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V. Laporte · D. Merdinoglu · P. Saumitou-Laprade
G. Butterlin · P. Vernet · J. Cuguen

Identification and mapping of RAPD and RFLP markers linked to a fertility restorer gene for a new source of cytoplasmic male sterility in *Beta vulgaris* ssp. *maritima*

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Abstract The present study shows that the recently described mitochondrial H haplotype is associated with cytoplasmic male-sterility (CMS). This new source of CMS appears to be different from the mitotype E-associated CMS most frequently found in natural populations. A mitotype H progeny with a sexual phenotype segregation was used to identify a gene restoring male fertility (*R1H*). Using bulk segregant analysis (BSA), nine RAPD markers linked to this restorer locus were detected and mapped. The comparison with other *Beta* genetic maps shows that the closest RAPD marker, distant from *R1H* by 5.2 cM, belongs to the same linkage group as the monogermy locus. In order to determine the position of *R1H* more precisely, four RFLP loci within this linkage group were mapped in the segregating progeny. It thus became possible to construct a linkage map of the region containing the RFLP, RAPD and *R1H* loci. The closest RFLP marker was located 1.7 cM away from *R1H*. However, a nuclear gene restoring the 'Owen' CMS which is currently used in sugar beet breeding is reportedly linked to the monogermy locus, raising the question of a possible identity between the new CMS system and the 'Owen' CMS.

Key words Gynodioecy · Cytoplasmic male sterility · Restorer gene · Bulk segregant analysis · *Beta vulgaris* ssp. *maritima*

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V. Laporte (✉) · P. Saumitou-Laprade · P. Vernet · J. Cuguen
Génétique et Evolution des Populations Végétales,
URA CNRS 1185, Université de Lille I, Bâtiment SN2,
F-59655 Villeneuve d'Ascq Cedex, France
Fax: +33 320 4369 79
E-mail: joel.cuguenauniv-lille1.fr

D. Merdinoglu · G. Butterlin
INRA, Station de Recherche sur les Grandes Cultures, 28 rue de Herrlisheim, 68021 Colmar Cedex, France

Introduction

Gynodioecy is a reproductive system in which both hermaphrodite (male-fertile) and female (male-sterile) individuals co-occur in natural populations (Darwin 1877). This system is known in more than 7.5% of the angiosperm species in Europe (Delannay 1978). In most of the cases, the inheritance of this system involves both cytoplasmic, maternally inherited, and nuclear, biparentally inherited, genes (Charlesworth 1981; Van Damme 1983; Boutin et al. 1987; Belhassen et al. 1991; Koelewijn and Van Damme 1995 a). In crop plants, cytoplasmic male sterility (CMS) is due to chimeric genes resulting from rearrangements of mitochondrial DNA sequences (Hanson 1991; Saumitou-Laprade et al. 1994; Vedel et al. 1994). Male fertility can be restored by nuclear genes which suppress the expression of the sterilising mitochondrial factors. Within a particular gynodioecious species, male sterility is generally due to different cytoplasmic types (CMS types), each type being associated with specific restorer genes (Kheyr-Pour 1981; Koelewijn and Van Damme 1995 a; De Haan et al. 1997 a). Theoretical models show that nucleo-cytoplasmic interaction is a key factor to understand the occurrence and the maintenance of gynodioecy (Charlesworth 1981; Delannay et al. 1981; Frank 1989; Gouyon et al. 1991). It is therefore essential to elucidate the genetic control of male sterility. Identification of both the different CMS types and their specific nuclear restorer genes requires cross analyses. These cross analyses are rather tedious due to three main reasons (Kaul 1988; Koelewijn and Van Damme 1995 b; De Haan et al. 1997 b). First, sexual phenotypes are often hard to assign because the trait can be continuously distributed from male-sterile to male-fertile. Second, there can be a large number of CMS systems. Third, several nuclear restorer genes are frequently involved in a single CMS system. Developing markers that allow fast identification of the CMS types and the

restorer alleles would facilitate genetic analysis. Moreover, such markers would also be useful for population-genetics investigations. In several gynodioecious species, such markers are in fact used to identify the different CMS types (Belhassen et al. 1991; Saumitou-Laprade et al. 1993; Cuguen et al. 1994; Ronfort et al. 1995; De Haan et al. 1997 a), but markers of the nuclear restorer genes are not yet available (though see Van Dijk 1984 for a loosely linked allozymic marker). However, such nuclear restorer gene markers are available for a few cultivated species such as rice (Zhang et al. 1994, 1997), rapeseed (Delourme et al. 1994), bean (He et al. 1995) and maize, for which a restorer gene has even been sequenced (Xiangqin et al. 1996).

Previous studies on wild beet populations growing along the French Atlantic coast have revealed that 42% of the populations are gynodioecious, with up to 80% of male-sterile individuals occuring locally (Boutin-Stadler et al. 1989; Cuguen et al. 1994). In order to differentiate CMS types, several authors have used molecular markers to study the cytoplasmic diversity of *Beta vulgaris* (Mikami et al. 1985; Halldén et al. 1988; Mann et al. 1989; Saumitou-Laprade et al. 1993). As a result, at least 12 mitochondrial haplotypes have been reported. In natural populations, male sterility was found to be associated mainly with two mitotypes, referred to as E and G, the E type being the most frequent (Cuguen et al. 1994). One male-sterile plant with another rare mitotype, termed H, was also found (Boudry et al. 1993). A different source of male sterility is used in sugar beet breeding programs. This CMS, originally described by Owen (1942), and called the 'Owen' CMS, is characterized by a specific mitochondrial haplotype *Svulg*, rarely found in natural populations. The nuclear component of male fertility restoration has received less attention in wild beets, just as in other gynodioecious species. The only system that has been investigated is the 'Owen' CMS type (Owen 1945). A plant having this CMS type is male-fertile only if it carries dominant alleles at both unlinked nuclear loci X and Z. These loci have been localized in chromosomes III and IV, respectively, according to the new nomenclature of *Beta* chromosomes (Schondelmaier and Jung 1997), but close flanking markers are still lacking. In the present study we report the first identification of a male fertility restorer locus in *B. vulgaris* ssp. *maritima*. We focused on the recently described H mitotype and found it to be associated with a new cytoplasmic source of male sterility, differing from the E mitotype-associated CMS most frequently found in natural populations. A progeny segregating for male sterility was the basis for identifying a gene restoring male fertility to plants having the H male-sterile cytoplasm. A bulk segregant analysis was employed to identify nine RAPD markers linked to this restorer locus. A linkage map of this region was established using both RAPD and RFLP loci and was compared to the genetic maps of *B. vulgaris* currently available (for a review see Schondelmaier and Jung 1997).

Materials and methods

Plant material

B. vulgaris ssp. *maritima* is a wind-pollinated, gynodioecious, short-lived perennial species widely distributed along the coasts of western Europe and around the Mediterranean Basin. It belongs to the same species as cultivated beet (leaf, table, fodder and sugar beets referred to as *B. vulgaris* ssp. *vulgaris*), and is the closest wild relative of these taxa. Open-pollinated progenies were collected from six natural populations located in Croze and Palavas les Flots (Hérault, Mediterranean coast), Lectoure and Bourgade (wild annual inland beets from south-western France, Gers), Marans and Brouage (Charente-Maritime, Bay of Biscay). Plants were grown under standard greenhouse conditions, classified for pollen fertility, and used to produce progenitors for subsequent crossing experiments.

Bulk segregant analysis and mapping

A family of 58 individuals was derived from a cross between a male-sterile plant having mitotype H and a male-fertile plant having mitotype A. These individuals originated from progenies collected in Marans and Palavas respectively. Within this family, sexual phenotypes were scored, and a BSA procedure was conducted using RAPD markers. Both parents (P_1 and P_2) of another progeny implicated in the construction of the RAPD map of Merdinoglu et al. (1995) were used to check the identity of markers between the RAPDs of the present study and those already mapped.

Reciprocal crosses

Five reciprocal crosses were conducted in the greenhouse under standard conditions (16 h light, 20°C). Contamination with foreign pollen was prevented by bagging the spikes before flowering occurred. *Beta maritima* is self-incompatible so that no precautions against self-pollination were needed when making reciprocal crosses.

Four crosses were conducted between male-fertile individuals having mitotypes-H and E. All four mitotype H parents were derived from a cross between two male-fertile individuals originating from Lectoure. All four mitotype E parents were derived from a cross between two male-fertile individuals originating respectively from Bourgade for the seed parent and Croze for the pollen parent. The fifth cross was conducted between a male-fertile mitotype-H individual issuing from the progeny used for the BSA, and a male-fertile individual originating from Brouage. All progenies were grown in the greenhouse under standard conditions (16 h light, 20°C) and reciprocal pairs were always grown together. At flowering, plants were classified according to pollen fertility.

Phenotypic classification and data analysis

Among the progenies involved in this study, three phenotypic classes were distinguished: male-sterile individuals with white or yellow empty anthers (ms), intermediate individuals with non-dehiscent anthers containing pollen (int), and male-fertile individuals with full and dehiscent anthers (mf). Within the progeny used for BSA, three kinds of observations were carried out on the sexual phenotype:

(1) Throughout the first flowering period, in April 1996, under inducing conditions (16 h light, 20°C), the sexual phenotype of each plant was noted daily, either as male-sterile, intermediate or male-fertile, on the basis of the examination of the anthers.
(2) Pollen viability was estimated on a sample of 53 plants by a staining procedure which differentially stains abortive and non-abortive pollen grains (Alexander 1969). Five to seven days after the first flower had opened, two flower buds were sampled on each plant, just before anthesis. Two stamens were then chosen from each bud and individually squashed into a drop of Alexander's stain. When pollen grains were present, a minimum of 300 grains was recorded per sample, and pollen viability was estimated for each plant as the mean of the four estimates.
(3) After flowering, stalks were cut down. The plants were then placed outside the greenhouse and flowered a second time in July (under natural conditions of light and temperature). During this second flowering period, one observation per plant was made to detect the presence of pollen.

For each cross, differences in the segregation of sexual phenotypes between reciprocal progenies were tested with two independent contingency tables: for the first test, all three phenotypic classes were considered (ms, int, mf), whereas in the second test intermediate individuals were pooled with male-fertile individulas (male-steriles ms, versus non-male-steriles int + mf). G-tests were performed as described by Sokal and Rohlf (1995), using BIOM package version 2.1 (Rohlf 1992).

Molecular analyses

RFLP analyses

Total DNA extraction, as well as restriction and blotting procedures, were carried out as described in Saumitou-Laprade et al. (1993). Three diagnostic probes were used on *Eco*RI DNA digests to discriminate between mitochondrial haplotypes. They were: ATPase subunit 6 (Dewey et al. 1985), pBv4 (Saumitou-Laprade et al. 1993) and Nvulg/N2 (Cuguen et al. 1994). Nuclear RFLP analyses were performed on the segregating progeny used for BSA with the help of four probes from chromosome IV according to the new nomenclature of Schondelmaier and Jung (1997): Koeln7, Koeln132 (Barzen et al. 1995), pKP753 and pKP823 (Pillen et al. 1993; Schondelmaier et al. 1995). Hybridization was performed on *Eco*RI digested DNA.

BSA and RAPD analyses

Total DNA extraction and amplification reactions were carried out as described in Pelsy and Merdinoglu (1996). The DNAs of six male-fertile and six male-sterile individuals were combined into two separate pools for BSA. These bulks were screened by 160 arbitrary 10-mer oligonucleotide primers (kits A, B, D, F, K, L, M, P; Bioprobe-System, Montreuil-sous-Bois, France), previously used to construct a RAPD map (Merdinoglu et al. 1995). Primers detecting polymorphism were then tested on the 12 individuals constituting the bulks. Markers that were definitely linked to the restorer locus were tested for linkage analysis in the 46 remaining F_1 individuals. The nomenclature for the markers conforms to the convention of primer designation, the fragment size being written down as a subscript (e. g. $K11_{100}$ designates a fragment of 1000 bp amplified by the K11 primer).

One RAPD fragment of 1000 bp amplified by the K11 primer in the present study had already been observed and mapped in a different segregating progeny (Merdinoglu et al. 1995). The identity between both fragments was checked by a Southern hybridization. To do this, the $K11_{1000}$ fragment, which had previously been amplified from the P_1 parent, was isolated from an agarose gel with a NUCLEOTRAP kit (Macherey-Nagel, Düren) and labelled with digoxygenin (Boehringer, Mannheim) by a PCR reaction that used the K11 primer. It then served as a probe to a Southern blot of 14 PCR products that had been amplified with the K11 primer for which the templates were the DNA from six male-fertile and six male-sterile individuals of the present mapping progeny and P_1 and P_2. The size and the segregation of the hybridized fragments were compared to the size and the segregation of the $K11_{1000}$ amplified fragment.

Genetic mapping

In order to locate the nuclear restorer gene on published genetic maps, three different maps of *Beta* were consulted. First, the map by Merdinoglu et al. (1995) was referred to in order to identify the linkage group that contained the markers linked to the restorer locus. Then the maps by Barzen et al. (1995) and Schondelmaier et al. (1995), including RFLP, RAPD and morphological markers, were used to produce a joint map of the region in which the restorer locus was situated.

Within the progeny used for BSA, a 1 : 1 ratio fitted the observed ratio of: (1) the sexual phenotypes, and (2) the co-segregant RAPD markers detected by BSA. To produce this ratio, the parents must be respectively heterozygous and recessive homozygous at these loci. It was therefore assumed that the sexual phenotype variation was determined by the polymorphism of one restorer locus. Linkage analysis between the restorer locus and linked-loci was performed using MAPMAKER version 3 (Lincoln 1992). Since the observed segregation was that of a test-cross design, data were encoded as recommended for such a cross. However, the genotypes observed for the RFLP markers had to be adapted to that design. In particular, when more than two alleles were segregating in the progeny, alleles were bulked to produce two synthetic classes, depending on the co-segregation with the sexual phenotype. Linkage groups were established with a LOD-score threshold of 3.0. The map order was based on maximum-likelihood estimation. Multipoint analysis was used to determine the genetic distance between markers using the mapping function of Kosambi (1994).

Results

Characterization of mitochondrial haplotype H-associated CMS

RFLP analyses using Nvulg/N2 and Pbv4 probes confirmed that mitochondrial type H is clearly different from the three other male sterile mitotypes (E, G or *Svulg*), as illustrated in Fig. 1. Among the families derived from the reciprocal crosses, 405 plants having a H mitotype were surveyed (Table 1). Among these, 88 were found to be male-sterile. The examination of sexual phenotypes within progenies derived from cross 1 revealed a highly significant difference between segregating reciprocal crosses: male-sterile individuals were systematically found to be associated with the H cytoplasmic type. These results show that mitotype H is associated with cytoplasmic male sterility. Within the eight other progenies involving mitotypes E and H, a segregation of sexual phenotypes was observed: male-sterile, intermediate and male-fertile individuals were scored. Nevertheless, the difference in segregation among reciprocal progenies was always highly significant. This

was true for each reciprocal pair, whatever the classification adopted (with two or three phenotypic classes). These results show that mitotypes E and H correspond to two different CMS systems. There were also significant differences among the various progenies with the same cytoplasm: $G = 85.9$, $df = 6$, $P = 0.001$ and $G = 44.7$, $df = 6$, $P = 0.001$ for progenies having cytoplasms H and E, respectively. This result indicates that even though parents, who were full-sibs, presented the same cytoplasms, they were not identical for the nuclear restorer components.

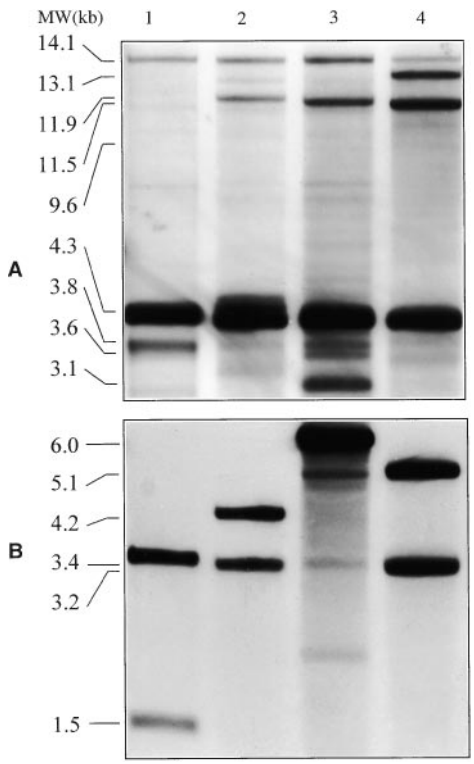

Fig. 1 Southern-blot analysis of the *Eco*RI-digested total DNA of *B. vulgaris* ssp. *maritima* (lanes 1, 2, 4) and *B. vulgaris* ssp. *vulgaris* (*lane 3*) with two probes: Nvulg/N2 (**A**) and Pbv4 (**B**). Four mitochondrial haplotypes are identified: G (*lane 1*), E (*lane 2*), S*vulg* (*lane 3*) and H (*lane 4*). Both probes clearly discriminate mitotype H from the other male-sterile mitotypes (G, E and S*vulg*)

Segregation of male sterility in the progeny used for BSA

A total of 23 male-sterile and 35 male-fertile individuals was observed in the progeny used for bulk segregant analysis. Various examinations of the sexual phenotype were consistent: no modification was observed, neither within nor between the two flowering periods. Alexander-staining also supported these observations: the pollen stainability of male-fertile individuals was very high (above 85%); on the other hand, the majority of male-sterile individuals had empty stamens: some exceptional grains were found, but they were always aborted. A 1:1 theoretical ratio fitted the observed ratio ($G = 2.14$, $df = 1$, $P = 0.144$). This result suggests that a single nuclear locus, referred to as *R1H*, is responsible for the sexual-phenotype variation in this progeny.

Identification and mapping of RAPD markers linked to the *R1H* locus

Repeatability of the RAPD reactions was satisfactory assessed, as similar profiles were observed during independent amplifications conducted for both the bulks and the individuals. Using 160 primers, the amplified fragments ranged in size from 300 to 2600 kb. Among these primers, 32 revealed a polymorphism between the bulks. They were utilized to individually analyze the DNA of the 12 individuals forming the bulks: 120 polymorphic bands were thus identified. Among them, only $K11_{1000}$ was fully associated with the male-fertile phenotype (Fig. 2), while eight other fragments were

Table 1 Reciprocal crosses conducted on *B. vulgaris* ssp. *maritima*. For each cross both parents are given as the seed parent, but each acted as the pollen parent in the reciprocal cross. For each cross, the mitochondrial DNA haplotype (mtDNA), the segregation of sexual phenotypes (ms, int, mf), and the total number of descendants observed on each seed parent (total) are indicated. Heterogeneity between the reciprocals is tested with two G tests: first, three phenotypic classes were taken into account (ms, int, mf); second, intermediates were bulked with the male-fertile individuals, as they both produce some viable pollen. The *G* values, their significance level, and the degrees of freedom (*df*) are given

Cross	Seed parent 1					Seed parent 2					f:int:h		f:(int + h)	
	mtDNA	ms	int	mf	Total	mtDNA	ms	int	mf	Total	*G*	*df*	*G*	*df*
1	H	15	0	9	24	+[a]	0	0	8	8	–	–	12.48 (***)	1
2	H	6	9	82	97	E	28	15	57	100	21.44 (***)	2	17.62 (***)	1
3	H	39	34	27	100	E	66	17	17	100	15.09 (***)	2	14.80 (***)	1
4	H	18	14	59	91	E	39	11	45	95	10.09 (**)	2	10.08 (**)	1
5	H	10	11	72	93	E	41	9	47	97	25.63 (***)	2	25.41 (***)	1
Total	H	88	68	249	405	E	174	52	174	400				

*** $P < 0.001$; ** $P < 0.01$

[a] This mitotype has not been determined

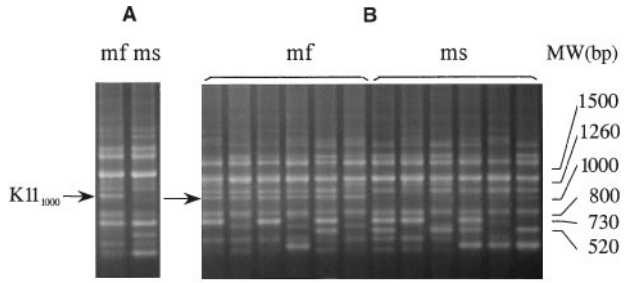

Fig. 2 RAPD-amplification profiles obtained with the K11 primer on the two bulks (**A**) and the 12 individuals of the two bulks (**B**). The sexual phenotypes are male-fertile (*mf*) or male-sterile (*ms*). The *arrows* indicate the polymorphic fragment of 1000 pb, $K11_{1000}$, associated with the male-fertile phenotype

only preferentially associated with one phenotype: within the 12 individuals tested, one potential recombinant was detected. Linkage between these nine markers and the sexual phenotype was confirmed when the remaining 46 individuals were amplified. No significant deviation was observed from the expected 1 : 1 segregation ratio. The fact that all these markers were located on the same linkage group as *R1H* was established (Fig. 3 A). The RAPD marker nearest *R1H* was $K11_{1000}$, located 5.2 cM away; six other markers were clustered 13.9 cM away, one was 17.4 cM away, and the most remote was situated 26.1 cM away from *R1H*. All of them were found on the same side of the *R1H* locus.

Location of the RAPD marker $K11_{1000}$, and mapping of RFLP markers

Hybridization showed that the present $K11_{1000}$ fragment was identical to the one previously mapped by Merdinoglu et al. (1995). Hybridization on amplified DNA revealed five major bands (data not shown). Two of them were polymorphic but did not co-segregate with the sexual phenotype. Three other bands were specific to the male-fertile individuals, including the smallest one (1000 bp), which is actually the original polymorphic RAPD marker linked to the restorer allele. The $K11_{1000}$ fragment is located on the same linkage group as the monogermy locus (Merdinoglu et al. 1995, see also Fig. 3D), identified as chromosome IV (Schondelmaier and Jung 1997). Four RFLP markers within this linkage group were then mapped in the progeny, and were found to be polymorphic (data not shown). Linkage analyses showed that they belonged to the same linkage group as the *R1H* locus, thus confirming its position (Fig. 3A). Two RFLP probes were mapped on the same side as the nine RAPD markers: the Koeln132 locus was mapped within the cluster of the six RAPD markers, 13.9 cM away from the *R1H* locus, and pKP823 co-segregated with the $A16_{1450}$ RAPD

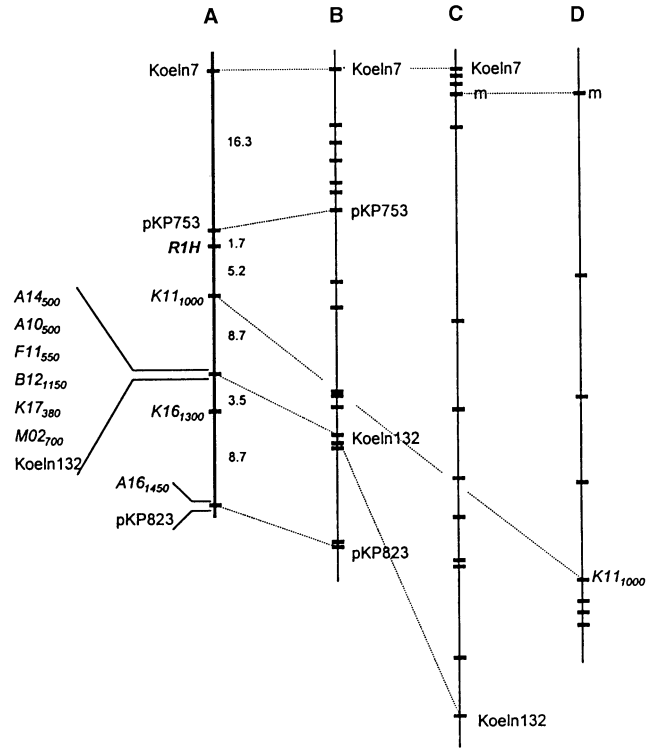

Fig. 3 Partial linkage map for the *R1H* region obtained from the segregating progeny used for BSA (**A**) and comparison with different genetic maps (**B**, **C** and **D**). **A** Genetic distances in cM are shown on the right of the vertical line. The restorer locus *R1H* (*in bold characters*), nine RAPD markers (*in italics*) and four RFLP markers were mapped. **B** Schondelmaier et al. (1995); **C** Barzen et al. (1995); **D** Merdinoglu et al. (1995). For a comparison between genetic maps, common markers are named; other mapped markers are indicated by *dashes*. The monogermy locus is indicated by *m*.

marker, 26.1 cM away from the *R1H* locus. The two other RFLP probes were mapped on the opposite side of the *R1H* locus: 1.7 cM away for the pKP753 probe, and 16.3 cM farther for the Koeln7 probe. The *R1H* locus is therefore flanked by molecular markers within a genetic window of 6.9 cM. The linear order of the four RFLP loci deduced from our progeny analysis was identical to that described by Schondelmaier et al. (1995). The genetic distances between the four RFLP loci inferred from our progeny analysis were relatively close to those estimated by Schondelmaier et al. (1995) but much lower than the estimates of Barzen et al. (1995) (Fig. 3 A, B, C).

Discussion

A new source of male sterility, genetically different from the type most commonly found in natural populations

In the present study, we showed that mitochondrial type H is clearly associated with cytoplasmic male

sterility, and identified one nuclear locus restoring male fertility in plants having this mitochondrial type. The expression of this gene seems to have a drastic effect on male fertility because no intermediate plants were found within the segregating progeny used for the bulk segregant analysis: plants were either fully male-sterile or fully male-fertile. This is different from what is observed in segregating progenies carrying the 'Owen' CMS, where wide variations in male fertility and many intermediates were reported among the offspring (Owen 1945). Such clear-cut phenotypes in segregating progenies are considered as characteristic of Mendelian male sterility (Owen 1952) and could suggest that *R1H* confers such sterility. However, a reciprocal cross involving offspring of the progeny used for BSA allows us to rule out the hypothesis of a purely nuclear inheritance of male sterility, since segregation of the sexual phenotypes was observed only in the maternal progeny carrying mitotype H.

Until now, three mitotypes were reportedly associated with cytoplasmic male sterility in beets (Powling 1982; Mikami et al. 1985; Boutin et al. 1987; Halldén et al. 1988; Mann et al. 1989; Halldén et al. 1990; Saumitou-Laprade et al. 1991; Saumitou-Laprade et al. 1993). Molecular characterization clearly discriminated them from mitotype H. These observations strongly suggest that mitotype H is a novel mitochondrial source of cytoplasmic male sterility. The analysis of reciprocal crosses also suggests that the CMS associated with type-H mitochondrial DNA is genetically different from the E mitotype-associated CMS most frequently found in natural populations.

The joint analysis of crossing experiments and the molecular mitochondrial polymorphism associated with male sterility suggests that five different CMS systems exist in *B. vulgaris*: the four male-sterile mitotypes, E, G, H and *Svulg*, could each represent one CMS system, and be distinct from the one associated with fertile mitotypes. Theoretical studies show that the number of CMS systems can strongly influence the conditions under which gynodioecy can be maintained: increasing the number of CMS systems results in relaxed maintenance conditions for male-sterile individuals (Frank 1989) and permits a higher male-sterile frequency at equilibrium (Manicacci 1993). In most gynodioecious species, polymorphism in the CMS systems has been found, but the extent to they vary is not precisely known. Simple crossing analyses allowed the discrimination of a minimum of two CMS systems in *Nemophila menziesii* (Ganders 1978), *Thymus vulgaris* (Belhassen et al. 1991), and *Plantago coronopus* (Koelewijn and Van Damme 1995 a). In *Daucus carota*, (Ronfort et al. 1995) and *Plantago lanceolata* (De Hann et al. 1997 a) a combination of mitochondrial DNA polymorphism studies and crossing experiments identified a minimum of three and four CMS systems, respectively.

Identification and mapping of the *R1H* restorer gene

One mitochondrial type-H family of *B. vulgaris* ssp. *maritima* enabled us to map the *R1H* nuclear locus, as well as nine RAPD and four RFLP markers, on chromosome IV, according to the nomenclature of Schondelmaier and Jung (1997). The closest flanking markers were located 5.2 and 1.7 cM away, on opposite sides of the restorer locus. Until now, nuclear genes restoring male fertility in *B. vulgaris* had only been identified for the cultivated 'Owen' CMS, but close flanking markers are not yet available. The present study reports the first case in which molecular markers tightly linked to a restorer locus have been found in beets. The Z restorer locus was reported to be loosely linked to the monogermy locus (Kinoshita and Takahashi 1968; Roundy and Theurer 1974) in chromosome IV. Restorer locus X is located in a terminal region of chromosome III, 11 cM away from the nearest marker (Pillen et al. 1993). Close markers of restorer genes have been described in other cultivated species. The BSA strategy was recently used in rapeseed (Delourme et al. 1994) and bean (He et al. 1995) to identify markers completely linked to previously located restorer genes. This strategy was also effective in identifying and mapping two unlinked loci governing the photoperiod-sensitive male sterility in rice (Zhang et al. 1994). However, due to the lack of close flanking markers for the nuclear restorer genes in any gynodioecious species, the nuclear component of male sterility has been little investigated, compared with the cytoplasmic component. The frequencies of restorer alleles and their distribution in natural populations are particularly poorly known. Until now, the only way to obtain this key information was to estimate the frequency of male-fertile individuals among plants with a given CMS type. This has been carried out for *T. vulgaris* (Manicacci et al. 1997), *P. lanceolata* (Van Damme 1986; De Haan et al. (1997 b), *B. vulgaris* ssp. *maritima* (Forcioli 1995; Laporte, unpublished), and *P. coronopus* (Koelewijn and Van Damme 1995 b), but it is a tedious and not very accurate procedure. Close flanking markers that allow a quick identification of the restorer genes will facilitate population-genetics investigations and an understanding of the maintenance and evolution of gynodioecy. For this purpose, continuation of the present work will focus on the search for markers even more tightly linked to the *R1H* locus. These markers can also be used to select individuals when designing the relevant crosses needed to investigate the genetic determination of male fertility restoration.

Comparison with the Owen CMS

Several authors have reported weak linkage between the monogermy locus and the *Z* restorer gene associated with the 'Owen' CMS (Kinoshita and Takahashi

1968; Roundy and Theurer 1974). In spite of numerous studies, male fertility restoration associated with 'Owen' CMS is still poorly known: the consensus model assumes that two unlinked loci act in epistasis to restore male fertility, but this does not explain all the crossing results reported (Owen 1945). Moreover, the precise position of the *Z* restorer locus is still unknown. As estimated by Roundy and Theurer (1974) from the segregation analysis of F_2 progenies, the recombination value between the monogermy locus and *Z* is 0.40 (SE = 0.037). In the present study, the monogermy locus did not segregate, but its closely linked marker, Koeln7, did (Barzen et al. 1995). The recombination value between Koeln7 and the *R1H* restorer gene was estimated to be 0.172 (SE = 0.050). Although situated in the same linkage group, the *R1H* and *Z* genes appear to be distinct. However, given the discrepancies observed between recombination frequencies in different mapping populations (Schumacher et al. 1997), the possibility of a single locus repressing both the 'Owen' CMS and the mitotype H-associated CMS cannot be excluded. Hence, two possibilities remain with respect to the CMS types. First, the H and the *Svulg* mitotypes could carry the same mitochondrial gene coding for male sterility. In this case, restoration of male fertility would be controlled by the same nuclear genes. To explain the observed segregation of the sexual phenotypes in our families, the offspring must then carry a restorer allele on the X locus. Second, mitotypes H and *Svulg* could be associated with different CMS types but share a common restorer gene. In this case, it should be possible to find other nuclear restorer genes specific to one or the other CMS type. The molecular characterization of the mitotypes argues for different CMS types, since large differences were found between them. Nevertheless, reciprocal crosses between male-fertile individuals carrying these mitotypes should be carried out in order to reach a conclusion.

Markers close to the other restorer genes are required to elucidate the control of nuclear restoration and to conduct population investigations. The BSA approach presented here turned out to be an efficient method to obtain markers linked to a restorer locus, and is a promising approach for further investigations.

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References

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

Barzen E, Mechelke W, Ritter E, Schulte-Kappert (1995) An extended map of the sugar beet genome containing RFLP and RAPD loci. Theor Appl Genet 90:189–193

Belhassen E, Dommée B, Atlan A, Gouyon PH, Pomente D, Assouad MW, Couvet D (1991) Complex determination of male sterility in *Thymus vulgaris* L.: genetic and molecular analysis. Theor Appl Genet 82:137–143

Boudry P, Mörchen M, Saumitou-Laprade P, Vernet P, Van Dijk H (1993) The origin and evolution of weed beets: consequences for the breeding and release of herbicide-resistant transgenic sugar beets. Theor Appl Genet 87:471–478

Boutin V, Pannenbecker G, Ecke W, Schewe G, Saumitou-Laprade P, Jean R, Vernet P, Michaelis G (1987) Cytoplasmic male sterility and nuclear restorer genes in a natural population of *Beta maritima*: genetical and molecular aspects. Theor Appl Genet 73:625–629

Boutin-Stadler V, Saumitou-Laprade P, Valero M, Jean R, Vernet P (1989) Spatio-temporal variation of male-sterile frequencies in two natural populations of *Beta maritima*. Heredity 63:395–400

Charlesworth D (1981) A further study of the problem of the maintenance of females in gynodioecious species. Heredity 46:27–39

Cuguen J, Wattier R, Saumitou-Laprade P, Forcioli D, Mörchen M, Van Dijk H, Vernet P (1994) Gynodioecy and mitochondrial DNA polymorphism in natural populations of *Beta vulgaris* ssp. *maritima*. Genet Sel Evol 26:87s–101s

Darwin C (1877) The different forms of flowers on plants of the same species. Murray, London

De Haan AA, Mateman AC, Van Dijk PJ, Van Damme JMM (1997 a) New CMS types in *Plantago lanceolata* and their relatedness. Theor Appl Genet 94:539–548

De Haan AA, Koelewijn HP, Hundscheid MPJ, Van Damme JMM (1997 b) Dynamics of gynodioecy in *Plantago lanceolata* L. II. Mode of action and frequencies of restorer alleles. Genetics 147:1317–1328

Delannay X (1978) La gynodioecie chez les angiospermes. Nat Belges 59:223–235

Delannay X, Gouyon PH, Valdeyron G (1981) Mathematical study of the evolution of gynodioecy with cytoplasmic inheritance under the effect of a nuclear gene. Genetics 99:169–181

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

Dewey RE, Schuster AM, Levings III CS, Timothy DH (1985) Nucleotide sequence of F_0-ATPase proteolipid (subunit 9) of maize mitochondria. Proc Natl Acad Sci USA 82:1015–1019

Forcioli D (1995) Differenciation génétique comparée des informations nucléaires et cytoplasmiques dans les populations naturelles de *Beta maritima*: importance des flux géniques et conséquences sur la stérilité mâle. Thesis Université de Paris VI

Frank SA (1989) The evolutionary dynamics of cytoplasmic male sterility. Am Nat 133:345–376

Ganders FR (1978) The genetics and evolution of gynodioecy in *Nemophila menziesii* (Hydrophyllaceae). Can J Bot 56:1400–1408

Gouyon PH, Vichot F, Van Damme JMM (1991) Nuclear-cytoplasmic male sterility: single point equilibria versus limit cycles. Am Nat 137:498–514

Halldén C, Bryngelsson I, Bosemark NO (1988) Two new types of cytoplasmic male sterility found in wild Beta beets. Theor Appl Genet 75:561–568

Halldén C, Lind C, Säll T, Bosemark NO, Bengtsson BO (1990) Cytoplasmic male sterility in *Beta* is associated with structural rearrangements of the mitochondrial DNA and is not due to interspecific organelle transfer. J Mol Biol 31:365–372

Hanson MR (1991) Plant mitochondrial mutations and male sterility. Annu Rev Genet 25 : 461–486
He SC, Lyznik A, Mackenzie S (1995) Pollen fertility restoration by a nuclear gene Fr in cms bean – nuclear-directed alteration of a mitochondrial population. Genetics 139 : 955–962
Kaul MLH (1988) Male sterility in higher plants. Springer Verlag, Berlin
Kheyr-Pour A (1981) Wide nucleo-cytoplasmic polymorphism for male sterility in *Origanum vulgare* L. J Hered 72 : 45–51
Kinoshita T, Takahashi M (1968) Genetic constitution of some male-sterile characters in sugar beets. 8th Res Meeting Sugar Beet Tech Coop, (Northen Japan), Bull Sugar Beet Res Suppl 10 : 56–60
Koelewijn HP, Van Damme JMM (1995 a) Genetics of male sterility in gynodioecious *Plantago coronopus*. 1. Cytoplasmic variation. Genetics 139 : 1749–1758
Koelewijn HP, Van Damme JMM (1995 b) Genetics of male sterility in gynodioecious *Plantago coronopus*. 2. Nuclear genetic variation. Genetics 139 : 1759–1775
Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12 : 172–175
Lincoln S, Daly M, Lander E (1992) Constructing genetic maps with MAPMAKER/EXP 3.0. Cambridge, Massachusetts, USA
Manicacci D (1993) Evolution et maintien de la gynodioecie: allocation sexuelle et structuration spatiale du polymorphisme nucleocytoplasmique. Etude théorique et approches expérimentales dans le genre *Thymus*. Thesis Université de Montpellier II
Manicacci D, Atlan A, Couvet D (1997) Spatial structure of the nuclear factors involved in sex determination in the gynodioecious *Thymus vulgaris* L. J Evol Biol 10 : 889–907
Mann V, McIntosh L, Theurer C, Hirschberg J (1989) A new cytoplasmic male-sterile genotype in the sugar beet *Beta vulgaris* (L.): a molecular analysis. Theor Appl Genet 78 : 293–297
Merdinoglu D, Pelsy F, Butterlin G, Soreau P (1995) Recherche de marqueurs génétiques de caratères d'intérêt agronomique par RAPD chez la betterave. Proc 58th Congr IIRB (Beaune): 45–51
Mikami T, Kishima Y, Sugiara M, Kinoshita T (1985) Organelle genome diversity in sugar beet with normal and different sources of male-sterile cytoplasms. Theor Appl Genet 71 : 166–171
Owen FV (1942) Male sterility in sugar beets produced by complementary effects of cytoplasmic and Mendelian inheritance. Am J Bot 29 : 692
Owen FV (1945) Cytoplasmically inherited male sterility in sugar beets. J Agric Res 71 : 423–440
Owen FV (1952) Mendelian male sterility in sugar beets. Proc Am Soc Sugar Beet Technol 7 : 371–376
Pelsy F, Merdinoglu D (1996) Identification and mapping of random amplified polymorphic DNA markers linked to a rhizomania resistance in sugar beet (*Beta vulgaris* L.) by bulked segregant analysis. Plant Breed 115 : 371–377
Pillen K, Steinrücken G, Herrmann RG, Jung C (1993) An extended linkage map of sugar beet (*Beta vulgaris* L.) including nine putative lethal genes and the restorer gene X. Plant Breed 111 : 265–272
Powling A (1982) Restriction endonuclease analysis of mitochondrial DNA from sugarbeet with normal and male-sterile cytoplasms. Heredity 49 : 117–120
Rohlf J (1992) Biom, a package of statistical programs to accompany the text Biometry. Applied Biostatistics Inc., Setanket, NY
Ronfort J, Saumitou-Laprade P, Cuguen J, Couvet D (1995) Mitochondrial DNA diversity and male sterility in natural populations of *Daucus carota* ssp. *carota* L. Theor Appl Genet 91 : 150–159
Roundy TE, Theurer JC (1974) Linkage and inheritance studies involving an annual pollen restorer and other genetic characters in sugarbeets. Crop Sci 14 : 230–232
Saumitou-Laprade P, Pannenbecker G, Boutin-Stadler V, Michaelis G, Vernet P (1991) Plastid DNA diversity in natural populations of *Beta maritima* showing additional variation in sexual phenotype and mitochondrial DNA. Theor Appl Genet 81 : 533–536
Saumitou-Laprade P, Rouwendal GJA, Cuguen J, Krens FA, Michaelis G (1993) Different CMS sources found in *Beta vulgaris* ssp. *maritima*: mitochondrial variability in wild populations revealed by a rapid rapid screening procedure. Theor Appl Genet 85 : 529–535
Saumitou-Laprade P, Cuguen J, Vernet P (1994) Molecular evidence from the nucleo-cytoplasmic conflict in plants. Trends Ecol Evol 9 : 431–435
Schondelmaier J, Jung C (1997) Chromosomal assignment of the nine linkage groups of sugar beet (*Beta vulgaris* L.) using primary trisomics. Theor Appl Genet 95 : 590–596
Schondelmaier J, Heller R, Pillen K, Steinrücken G, Jung C (1995) A linkage map of sugar beet. Proc 58th Congr IIRB (Beaune) : 37–44
Schumacher K, Schondelmaier J, Barzen E, Steinrücken G, Borchardt D, Weber WE, Jung C, Salamini F (1997) Combining different linkage maps in sugar beet (*Beta vulgaris* L.) to make one map. Plant Breed 116 : 23–38
Sokal RR, Rohlf J (1995) Biometry. Freeman and Co., San Francisco
Van Damme JMM (1983) Gynodioecy in *Plantago lanceolata* L. II. Inheritance of three male-sterile types. Heredity 50 : 253–273
Van Damme JMM (1986) Gynodioecy in *Plantago lanceolata* L. V. Frequencies and spatial distribution of nuclear and cytoplasmic genes. Heredity 56 : 355–364
Van Dijk H (1984) Allozyme genetics, self-incompatibility and male sterility in *Plantago lanceolata*. Heredity 54 : 53–63
Vedel F, Pla M, Vitart V, Gutierres S, Chétrit P, De Paepe R (1994) Molecular basis of nuclear and cytoplasmic male sterility in higher plants. Plant Physiol Biochem 32 : 601–618
Xiangqin C, Wise RP, Schnable PS (1996) The *rf2* nuclear restorer gene of male-sterile T-cytoplasm maize. Science 272 : 1334–1336
Zhang G, Bharaj TS, Lu Y, Virmani SS, Huang N (1997) Mapping of the *Rf-3* nuclear fertility restoring gene for WA cytoplasmic male sterility in rice, using RAPD and RFLP markers. Theor Appl Genet 94 : 27–33
Zhang Q, Shen BZ, Dai XK, Mei MH, Saghai Maroof MA, Li ZB (1994) Using bulking extremes and recessive class to map genes for photoperiod-sensitive genic male sterility in rice. Proc Natl Acad Sci USA 91 : 8675–8679