does not provide a reliable estimate of the physical distance between the two genes. Recombination preferentially takes place in the homologous parts of the genomes and this reduces recombination in the homoeologous parts (Poisson 1970). The recombination rate should be studied in radish itself in order to assess the minimum length of the introgressed segment. This task will be difficult because of the fact that several restorer genes are present in radish.

We have deduced the presence of a null allele at one *Pgi-2* locus for plant II. Spring restorer material was originally crossed to the variety 'RP1' which carries a *Pgi-2* null allele. This null allele might have been retained from that cross.

Recombination events could not explain the occurrence of a restored plant lacking the radish isozyme marker in the progeny of a plant which was homozygous for the *Pgi-2* gene and the restorer gene. A deletion or an unequal cross-over might be the cause of the loss of the radish isozyme marker in this case. Deletion of the restorer gene in the progeny of plants which were homozygous for this gene has also been observed, with a frequency of 2‰ (Delourme et al., unpublished).

Linkage between the restorer gene and the radish isozyme marker proved very useful for understanding the genetic structure of restored plants. Moreover, this linkage is of great value for screening the restored plants at the plantlet stage. This was possible even when the radish isozyme marker was eliminated but was more difficult since the difference between the three-banded patterns relies on the intensity of one band. What is more interesting is that it was also possible to screen homozygous restored plants. Thus, this isozyme marker is now currently used in restorer line breeding schemes.

Additional markers, such as RFLP (restriction fragment length polymorphism) and RAPD (random amplified polymorphic DNA) markers, will now be analyzed for the location of the restorer gene on an existing *B. napus* map (Landry et al. 1991). Furthermore, the saturation of the *Pgi-2*-restorer chromosome segment with additional markers may ultimately lead to the cloning of the restorer gene via the technique of chromosome walking.

Acknowledgements. We are grateful to R. Horvais for her excellent technical assistance. We thank C. F. Quiros for his critical review of the manuscript.

References

Arus P, Orton TJ (1983) Inheritance and linkage relationships of isozyme loci in *Brassica oleracea*. J Hered 74:405–412

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

Dolstra O (1982) Synthesis and fertility of × *Brassicoraphanus* and ways of transferring *Raphanus* characters to *Brassica*. Agric Res Rep (Wageningen), no. 917

Gale MD, Scott PR, Law CN, Ainsworth CC, Hollins TW, Worland AJ (1984) An α-amylase gene from *Aegilops ventricosa* transferred to bread wheat together with a factor for eyespot resistance. Heredity 52:431–435

Heyn FW (1976) Transfer of restorer genes from *Raphanus* to cytoplasmic male-sterile *Brassica napus*. Cruciferae Newslett 1:15–16

Landry BS, Hubert N, Etoh T, Harada JJ, Lincoln SE (1991) A genetic map for *Brassica napus* based on Restriction Fragment Length Polymorphisms detected with expressed DNA sequences. Genome 34:543–552

McMillin DE, Allan RE, Roberts DE (1986) Association of an isozyme locus and strawbreaker foot rot resistance derived from *Aegilops ventricosa* in wheat. Theor Appl Genet 72:743–747

Pellan-Delourme R (1986) Étude de deux systèmes de stérilité mâle cytoplasmique introduits chez la colza (*Brassica napus* L.) par croisements intergénériques avec *Raphanus* et *Diplotaxis*. Thèse de Doctorat, Université de Rennes I, France

Pellan-Delourme R, Renard M (1988) Cytoplasmic male sterility in rapeseed (*Brassica napus* L.): Female fertility of restored rapeseed with "Ogura" and cybrids cytoplasms. Genome 30:234–238

Pelletier G, Primard C, Vedel F, Chétrit P, Rémy R, Rousselle P, Renard M (1983) Intergeneric cytoplasmic hybridization in *Cruciferae* by protoplast fusion. Mol Gen Genet 191:244–250

Pelletier G, Primard C, Vedel F, Chétrit P, Renard M, Pellan-Delourme R, Mesquida J (1987) Molecular, phenotypic and genetic characterization of mitochondrial recombinants in rapeseed. Proc 7th Int Rapeseed Conf, Poznan, Poland, pp 113–118

Poisson C (1970) Contribution à l'étude de l'hybridation interspécifique dans le genre *Gossypium*: Transfert de matériel génétique de l'espèce sauvage diploïde *Gossypium anomalum* à l'espèce cultivée tétraploïde *G. hirsutum*. Thèse de Doctorat, Université de Paris, Centre d'Orsay, France

Rousselle P (1982) Premiers résultats d'un programme d'introduction de l'androstérilité "Ogura" du radis chez le colza. Agronomie 2:859–864

Rousselle P (1983) Obtention et étude cytogénétique de l'hybride Radis × Colza. Comptes Rendus Groupe de Travail 'Cytologie et Cytogénétique', INRA, pp 26–27

Shields CR, Orton TJ, Stuber CW (1983) An outline of general resource needs and procedures for the separation of active enzyme from plant tissue. In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding, part A. Elsevier Science Publishers, Amsterdam, pp 443–468

Vallejos CE (1983) Enzyme activity staining. In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding, part A. Elsevier Science Publishers, Amsterdam, pp 469–516

Wendel JF, Stuber CW (1984) Plant Isozymes: enzymes studied and buffer systems for their electrophoretic resolution in starch gels. Isozyme Bull 17:4–11

Young ND, Tanksley SD (1989) RFLP analysis of the size of chromosomal segments retained around the *Tm-2* locus of tomato during backcross breeding. Theor Appl Genet 77:353–359

Yu MQ (1991) Transfert des gènes de résistance aux nématodes *Meloidogyne naasi* et *Heterodera avenae* d'*Aegilops variabilis* dans le blé tendre. Thèse de Doctorat, Université de Rennes I, France

Zeven AC, Knott DR, Johnson R (1983) Investigation of linkage drag in near isogenic lines of wheat by testing for seedling reaction to races of stem rust, leaf rust and yellow rust. Euphytica 32:319–327