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Genetic analysis of male fertility restoration in wild cytoplasmic male sterility *G* of beet

Abstract Cytoplasmic male sterility (CMS) has been used in the breeding of sugar beet for decades but is also more generally an important feature of the reproductive system in its wild relative, *Beta vulgaris* ssp. *maritima*. Among the several CMSs found in wild populations, the *G* CMS is a mitochondrial variant of the respiratory chain. The segregants derived from a cross between a restored plant and a female (male-sterile) plant on *G* cytoplasm exhibited three sexual phenotypic classes: female, hermaphrodite and intermediate. The pattern of segregation suggests a genetic inheritance with two loci in epistatic interaction. Nevertheless, it was possible to apply a bulk segregant analysis approach to search for AFLP and microsatellite markers linked to the restorer locus (RfG_1) which controls the capacity to produce pollen [female versus non female (i.e. intermediates and hermaphrodites)] in the segregating population. A linkage group was constructed with four AFLP markers and nine microsatellites, and a total size of 40 cM (Kosambi). The closest marker, a microsatellite, was totally linked to *RfG1*, which was also flanked by two AFLP markers delimiting a 5 cM window. This linkage group was identified as being chromosome VIII where neither of the restorer loci of the *Owen* CMS are located.

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Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait which results in the abortion of pollen development. This phenomenon has been described in many higher plant species and is called gynodioecy when it occurs in wild populations. Plants with sterilizing mitochondria can eventually produce pollen grains thanks to the "counteraction" of nuclear genes which restore male fertility, the so-called restorer loci (*Rf*). CMS has been advantageously used in plant breeding for numerous species in order to facilitate hybridization at a large scale for hybrid production. Apart from this practical interest for plant breeding, CMS has generated extensive investigations in two areas: molecular genetics, as an example of nucleo-cytoplasmic interactions; and evolutionary biology, as an example of genomic conflict between two genomes that do not share the same pattern of inheritance (Cosmides and Tooby 1981).

Molecular genetic studies have shown that in most cases, CMS has been associated with chimeric mitochondrial genes believed to result from intragenomic recombination (Saumitou-Laprade et al. 1994; Schnable and Wise 1998). On the nuclear side, the effects of the restorer loci generally have direct effects on the novel gene expression, either at the transcript or at the protein level (Budar et al. 2003). As of today, only three restorer loci have been cloned: *Rf*2, restoring CMS-T in maize (Cui et al. 1996, but see Touzet 2002), *Rf* in Petunia (Bentolila et al. 2002) and *Rfo/Rfk1* in Brassica (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003). The Brassica and Petunia loci both encode proteins containing a PPR motif, and may reveal a general feature of *Rf* genes (Wise and Pring 2002) that could belong to the large gene family assumed to be involved in organellar gene expression (Small and Peeters 2000).

In order to understand the maintenance of male sterility in natural populations, theoretical models have emphasized two key parameters: firstly, male sterility must confer an advantage in seed production (it is commonly called the female advantage), this is a character

which is also of interest in plant breeding (for reviews see Budar et al. 2003); and secondly, restorer alleles must be counter-selected in the absence of the corresponding sterilizing cytoplasm (through a negative pleiotropic effect commonly called "cost of restoration") (Charlesworth 1981, Frank 1989, Gouyon et al. 1991).

In beet (*Beta vulgaris*) several CMSs have been described: the *Owen* CMS which has been widely used in plant breeding of sugar beet for decades (Owen 1942), and three others called *E*, *G* and *H*, found in wild beet populations (Mikami et al. 1985; Halldén et al. 1988; Cuguen et al. 1994; Laporte et al. 2001). To our knowledge, the molecular sterilizing factors of *Owen* CMS have not yet been isolated (but see Yui et al. 2003). For the other wild sources of sterility, we recently showed that CMS *G* was a variant of the mitochondrial respiratory chain and hypothesized that there may be a link between male sterility and mitochondrial complex modifications (Ducos et al. 2001). Male fertility restoration of *Owen* CMS seems to imply at least two loci, *X* and *Z*, which have been mapped to chromosomes III and IV, respectively (Schondelmaier and Jung 1997, following Butterfass 1964). A restorer locus for CMS *H*, the least frequent "wild" CMS in populations from Western Europe, has also been mapped to chromosome IV (Laporte et al. 1998).

The recent development of mitochondrial markers in the context of CMS, and nuclear markers genetically linked to restorer loci, opens approaches for linking molecular genetics to evolutionary biology to give a better understanding of this dynamic trait in populations. In the present study, we investigated the genetic architecture of male fertility restoration in CMS *G* on a segregating population that was produced by crossing two plants on CMS *G*, a male-sterile (or female) and a restored plant (thus hermaphrodite). We present the characteristics of the resulting population, the genetic hypothesis to fit the observed ratio, as well as the genetic mapping of one of the restorer loci, following bulk segregant analysis (Michelmore et al. 1991). This is the first report of male fertility restoration of this newly characterized CMS, which could be valuable in sugar beet breeding.

Materials and methods

Plant material

Beta 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 is part of the same complex species as the cultivated beet (*Beta vulgaris* ssp. *vulgaris*).

An open-pollinated progeny was collected from a male sterile plant in a gynodioecious population in Bages in 1995 (Aude, South of France), composed of 14.5% of male sterile plants. The plant on which the seed was set was identified as having the *G* cytoplasm by an RFLP procedure as described in Cuguen et al. (1994). Two half-sibs from this progeny, one male sterile and one hermaphrodite (restored) were crossed to produce a family segregating for the restoration of male fertility. The cross was made on the hypothesis that restoration alleles were dominant over the non-restorer ones.

The progeny was sown in two consecutive years, 1999 and 2000. The plants were vernalized in order to observe the flowers the first year. Seeds were sown in December, seedlings planted in 17-cm-diameter pots and grown in standard greenhouse conditions, and the two-month-old plants transferred to a temperate greenhouse close to outside conditions but which maintained the temperature above freezing. They were then transferred outside in May, for subsequent determination of their male fertility phenotype.

The segregating progeny was composed of 179 plants, of which 95 flowered for the first time in 2000, and 84 in 2001.

Male fertility phenotyping

The 179 plants of the progeny were phenotyped for male fertility in 2001 (between May and August). Male fertility was characterized via two approaches: firstly, the anthers were described by the presence or absence of pollen, their color (white to yellow), their fullness, their dehiscence; and secondly, pollen viability was estimated by a staining procedure which differentially stains viable and non-viable pollen grains (Alexander 1969). Two to three buds per plant were sampled just before anthesis. Two stamens were then chosen from each bud and squashed together into a drop of Alexander's stain, and when pollen grains were present, a minimum of 300 grains per sample were scored for viability.

Dates of flowering

The date of flowering was recorded for each plant between May and July 2001. It should be noted that the plants that had been sown in December 1999 were flowering for the second time, whereas those sown in December 2000 flowered for the first time after cold treatment. The groups were treated separately in the statistical analyses. The date of flowering is given in calendar weeks from week 21 (22 May) to week 29 (17 July).

Molecular analysis

Extraction and purification of total DNA was performed using the DNeasy 96 plant kit following the standard protocol for isolation of DNA from dried plant leaf tissue outlined in the DNeasy 96 plant protocol handbook (QIAGEN).

The AFLP procedure was performed according to a standard protocol (Vos et al. 1995). One hundred nanograms of total DNA were digested with *Eco*RI (New England Biolabs) and *Mse*I (New England Biolabs) and ligated to the *Eco*RI and *Mse*I adapters (T4 DNA ligase, Amersham Pharmacia). For pre-amplification, ligated DNA was amplified using the *Eco*RI primer +A and the *Mse*I primer +C. The PCR reaction was carried out in a Perkin Elmer 9600 thermocycler. Amplifications were then performed using primers with three selective bases (IRD-labelled *Eco*RI 32, 33, 35–38, 40, 41; unlabelled *Mse*I 47–51, 55, 59–62).

AFLP products were mixed with an equal volume of tracking dye (98% formamide, 10 mM of EDTA, ph 8.0, xylene cyanol and 0.1% bromophenol blue). Amplicons were denatured at 94°C for 3 min and chilled on ice prior to gel electrophoresis, which was performed on a LI-COR automated DNA sequencer model 4200s (LI-COR, Neb., USA). Aliquots (1 μl) of sample solutions were loaded onto a denaturing gel (6% Long Ranger, urea 7 M, 1.2×TBE). Polymorphisms were scored visually using the program Image IR (LI-COR). The size of the fragments was determined with the program RFLP scan 3.12 (Scanalytics, Mass., USA).

Bulks were composed of ten individuals sharing the same phenotype, i.e. male-sterile or hermaphrodite. For a given bulk, DNAs were pooled (10 ng per individual) prior to restriction-ligation.

Ninety-six microsatellite markers, chosen to cover the *Beta vulgaris* genome were also applied to the bulks, to identify the linkage group(s) associated with the trait. Additional markers from the same group(s) were then applied to individual plants. Ampli-
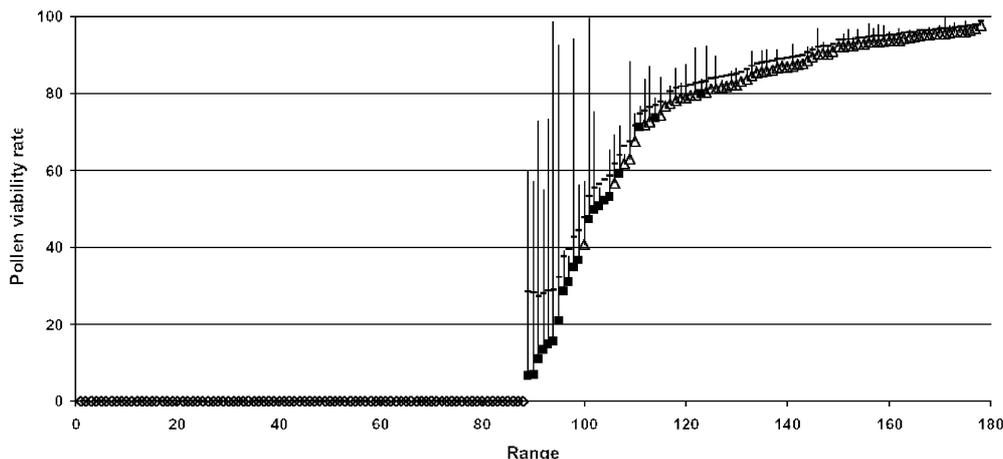
Fig. 1 Sexual phenotyping and pollen viability of the segregating descent. The individuals of the whole progeny were ranked from the lowest to the highest pollen viability rates. The three classes determined by visual phenotyping (see text) are indicated with different symbols: *empty diamonds* for females, *full squares* for intermediate and *empty triangles* for hermaphrodites. For non-female plants for which a coefficient of variation could be determined, a *vertical bar* indicates the coefficient of variation of pollen viability for a given plant in %. The expected values in the case of a binomial distribution of pollen viability are represented by *horizontal bars* (pollen viability rate + expected coefficient of variation)

fication was essentially as described by Rae et al. (2000), except that fluorescently labelled primers were used in conjunction with electrophoresis on an ABI 3100 sequencer (Applied Biosystems).

Statistical analysis

We compared the observed coefficient of variation of pollen viability of a given individual (intermediate or hermaphrodite) with the expected coefficient of variation under a binomial distribution for the same value of pollen viability estimated out of 300 counted pollen grains. A Wilcoxon's signed-ranks test for two groups was conducted with the observed and theoretical coefficients of variation for each rate of viability measured being arranged as paired observations (Sokal and Rohlf 1995). Differences between the observed and expected values were computed and compared between the intermediate and hermaphrodite classes by a Kruskall-Wallis test.

Kruskal-Wallis tests were also conducted to assess the effect of male phenotype on the date of flowering, as well as the effect of the GC content in the selective primers on the number of AFLP bands revealed, using the program Minitab 13.2 (Minitab).

For testing genetic hypothesis of the observed segregation, chi-square tests were performed as described by Sokal and Rohlf (1995).

Linkage analysis

Linkage analysis for the markers used on the whole progeny was performed using the program Mapmaker v3 (Lander et al. 1987). The linkage group was established with a LOD-score threshold of 3.0 and using the Kosambi mapping function.

Results

Male fertility phenotyping

The sexual phenotype of each individual of the population was assessed in two ways: firstly, a visual score was given, placing individuals into three classes: (1) female flowers with empty white or yellowish anthers, (2) hermaphrodite flowers with golden yellow anthers, full of pollen, dehiscent, and (3) intermediate flowers with light yellow anthers, variable pollen content, late dehiscence. Secondly, pollen staining was used to estimate the pollen viability of flowers, combining two anthers from each of two to three flowers per plant. The combination of both classifications is summarized in Fig. 1, which shows good correspondence between the two methods. In particular, the three classes that could be defined visually corresponded to individuals with different levels in pollen viability.

The intermediate phenotypic class appears to be heterogeneous for pollen viability (from 7% to 80%). Considering the intra-individual variations of pollen viability (intermediates and hermaphrodites), the observed coefficients of variation were significantly higher than those expected under a binomial distribution (Wilcoxon's signed-ranks test, $P = 3.3 \times 10^{-7}$; see Materials and methods). Moreover, the intermediates exhibited a higher intra-individual variation in the pollen viability rate, when compared to the full hermaphrodites (Kruskal-Wallis test on the differences between the observed and the expected coefficients of variation, $H = 13.14$, $P < 0.001$).

Flowering dates and sexual phenotype

The flowering date was assessed for each individual of the population, in order to test whether there was a significant difference in earliness between the three sexual classes. The population was composed of plants that flowered for the first time since May 2001 after cold treatment (see Material and methods) and plants that had been sown the year before (December 1999), and thus that flowered for the second time. These two groups differed significantly

Table 1 Mean values of flowering date for the three sexual phenotypic classes. *N* Number of individuals for each phenotypic class. The dates are given in weeks of the calendar

Phenotypic class	N	Mean (weeks)	Standard deviation (weeks)
Female flowering for the first time	43	23.44	1.12
Intermediate flowering for the first time	11	23.46	1.21
Hermaphrodite flowering for the first time	30	24.40	1.00
Female flowering for the second time	45	24.31	1.38
Intermediate flowering for the second time	9	25.00	1.12
Hermaphrodite flowering for the second time	41	25.54	1.31

Table 2 Genotypes at the restorer loci of the three phenotypic classes, under the two locus additive or epistatic models. RfG_i is the dominant restorer allele at the locus *i*. The genotype RfG_i indicates that for a given phenotypic class the nature of the second allele is either RfG_i or rfG_i

Model	Female	Intermediate	Hermaphrodites
Two loci, additivity	$rfG_1\ rfG_1$; $rfG_2\ rfG_2$	RfG_1 -; $rfG_2\ rfG_2$ or $rfG_1\ rfG_1$; RfG_2 -	RfG_1 -; RfG_2 -
Two loci, epistasis	$rfG_1\ rfG_1$; - -	RfG_1 -; $rfG_2\ rfG_2$	RfG_1 -; RfG_2 -

for the mean flowering date (Kruskal-Wallis test, H =24.30, P <0.001). The plants flowering for the second time were later, on average, than those flowering for the first time.

The flowering dates were compared between the three phenotypic classes: female, intermediate and hermaphrodite within both groups. A Kruskal-Wallis test revealed a significant effect of the sexual phenotype on the flowering date (H =17.49, P <0.001 for plants flowering for the second time, H =12.37, P =0.002 for the plants flowering for the first time). In both groups, the female plants appeared to flower earlier than the hermaphrodites, whereas the intermediate plants were also intermediate for this character (Table 1).

Segregation analysis of male fertility

The segregating population was composed of 49.2% plants with male sterile flowers and 50.8% plants with flowers producing at least some viable pollen grains. A 1:1 ratio could not be rejected by a Chi-square goodness of fit test (χ^2 =0.050, df =1, P =0.822). A monogenic inheritance could thus explain the observed segregation. The non-female class could be further divided into intermediate plants (12.3%) and full hermaphrodites (38.5%) according to morphological characteristics.

The occurrence of an intermediate class suggested that more than one restorer locus was segregating in the population. We tested the most parsimonious genetic models for goodness of fit with the observed ratios: a two locus model with additive effects of dominant alleles at both loci (two locus additive model), a two locus epistatic model and a three locus additive model. All the RfG_i restorer loci were assumed to be genetically unlinked and the restorer allele was considered to be dominant over the maintainer allele at each locus (Table 2).

In the additive models, hermaphrodites must have at least one copy of the dominant restorer allele at each locus. The intermediate individuals must have at least one copy of the dominant restorer allele at one locus and be homozygous for the recessive non-restorer allele at a minimum of one of the other loci. Finally, the female individuals must be homozygous recessive at all restorer loci.

In the case of the epistatic model, the hermaphrodite must have the same genotype as in the additive model. Considering that RfG_1 is epistatic to RfG_2, i.e. the expression of RfG_2 depends on the allelic status at RfG_1, intermediates must have at least one copy of the dominant restorer allele RfG_1, and must be homozygous for the non-restorer recessive allele rfG_2. Females must be homozygous for the non-restorer allele rfG_1 whatever the allelic state at the RfG_2 locus.

Assuming the hermaphrodite parent to be heterozygous at each locus, Table 3 presents the expected ratio as well as the significance level of the Chi-square tests; the only model that could not be rejected was the epistatic model with a heterozygous female parent. Two other crosses between a male sterile plant and a hermaphrodite from the studied segregating population gave ratios that fitted the same model (data not shown).
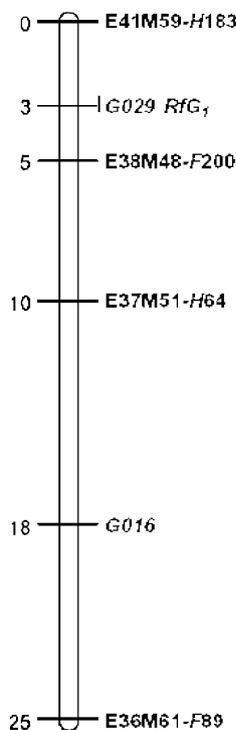
Considering the 1:1 ratio between female and non female plants, which reflected the segregation on RfG_1, we conducted a bulk segregant analysis in order to find markers genetically linked to the restorer locus. Note that theoretically, the same methodology could have been applied for the second locus RfG_2 by constituting intermediate ($rfG_2\ rfG_2$) and hermaphrodite ($RfG_2\ rfG_2$) bulks. However, we decided not to undertake it because of the lack of intermediates in the segregating population (20), which would have limited the power of the method.

Bulk segregant and linkage analysis

Eighty primer combinations (PC) were used to compare the male-sterile and the fertile bulks. Approximately 3,050 bands were scored, giving an average of 38 bands per PC, with a range of 4–75 bands. The number of bands revealed was positively correlated to the number of A/T bases of the selective primers (Kruskal-Wallis test,

Table 3 Goodness of fit tests for the observed segregating ratio of the sexual phenotypes of the segregating population, with four expected distributions. The observed and the expected frequencies (expected ratios between brackets) are given for each genetic model. In all cases, the male parent was assumed to be heterozygous at each locus, and the female parent homozygous recessive at each locus, but in the case of the epistatic model (the third distribution in the table). *P* Probability of χ^2 with 2 degrees of freedom

Genetic model		Female	Intermediate	Hermaphrodite	χ^2	*P*
Two loci, additivity	Observed	88	20	71		
	Expected	44.75	89.50	44.75	111.17	7.3×10^{-7}
		(0.25)	(0.5)	(0.25)		
Two loci, epistasis	Observed	88	20	71		
Female parent:	Expected	89.5	44.75	44.75	29.11	4.8×10^{-7}
$rfG_1\ rfG_1;\ rfG_2\ rfG_2$		(0.5)	(0.25)	(0.25)		
Two loci, epistasis	Observed	88	20	71		
Female parent:	Expected	89.5	22.37	67.12	0.50	0.78
$rfG_1\ rfG_1;RfG_2\ rfG_2$		(0.5)	(0.125)	(0.375)		
Three loci, additivity	Observed	88	20	71		
	Expected	22.375	134.25	22.37	395.38	1.4×10^{-10}
		(0.125)	(0.75)	(0.125)		

Fig. 2 Graphical representation of the linkage group of RfG_1. A focus on the chromosomal region around RfG_1 is represented with the four AFLP markers and the two closest SSRs, *G029* and *G016*. For the AFLP markers, the PC is indicated, and then the letters *H* or *F* when the dominant band is associated with the hermaphrodite or female phenotypes respectively, then the size of the polymorphic band in base pairs. The codes of marker loci are listed on the *right*, and the map distance on the *left* (Kosambi, cM)

H =18.87, *P* <0.001). This phenomenon, suggesting that the *Beta* genome is rich in A/T, was previously described by Hansen et al. (1999). A total of 171 bands were polymorphic between the bulks, with an average of 2.1 polymorphic bands per PC (10.3 between the parents). Forty-three PCs were used to separately genotype the individuals of the bulks, and 52 polymorphic bands were revealed with a recombination that was lower or equal to 5/20, while eight had a recombination rate lower than or equal to 2/19. Finally, four PCs (E36 ACC-M61 CTG,

E37 ACG-M51 CCA, E38 ACT-M48 CAC, E41 AGG-M59 CTA) were chosen to genotype the whole segregating population.

In parallel, the bulks were genotyped with 96 microsatellite markers chosen to cover all nine linkage groups of the beet genome, in order to identify the linkage group (Rae et al. 2000).

Analysis of all members of the segregating population with the AFLP and microsatellite markers revealed a single linkage group associated with the trait: this group comprised four AFLP markers and nine SSR markers, with a total size of 40 cM (Kosambi). *G029*, the closest marker, was 0 cM from RfG_1 (no recombination on 111 individuals, 95% *CI* [0; 2.66]). RfG_1 was flanked by two AFLP markers (E41M59-*H*183 and E38M48-*F*200) which delimited a 5 cM window (Fig. 2).

The RfG_1 linkage group was identified as linkage group VII (Rae et al. 2000), which corresponds to chromosome VIII (Butterfass's nomenclature 1964). Therefore, RfG_1 is not located on the chromosomes where the two restorer loci of the CMS *Owen*, *X* and *Z* are located: chromosomes III and IV respectively (Schondelmaier and Jung 1997).

Discussion

An alternative CMS type in *Beta vulgaris*

A large survey of sea beet populations along the French coasts had revealed three mitochondrial types associated with male sterility, named *E* (most frequent) *G,* and *H* (least frequent) (Cuguen et al. 1994). An additional type, *Svulg*, rare in wild populations, is associated with the *Owen* CMS (Owen 1942) which is widely used in hybrid production of sugar beet. Recently, we described CMS *G* as a variant in the mitochondrial respiratory chain, a feature not found in *E*, *H* or *Svulg* mitochondria (Ducos et al. 2001).

In this study we report the genetic mapping of one restorer locus of CMS *G* which is not co-located with any of the *Owen* restorer loci, and consequently neither on the chromosome where a restorer locus of the CMS *H* has

been reported (Laporte et al. 1998). It can be argued that the different locations of restorer loci are not sufficient to establish a new CMS: in maize CMS-*T* (Dill et al. 1997) and in *Plantago* (*P. coronopus*, Koelewijn and Van Damme 1995; *P. lanceolata*, De Haan et al. 1997a), different loci can provide alleles capable of restoring the same CMS. However, an additional argument can support the hypothesis that *G* and *Owen* are two different CMSs: CMS *G*, when introduced in sugar beet lines, is not maintained by the lines that maintain the *Owen* CMS; in addition we have found a maintainer line of CMS *G* that could restore CMS *Owen* (unpublished results), demonstrating that restorer loci of CMS *Owen* can not restore male fertility of CMS *G*. Therefore, *G* can be considered as a new CMS in beet. The development of markers linked to *RfG₁* could facilitate the introduction of this novel source of sterility by choosing, through marker assisted selection, the germplasm that maintains male sterility, a critical point in sugar beet hybrid production.

Genetic architecture of male fertility restoration

The present study is a part of a larger program aiming to investigate the genetic architecture of fertility restoration of the different CMSs in beet. As an evolutionary process, CMS spread and maintenance is expected to be under the influence of the genetic factors controlling the trait, as well as demographic dynamics. CMS *G* is the second most common source of sterility in populations from western France (Cuguen et al. 1994). The segregation of male fertility in the progeny studied suggested a determinism involving more than one locus: the best fitting model involved two loci in epistasis. In the *Owen* CMS, two loci have been proposed to explain male fertility restoration, *X* and *Z* (Owen 1942, 1945); a recent QTL mapping revealed a third locus genetically linked to chromosome IV (Hjerdin-Panagopoulos et al. 2002). In crop species, restoration has been described to be monogenic in species like petunia (*Rf*, Pruitt and Hanson 1991), bean (*Fr2*, Abad et al. 1995; *Fr*, He et al. 1995), maize (*Rf*3 for CMS-S, Laughan and Gabay-Laughan 1983), Brassica (*Rfo* for CMS-Ogura, Delourme et al.1994; *Rfn* for CMS-nap, *Rfp* for CMS-pol, Li et al. 1998), rice (*Rf1* for cms-bo Iwabuchi et al. 1993), digenic in maize (*Rf₁* or *Rf₈* or *Rf** and *Rf₂* for CMS T, Laughan and Gabay-Laughan 1983; Dill et al. 1997), rice (for WA-type CMS Yao et al. 1997), sunflower (for CMS-PET1, Leclercq 1984) and sorghum (*Rf*3 and *Rf*4 for CMS IS1112C Tang et al. 1998). In gynodioecious species, complex genetic determinisms seem to be the rule as exemplified in *Thymus vulgaris* and *Silene vulgaris* (Charlesworth and Laporte 1998), in *P. lanceolata* (De Haan et al. 1997a) and in *P. coronopus* (Koelewijn and Van Damme 1995). Evolutionary dynamics of male sterility has been predicted by several theoretical models that suppose a monogenic determinism of male fertility restoration (Charlesworth 1981, Gouyon et al. 1991, Bailey et al. 2003). More complex determinisms and in particular

epistasis might influence the evolutionary dynamics of CMS and call for new theoretical developments (Charlesworth and Laporte 1998; Bailey 2002).

Intermediate types

Intermediate types have been described in a number of gynodioecious species. They can be either plants with female and hermaphrodite flowers as seen in *P. lanceolata* (De Haan et al. 1997a), *P. coronopus* (Koelewijn 1996), *Silene acaulis* (Shykoff 1988) and *Dianthus sylvestris* (Collin and Shykoff 2003) or with intermediate flowers (more or less aborted anthers) as in thyme (Gigord et al. 1999) and also in *P. coronopus* (Koelewijn and Van Damme 1995). In beet, the presence of plants with intermediate flowers has been described for the *Owen* CMS (Owen 1945), CMS *E* (Boutin-Stadler et al. 1989) and CMS *H* (Laporte et al. 1998). CMS *G* follows the same rule. This phenotypic class appears to be heterogeneous for the pollen viability rate (from 7 to 80%), from almost female types to almost full hermaphrodites. This variation can be observed by visual phenotyping through the shape of anthers (almost empty to full), though dehiscence is late compared to full hermaphrodites. Even though a genetic model fitting the observed segregation has been proposed, the underlying cause of this heterogeneity is still unknown and might be a combination of environmental and additional genetic factors.

Flowering dates

Female plants flowered earlier than hermaphrodites. In this study, it is not possible to distinguish between a pleiotropic effect of the restorer alleles and linkage disequilibrium with genes affecting the flowering date. It must be noted that we observed the same tendency in the open-pollinated progeny from which both parents of the studied cross issued, and a significant difference ($P < 0.001$) between female and non-females in a segregating population from a cross between a hermaphrodite plant from the population studied and a female on *G* from a population of the northern part of France (data not shown). Studies to assess whether this phenomenon is general in wild beet populations must be conducted. Theoretical models point out that the maintenance of females in populations is dependant on the gain of seedling production by plants that have lost their pollen production, via a possible reallocation of resources or due to inbreeding avoidance (see review in Thompson and Tarayre 2000). A possible aspect of the so-called female advantage could be related to an earlier capacity of the females to receive pollen as soon as it is available in this protandrous species, especially if there is a peak in the pollen production early in the flowering season, as is observed in *P. coronopus,* the females of which also flower earlier (H. Koelewijn, personal communication).

Cost of restoration

In order to understand the maintenance of male sterility in natural populations, theoretical models have emphasized two key parameters, a female advantage of male sterile cytoplasm, and a cost of restoration, i.e. restorer alleles are counter-selected in the absence of the corresponding sterilizing cytoplasm (Charlesworth 1981, Frank 1989, Gouyon et al. 1991). The last parameter has been barely assessed in experimental studies because of the necessity of doing time-consuming test crosses to identify plants that carry the restorer allele(s) (see De Haan et al. 1997b, Bailey 2002). The markers genetically linked to RfG_1, developed in the present study and one of which is located at 0 cM from RfG_1, will be used to identify the plants that bear the restorer allele (except if recombination occurs) and thus will greatly facilitate the study of the cost associated with the presence of the restorer allele.

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