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Different CMS sources found in *Beta vulgaris* ssp *maritima*: mitochondrial variability in wild populations revealed by a rapid screening procedure

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Summary. Mitochondrial DNA (mtDNA) variation in natural *Beta maritima* populations has been characterized by way of Southern blot hybridizations of total DNA using non-radioactive probes and chemiluminescent detection. It was found that the previously described N ("normal") mitochondrial type could be subdivided into three subtypes. A new mitochondrial genotype (type R) was distinguished in addition to the previously described type S. Both are male-sterile cytoplasms and can produce a segregation of sexual phenotypes in their progenies depending on the nuclear background. The populations contained at least two to four different mitochondrial genotypes.

Key words: CMS – Wild beet – Cultivated sugar beet – RFLP – MtDNA variability

Introduction

Cytoplasmic male sterility (CMS) is a common feature observed in many plant species that results in the lack of functional pollen production (reviewed by Pring and Lonsdale 1985; Kaul 1988; Hanson 1991). This trait has proven to be useful in emasculating the female parent of hybrid crosses (Edwardson 1970; Hanson and Conde 1985).

The study of cytoplasmic male sterility in *Beta* is motivated by applied and fundamental interests. Hitherto only one cytoplasm leading to male sterility has been available for sugar beet breeding programs. Therefore, it would be interesting to obtain new male-sterility sources that could be used as an alternative to the Owen CMS source (Owen 1942). New accessions of male sterility can be obtained from *Beta vulgaris* seed collections (Duchenne et al. 1989; Komarnitsky et al. 1990; Weihe et al. 1991), mutagenesis experiments, or from natural populations of *Beta maritima* (Mikami et al. 1985; Boutin et al. 1987; Halldén et al. 1988; Mann et al. 1989).

There is abundant evidence for linking the cytoplasmically inherited component of CMS and mitochondria. Restriction fragment analysis of the cytoplasmic genomes of fertile and male-sterile plants from many species – including *B. vulgaris* and *B. maritima* – has shown that mitochondrial gene rearrangements are highly correlated with CMS (for a review see Hanson 1991). Detailed analyses of the molecular mechanisms of CMS have shown that the lack of the male function is probably caused by the expression of new mitochondrial chimeric genes. Examples for such chimeric genes are the *urf-T13* in *Zea mays* L. (for review see Levings 1990), the *Pcf* gene in *Petunia* (Nivison and Hanson 1989; Pruitt and Hanson 1991), and the novel reading frame generated in the mitochondria of the cytoplasmic male-sterile radish (Makaroff et al. 1991) and sunflower (Köhler et al. 1991). In *Phaseolus vulgaris* a permanent restoration of male fertility is obtained by the loss of a specific fragment of the mitochondrial genome (Mackenzie and Chase 1990). All of these results substantiate the role of the mitochondrial genome in CMS.

We have investigated cytoplasmic variability in wild populations of *Beta maritima* growing at the French coast of the English Channel and containing up to 60% of female plants (Boutin et al. 1987). The *B. maritima* plants were classified according to the segregation of sexual phenotypes in their progenies. Two groups could be distinguished: plants producing homogeneous progenies of hermaphrodites and plants showing a segregation of females (Fe, male-sterile plants), intermediate-fe-

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males (IFe, semi-male-sterile plants), and hermaphrodites (H, male-fertile plants) in their progenies. Plants of these two groups were found to differ in the *SalI* restriction profile of their mitochondrial DNA (mtDNA). Segregating plants contained a mtDNA type called S, whereas non-segregating plants exhibited a mtDNA type denoted N. These two *B. maritima* mtDNA profiles differ from those of *B. vulgaris* S_{vulg} and N_{vulg} mtDNAs, which are present in CMS Owen and maintainer (O type) plants, respectively.

Restriction fragment analysis of purified mtDNA is too laborious to be easily used for population screening. However, a method using total DNA as a target followed by discrimination with mtDNA sequences as non-radioactive probes would be able to meet desirable criteria such as low amount of plant material, economy of time, and specificity. In the course of the study described herein such a rapid method was developed and applied to *Beta*. Seven different mtDNAs could be distinguished with only two probes. One of the mtDNAs found in *B. maritima* is highly associated with the male-sterility trait; it differs from previously described *B. vulgaris* and *B. maritima* male-sterile mtDNA types (S_{vulg} and S_{mar}, respectively) and could represent a new source of cytoplasmic male sterility. Both *B. maritima* CMS types are potentially useful in diversifying sugar beet germ plasm.

Materials and methods

Plant material

The original *Beta vulgaris* (L.) and *Beta maritima* (wild beet) seeds were collected from individual plants from four natural populations located at the French coast of the English Channel: in the Canche estuary near Etaples in 1970 and 1984 and in the Somme estuary near Le Crotoy in 1986. The original plants were labelled and classified according to their cytotype by the analysis of the sexual phenotype of their progeny as described in Boutin et al. (1987).

Beta vulgaris (L.) *ssp vulgaris* (sugar beet) clones 5A3031 (male sterile; CMS) and 5B3031 (male fertile; O type) were kindly provided by Kleinwanzlebener Saatzucht AG, Einbeck (FRG).

Inheritance of sexual phenotype

Details are described in Boutin-Stadler et al. (1989) and Saumitou-Laprade et al. (1989).

Isolation of mitochondria and purification of mtDNA

Mitochondrial DNA was isolated from 200 g of green leaves from *B. maritima* and *B. vulgaris* according to Boutry and Briquet (1982) with some modifications. The final purification of mitochondria was performed by centrifugation on a discontinuous gradient of 10 ml 30% percoll on the top of a 2 ml 42% percoll layer. The 30% percoll layer contained a 0–10% linear gradient of polyvinylpyrrolidone 25000 (top to bottom). Following centrifugation for 15 min at 20 000 rpm in a Sorvall SS34 rotor, the purified mitochondria were collected from the interface of the 30% and 42% layers and pelleted by centrifugation at 9000 rpm for 10 min in a Sorvall HB4 rotor. The pellet was treated with DNAse I (100 µg/ml) for 1 h at 0 °C. The mitochondrial suspensions were added to 2% sarcosyl, 0.5% SDS, and 100 µg/ml proteinase K and incubated at 37 °C for 1 h. The mitochondrial DNA was purified by centrifugation through a discontinuous CsCl gradient as described by Kolodner and Tewari (1975).

Mitochondrial DNA probes

The mitochondrial genes used as probes code for ATPase subunit α (Schuster and Brennicke 1986), ATPase subunit 6 (Dewey et al. 1985a), ATPase subunit 9 (Dewey et al. 1985b), cytochrome c oxidase subunit I (Isaac et al. 1985), cytochrome c oxidase subunit II (Hiesel and Brennicke 1983), cytochrome c oxidase subunit III (Hiesel et al. 1987), and apocytochrome b (Dawson et al. 1984). The probes *pBv3* and *pBv4* were isolated from a pUC19-library of *Eco*RI-digested *B. vulgaris* CMS mtDNA.

Isolation of total DNA

Total DNA was isolated essentially as described by Dellaporta et al. (1983).

Restriction of DNA

Restriction enzymes from various manufacturers were purchased and used according to their instructions: for the digestion of total DNA, twice the recommended amount of enzyme was used during a 2-h incubation period. In addition, the restriction reaction buffer was supplemented with spermidine to 2.5 m*M*.

Southern blotting and hybridization

After separation of the restriction fragments on 0.8% agarose gels (40 m*M* TRIS-acetate, pH 7.8, 1 m*M* EDTA, and 0.5 µg/ml EtBr), the DNA was transferred to nylon membranes (Biodyne A from Pall) using the vacuum-blot system of Pharmacia. After transfer the DNA was UV cross-linked (1.2 J/cm^2) to the nylon support.

Plasmid DNA was isolated as described by Birnboim and Doly (1979) and modified by Sambrook et al. (1989). This DNA was labelled with digoxigenin-dUTP without prior linearization by restriction endonuclease digestion. The resulting probe was hybridized overnight at 68 °C as recommended by the supplier, and the hybridization signals were detected according to the chemiluminescence method of Allefs et al. (1990).

Results

Five variants of mtDNA from B. maritima plants of the Canche estuary: association with CMS

In a survey of *B. maritima* populations found on the French coast of the Channel, seeds were collected from 56 plants in the Canche and Somme estuaries in 1984 and 1986, respectively. MtDNA of progenies from the sampled plants was digested with various restriction enzymes and analyzed by agarose gel electrophoresis. Male-fertile plants with the N mitochondrial type – as previously defined by crossing behavior and restriction analysis with the *SalI* restriction enzyme – were found to be non-homogeneous with respect to mtDNA composition; *Eco*RI digestion revealed three different restriction patterns (Fig. 1, lanes 2–4), whereas no variation could be detect-

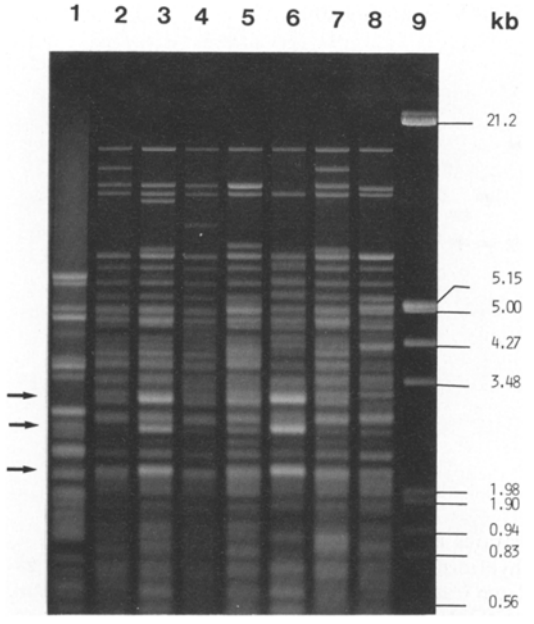

Fig. 1. *Eco*RI digest of cpDNA (*lane 1*) and mtDNA (*lanes 2–8*) from various *Beta maritima* (*B.m.*) and *Beta vulgaris* (*B.v.*) plants. *1 Eco*RI digest of chloroplast DNA from *B. maritima*; *2 B.m.* from Canche B population, mtDNA type N_1; *3 B.m.* from Canche B population, mtDNA type N_2; *4 B.m.* from Canche B population, mtDNA type N_3; *5 B.m.* from Canche A population, mtDNA type S; *6 B.m.* from Canche (Wageningen), mtDNA type R; *7 B.v.* male-fertile O-type line 5B 3031 from KWS; *8 B.v.* cytoplasmic male-sterile line 5A 3031 from KWS; *9 lambda* DNA digested with *Eco*RI and *Hin*dIII was used as a molecular weight marker. *Arrows* denote three fragments of the 10.4-kb linear plasmid present in some of the *B. maritima* plants

ed among the S-type plants (Fig. 1, lane 5). However, a fifth restriction pattern type (Fig. 1, lane 6) was discovered in the progeny of a male-sterile *B. maritima* plant sampled in the Canche estuary in 1970 and maintained at the CPRO-DLO Institute. As a result of these and additional mtDNA differences detected by *Sma*I and *Sal*I restriction profiles (data not shown), this cytotype has been classified as a new category and is henceforth referred to as R.

The classification of five mtDNA types based on *Eco*RI restriction profiles is confirmed by *Sma*I restriction fragment analysis (data not shown), whereas *Sal*I digestion merely distinguishes types N, S, and R but not subtypes N_1, N_2, and N_3.

A comparison of the mtDNA restriction patterns with the *B. maritima* chloroplast DNA (cpDNA) restriction pattern (Fig. 1, lane 1) demonstrates that the mtDNA is not contaminated with cpDNA. The fragments appearing at 3.3, 2.7, 2.2 (Fig. 1, see arrows), 1.1, and 0.9 kbp (not clearly visible in Fig. 1) in the *Eco*RI profiles are derived from a 10.4-kbp linear plasmid (Saumitou-Laprade et al. 1989) and occur independently of the cytoplasmic type. The five mitochondrial genotypes of *B. maritima* presented here can be clearly distinguished from the mtDNA of the cultivated sugar beet O type (Fig. 1, lane 7) and CMS type (Fig. 1, lane 8).

The association between mitochondrial genotype and the ability of plants to produce segregating progeny of male-sterile (female: Fe), semi-male-sterile (intermediate-female: IFe), and hermaphrodite (H) plants has been examined. The male-sterile plant with the type-R mtDNA that was discovered in 1970 in the Canche estuary gave a segregating G_1 progeny. The male-sterile plants of this progeny were pollinated by a *B. vulgaris* maintainer line. The resulting G_2 generation presented high frequencies of male-sterile plants. Similarly, all of the 25 plants with a type-S mtDNA that were collected in 1984 in the same estuary produced a segregating G_1 progeny. Therefore, as plants with either of these mtDNA types, R or S, consistently segregate for sexual phenotype, both of these mitochondrial genomes can be said to confer cytoplasmic male sterility.

On the other hand, among the 25 families containing the N_1 mtDNA profile, 22 produced homogeneous progenies consisting of hermaphrodites only. Unexpectedly, the remaining 3 progenies segregated for sexual phenotype in generations G_1 or G_2. The single plant with the N_2 cytoplasm was hermaphrodite in G_0 and G_1, but segregated in G_2. Up to now, the plants with the N_3 mitotype have produced homogeneous progenies of hermaphrodites.

In conclusion, plants with the N_1, N_2, and N_3 mtDNA show a low level of segregation, whereas plants with the S and R mtDNA systematically segregate for the sexual phenotype.

A rapid screening procedure for population analysis: discriminating probes and non-radioactive detection

In the previously described analyses the various *Beta* cytoplasms were characterized with purified mtDNA. Since this laborious procedure may require up to 100 g of plant material (fresh weight), we set out to develop a more efficient method that would be suitable for analyzing large numbers of plants. Ideally, simple purification steps using minor amounts of plant material should yield sufficient DNA for such subsequent analyses as Southern blot hybridizations.

In order to select probes discriminating the seven types of purified mtDNAs distinguished by restriction fragment analysis, the mtDNAs were further characterized by Southern blot analysis. Non-radioactive hybridization and detection were performed with nine different mtDNA probes from various higher plants (Table 1). Three of these probes [*atpA*, *cob*, and *cox*III (Fig. 2a)] failed to detect any difference at all between the seven *Eco*RI-restricted mtDNAs. Five probes (*atp6*, *atp9*, *coxI*,

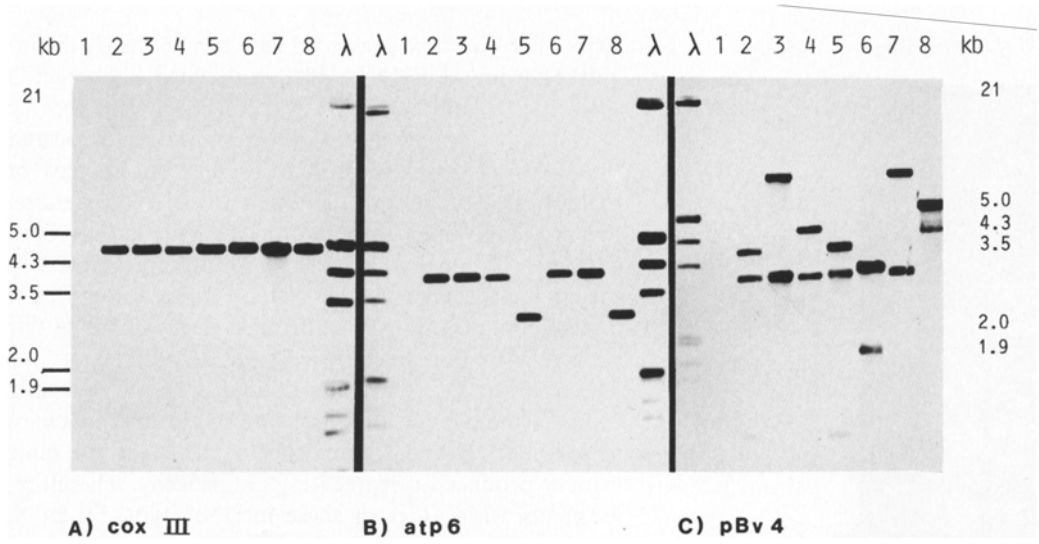

Fig. 2A–C. Southern blot analysis of the *Eco*RI digests of mtDNA from the various *Beta maritima* (*B.m.*) and *Beta vulgaris* (*B.v.*) plants presented in Fig. 1. ***Panel A*** hybridization with the *coxIII* probe, ***Panel B*** hybridization with the *atp6* probe, ***Panel C*** hybridization with the *pBv4* probe. *1 Eco*RI digest of cpDNA from *B. maritima*; *2 B.m.* from Canche B population, mtDNA type N_1; *3 B.m.* from Canche B population, mtDNA type N_2; *4 B.m.* from Canche B population, mtDNA type N_3; *5 B.m.* from Canche A population, mtDNA type S; *6 B.m.* from Canche (Wageningen), mtDNA type R; *7 B.v.* male-fertile O-type line 5B 3031 from KWS; *8 B.v.* cytoplasmic male-sterile line 5A 3031 from KWS; *9 lambda* DNA digested with *Eco*RI and *Hin*dIII was used as a molecular weight marker

Table 1. Mitochondrial variation in *Beta* ssp. as revealed by Southern blot analysis of *Eco*RI-digested mtDNA

Probes	Molecular weight of hybridization fragments (kbp)	Hybridization patterns of the different cytotypes						
		B. maritima					*B. vulgaris*	
		N_1	N_2	N_3	S	R	N_{vulg}	S_{vulg}
atpA	1.7	+	+	+	+	+	+	+
cob	2.0	+	+	+	+	+	+	+
coxIII	5.1	+	+	+	+	+	+	+
coxI	1.6	+	+	+	+		+	+
	2.7					+		
atp6	4.0	+	+	+		+	+	
	3.0				+			+
atp9	1.2							+
	1.2, 3.5	+	+	+	+		+	
	1.2, 2.0					+		
pBv3	0.7, 1.3, 1.7	+	+	+	+		+	+
	0.7, 2.9					+		
coxII	(1.1)[a], 1.5, 2.0, (10.8)	+	+	+	+		+	
	(1.1), 1.8, 2.0, 2.2, 6.8, (10.8)							+
	(1.1), 2.0, 2.8, 6.8					+		
pBv4	3.0, 7.2		+				+	
	4.2, 5.1							+
	0.8, 3.0, 3.7	+			+			
	1.5, 3.0					+		
	3.0, 4.4			+				

[a] ( ) Low signals observed on pure mtDNA profiles

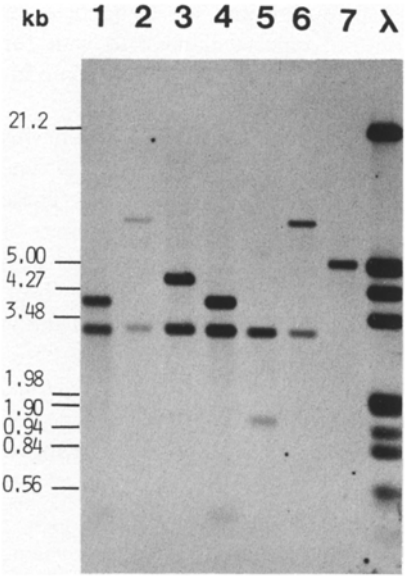

Fig. 3. Southern blot analysis of the *Eco*RI digests of total DNA from various *Beta maritima* (*B.m.*) and *Beta vulgaris* (*B.v.*) plants. *1 B.m.* from Canche B population, mtDNA type N_1; *2 B.m.* from Canche B population, mtDNA type N_2; *3 B.m.* from Canche B population, mtDNA type N_3; *4 B.m.* from Canche A population, mtDNA type S; *5 B.m.* from Canche (Wageningen), mtDNA type R; *6 B.v.* male-fertile O-type line 5B 3031 from KWS; *7 B.v.* cytoplasmic male-sterile line 5A 3031 from KWS; *8 lambda* DNA digested with *Eco*RI and *Hin*dIII was used as a molecular weight marker. The hybridization was performed with the *pBv4* probe

Table 2. Distribution of the different *Beta maritima* mtDNA types in three natural populations located in two estuaries on the French coast of the English Channel (1984 and 1986 collection)

Populations	MtDNA types				*n*
	N_1	N_2	N_3	S	
Canche A	7	0	0	14	21
Canche B	2	1	2	9	14
Somme A	16	0	4	1	21
Total	25	1	6	24	56

n = Number of plants analyzed

coxII, pBv3) distinguished two or three types of mtDNA (e.g. *atp6* in Fig. 2b). However, probe *pBv4* – a S_{vulg} mtDNA *Eco*RI fragment – was the most informative probe as it revealed extreme variation and discriminated five restriction patterns (Fig. 2c).

None of the probes distinguished between the *B. maritima* N_2 and *B. vulgaris* maintainer N_{vulg} cytoplasms although their restriction profiles on agarose gel were clearly different (Fig. 1 cf. lanes 3 and 7). Therefore, our results demonstrate that the combination of the two probes *atp6* and *pBv4* is sufficient to identify six out of seven mtDNA types encountered in this study: the five mitochondrial types N_1, N_2, N_3, S and R of *B. maritima*, and the two N_{vulg} and S_{vulg} of sugar beet.

As the nine probes were derived from mtDNA and gave no hybridization signals with the cpDNA, hybridization signals using total DNA or pure mtDNA (Figs. 2c and 3) were expected to be similar, which has been confirmed. Thus, Southern blot hybridization of *Eco*RI-digested total DNA employing the two probes *atp6* and *pBv4* together with the non-radioactive hybridization and detection system provides a rapid, sensitive, and convenient way to identify mtDNA types in *B. maritima* populations.

As an example of the efficiency of the method, the screening of the 56 families used in this study by means of this rapid procedure based on total DNA analysis revealed mtDNA polymorphism within and among populations (Table 2). In the Canche A population two mitochondrial types, N_1 and S, were present among the 21 plants tested, while four types were found among the 14 families checked in Canche B. In the Somme population two of the three N types, N_1 and N_3, were observed, but only one S-type family showed up among the 21 families tested.

Discussion

In this study we have worked out a rapid assay for the detection of mtDNA polymorphism in *Beta*. Such an assay is of great value in the study of cytoplasmic male sterility in cultivated and wild beets.

The purification of mtDNA from green leaves is tedious and requires a large amount of fresh material. In contrast, the isolation of total DNA can be done with a small amount of frozen leaves in a relatively simple procedure. For example, 1 g of leaves would yield total DNA sufficient for at least 50 blots using non-radioactive labelling of probes and chemiluminescent detection. Each blot can be rehybridized several times with different probes. Thus, small samples can be taken in a non-destructive way from a large number of individuals and stored frozen at −70 °C until a later experiment.

The use of total DNA – instead of mtDNA – did not result in the detection of additional signals with the nine different probes used. The probes *coxIII*, *cob*, and *aptA* displayed extreme uniformity, since each of them hybridized to an invariant *Eco*RI fragment in each of the cytoplasms tested. The two probes *atp6* and *pBv4* have been shown to be specific for mtDNA, and they allow characterization of the five cytoplasmic variants found in *B. maritima* plants from natural populations. The *pBv4* fragment has not been found to be transcribed (data not shown). This non-expressed region seems to be situated

in a highly recombinogenic part of the mitochondrial genome, since homologous sequences are located in cytoplasm-specific genomic environments in five out of seven cytoplasms identified in this study. The diversity of the hybridization profiles could be generated by the involvement of adjacent repeats in intramolecular recombination (Palmer and Shields 1984; Lonsdale et al. 1984). Five different repeats have been reported for the mitochondrial genome of the Owen male-sterile cytoplasm (Brears and Lonsdale 1988).

Three main groups of mtDNA can be distinguished by the molecular analyses presented in this article. Plants containing the S or R mitochondrial types produced systematically high frequencies of male-sterile offspring. The S type has been previously described by Boutin et al. (1987) and seems to be identical to that presented by Mann et al. (1989) and Halldén et al. (1988). Our analysis suggests that mitotype R could correspond to a new *Beta* CMS system that has not been described up to now. This mitochondrial genotype is highly different from the S type because it can be distinguished by Southern blot hybridization with five out of the nine probes tested.

The N type has previously been shown to be associated with the non-segregating character of the sexual phenotype. This type can now be divided into three subtypes – N_1, N_2, and N_3 – based on small differences at the mtDNA level. In subtypes N_1 and N_2 some plants yielded male-sterile progeny. This could be due to nuclear genes coding for male sterility like the "*a1*" and "*a2*" genes discovered in *B. vulgaris* by Owen (1952). Alternatively, the concept of a "normal" cytoplasm producing only fertile plants should be modified. For example, each cytoplasm of *Beta* could yield sterile or fertile progenies depending on the content of nuclear restorer genes, as has been described for *Plantago lanceolata* (Van Damme 1983).

In view of their discriminating capacities probes *atp6* and *pBv4* were chosen to examine the mitotypes of *Beta* plants from several natural populations. Two populations of the same estuary (Canche) were already known to differ with respect to mitochondrial type composition and frequencies. In the Canche A population, N_2 and N_3 were not found, while they were present in Canche B. These two populations contained N_1 and S plants, although the male-sterile S mitochondrial type was very rare in the sample from the Somme estuary. Surprisingly, in the Canche populations studied in 1984 no plants containing the R cytoplasm were found, while a plant with this mitochondrial type was isolated from this estuary 14 years before. Either the 1984 collection was too small or the S cytoplasm has replaced R with time. It should be mentioned that the R cytoplasm has also been found among plants collected at the English coast of the channel in 1982 and at the coast of Batz island in 1988 (unpublished observations). However, definitive conclusions regarding the distribution and the frequencies of the various mitochondrial types will have to wait for large-scale screenings performed over different time intervals.

The various *B. maritima* cytoplasms described in this study differ from the two cytoplasms CMS and O type used in the hybrid seed production of sugar beets. These differences are based on restriction profile differences of the mtDNA and differential segregation in crosses with different restorer and maintainer lines of sugar beet. Work is in progress to introduce the *B. maritima* S and R cytoplasms into commercial *B. vulgaris* lines by backcrosses. A *B. vulgaris* maintainer line for the *B. maritima* S cytoplasm has already been selected in the O-type collection of the French Agronomy Research Institute (INRA).

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