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Title:

**A new source of CMS found in wild beet and its relationship to other CMS types**

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## Abstract

We found a number of male sterile plants in a wild beet (*Beta vulgaris* L. ssp. *maritima*) accession line FR4-31. The inheritance study of the male sterility indicated the trait to be of the cytoplasmic type. The mitochondrial genome of FR4-31 proved to lack both of the two male-sterility-associated genes, *preSstp6* and *orf129*, which are characteristic of the Owen CMS and I-12CMS(3) cytoplasms of beets, respectively. Instead, the truncated *cox2* gene, involved in *G* CMS originating from wild beets, was present in the FR4-31 mitochondrial genome. In Southern hybridization using four mitochondrial gene probes, the FR4-31 cytoplasm showed the patterns similar to those typical of the *G* cytoplasm. It is thus likely that the FR4-31 cytoplasm has a different CMS mechanism both from Owen CMS and I-12CMS(3), and that the FR4-31 and *G* cytoplasms resemble each other closely. A restriction map of the FR4-31 mitochondrial DNA was generated and aligned with those published for the Owen and normal fertile cytoplasms. The FR4-31 mitochondrial genome was revealed to differ extensively in arrangement from the Owen and normal genomes, and the male-sterile Owen and FR4-31 genomes seem to be derived independently from an ancestral genome.

**Key words:** Sugar beet, cytoplasmic male sterility, plant mitochondria, mitochondrial genome.

## Introduction

The discovery by Owen (1942, 1945) of cytoplasmic male sterility (CMS) in sugar beet (*Beta vulgaris* ssp. *vulgaris*), and subsequent identification and development of the sterility-maintainer lines resulted in the widespread production of sugar beet hybrids using this CMS source (Owen CMS) (Bosemark 1993). Due to its importance for breeding, the molecular basis of Owen CMS has been carefully studied (McGrath 2005). Consequently, a mitochondrial open reading frame termed *preSatp6*, which encodes 39 kDa protein (formerly described as 35 kDa), was found to be responsible for the male sterile phenotype (Satoh et al. 2004; Yamamoto et al. 2005). Additional sources of male-sterile cytoplasm have been discovered, some of which are considered to have different CMS mechanisms from Owen CMS. For example, in the sterile anthers carrying the sterilizing I-12CMS(3) cytoplasm from wild beets collected in Pakistan, the 39-kDa preSATP6 protein was missing and instead a male sterility-associated ORF129 protein of 12 kDa appeared (Yamamoto et al. 2008). The association of ORF129 with pollen disruption has recently been found in a different source of CMS [I-12CMS(2)] derived from wild beets distributed in Turkey as well (Y. Onodera et al., manuscript in preparation). Ducos et al. (2001) reported that an alteration of mitochondrial *cox2* gene might be involved in male sterility caused by the *G* cytoplasm discovered in wild beet populations.

Attempts have been made to unravel the phylogenetic relationship among different sources of male-sterile cytoplasm and normal fertile cytoplasm. The nucleotide sequence diversity of chloroplast DNA was used to demonstrate that four CMS sources (Owen, *G*, *H* and *E*) did not constitute a single evolutionary lineage but occurred independently from an ancestral non-sterilizing cytoplasm (Fenart et al. 2006). The sequence analysis of the downstream flanking region of *cox2* gave 26 mitochondrial haplotypes (named seq01 through seq26) in cultivated and wild beets, of which three

(seq07, Owen; seq08, I-12CMS(2); and seq09, I-12CMS(3)) were associated with male sterility and one (seq01) with normal cytoplasm (Nishizawa et al. 2007). Moreover, the analysis indicated the independent occurrence of the three CMS haplotypes from the core haplotype seq01. We have most recently noticed the haplotype seq06 to be also associated with male sterility.

In this paper, we describe the results of an inheritance study of the male sterility in a wild beet (*B. vulgaris* ssp. *maritima*) accession FR4-31 with seq06. We also report the characterization of the mitochondrial genome of acc. FR4-31.

## Materials and methods

### Plant materials

*Beta vulgaris* ssp. *maritima* acc. FR4-31 was collected in France by USDA Agriculture Research Service, U. S. Agricultural Research Station (M. H. Yu, personal communication). Sugar beet CMS line TK81-MS and its maintainer TK81-O are nearly isogenic except for the type of cytoplasm: TK81-MS and TK81-O carry the sterilizing Owen and normal cytoplasm, respectively. A fertility restorer line NK198 was also included in this study. Sugar beet CMS line I-12CMS(3) possesses different source of CMS cytoplasm derived from wild beets in Pakistan (Yamamoto et al. 2008). Plants were grown in the experimental field of Hokkaido University, and classified for male fertility based on the three criteria (viz. anther color, pollen stainability, and pollen shed) as described in Hagihara et al. (2005).

### Isolation of nucleic acids

Mitochondrial (mt) DNA was isolated from fresh green leaves according to Mikami et al. (1985). Total cellular DNA was isolated as described in Doyle and Doyle (1990). Mt RNA was prepared as previously described (Kubo et al. 2000).

#### Polymerase chain reaction and nucleotide sequencing

Genomic PCR amplification was done with Go-Taq DNA polymerase (Promega, Madison, WI, USA) according to the instruction manual, using 10 ng of mtDNA as templates. Primers for genomic PCR are 5'-GGAGCAGTCAAAGAATGAACCAA-3' and 5'-TTCACCCTATTCTTTTCATTTC CG-3'. The PCR products were directly sequenced using ABI3700 platform (Applied Biosystems, Foster City, CA, USA). Sequence data were deposited in DDBJ/EMBL/GenBank under accession number of AB521205.

#### Construction of genomic library and chromosome walking

Twenty µg of mtDNA was partially digested with *Mbo*I and laid onto continuous sucrose density gradients, followed by centrifugation as described in Sambrook et al. (1989). DNA fragments of 15-23 kb were collected and ligated into the Lambda DASH II vector (Stratagene, La Jolla, CA, USA). Chromosome walking was conducted as described in Kubo et al. (1995, 1999) and Nishizawa et al. (2007). Probes (see Supplementary Table S1) were labeled with AlkPhos Direct DNA labeling system (GE Healthcare UK, Amersham Place, England) and used for screening the recombinant phage clones by plaque hybridization. Hybridization signals were detected on X-ray films according to the

manufacturer's instruction.

### Southern hybridization

One  $\mu\text{g}$  of mtDNA or five  $\mu\text{g}$  of total cellular DNA was digested with restriction endonucleases purchased from Takara Bio (Ohtsu, Japan) and electrophoresed in a 1% agarose gel. DNA fragments were transferred onto Hybond N+ membrane (GE Healthcare UK, Amersham Place, England) according to the manufacturer's instructions.

### Immunoblot analysis

Total proteins were extracted from young fresh leaves as described in Cheng et al. (2009). Proteins were fractioned by 12% or 15% SDS polyacrylamide gel electrophoresis, transferred to Hybond-P membranes (GE Healthcare UK, Amersham Place, England), and probed with antisera according to Yamamoto et al. (2005, 2008).

### Results and discussion

#### Male sterility in acc. FR4-31

A number of male sterile plants were observed in a field planting of *B. vulgaris* ssp. *maritima* acc. FR4-31. When the male sterile plants were open-pollinated, the subsequent generation presented high frequencies of male sterile plants. A male sterile plant from FR4-31 was crossed as female parent with TK81-O, a maintainer for Owen CMS. The

resulting three F1 plants were all male sterile (Table 1). The same female parent was pollinated with pollen from NK198 (a restorer for Owen CMS), yielding sterile and partial-fertile as well as fertile progenies (Table 1). Furthermore, crossing between a male sterile F1 plant from the cross FR4-31 x TK81-O and a male fertile F1 of FR4-31 x NK198 resulted in almost exclusively male sterile (sterile + partial-fertile) progenies (Table 1). TK81-O and NK198 do not carry nuclear male sterility gene(s) because sibmatings or self pollinations of them have produced progenies entirely free of male sterile offspring. Hence, the results suggest that the male sterility in question is inherited through the maternal parent and is cytoplasmic.

#### Sterilizing FR4-31 cytoplasm

Mitochondrial genes, *preSatp6* and *orf129*, are involved with Owen CMS and I-12CMS(3), respectively (Yamamoto et al. 2005, 2008). In this study, expression of *preSatp6* and *orf129* was examined by Western blot analysis using anti-preSATP6 and anti-ORF129 antisera. As shown in Fig. 1, both antisera failed to react with FR4-31 total cellular protein from leaf, which indicates that molecular basis of male sterility in FR4-31 is different from those in Owen CMS and I-12CMS(3).

Ducos et al. (2001) described that CMS *G* discovered in wild sea beet (*B. vulgaris* ssp. *maritima*) was associated with a mitochondrial *cox2* gene missing eight highly conserved, C-terminal amino acids. In order to ascertain whether such a truncated *cox2* is encoded in FR4-31, the *cox2* region was PCR-amplified from FR4-31, followed by directly sequencing of the amplification products. The coding sequences of FR4-31 *cox2* and *G cox2* proved to be perfectly identical (see Supplementary Fig. S1). The *G* mitochondrial genome was also reported to carry a 390-bp deletion in the *nad9-trnP-UGG-trnW-CCA* gene cluster (Ducos et al. 2001). The deletion contains the 3'



region of *nad9* and downstream chloroplast-derived *trnP*-UGG. The same deletion was actually detected in the FR4-31 mtDNA by the size of PCR product amplified from *nad9-trnP*-UGG-*trnW*-CCA cluster (data not shown).

Saumitou-Laprade et al. (1993) previously showed that several mitochondrial gene probes distinguished *G* cytoplasm from Owen cytoplasm. For instance, hybridization of *Eco*RI-digested total DNA to a *cox1* probe was described to detect a 2.7-kb fragment in *G* cytoplasm plants, but not in Owen cytoplasm plants, where a unique fragment of 1.6 kb was identified (Table 2). Polymorphism of the *Eco*RI restriction pattern was also observed by using the *atp6*, *atp9* and *nad9* probes (Saumitou-Laprade et al. 1993, see Table 2). Here, we found that FR4-31 displayed the RFLP patterns similar to those characteristic of *G* CMS. (Table 2). Summing up the results mentioned above, it can be concluded that the male-sterile FR4-31 and *G* cytoplasms resemble each other closely.

#### Physical mapping of the FR4-31 mitochondrial genome

A physical map of the mitochondrial genome from acc. FR4-31 was constructed using a library produced in the lambda phage vector Lambda DASH II. Three enzymes were used in the restriction mapping: *Xho*I, *Sal*II and *Xba*I. Families of overlapping phages were identified by screening the clone library with 38 mitochondrial gene probes as well as with the subfragments of fertile sugar beet (cv. TK81-O) mtDNA (see Supplementary Table S1). These were extended subsequently by chromosome walking, and as a result, the entire genetic information was arranged into a circular master chromosome of 542.4 kb which included two major families of repeats: a four-copy repeat family (named R1, 22.7 to 77.3 kb in length) and a three-copy repeat family (R2, 13.1 to 95.0 kb) (Fig. 2).

### Comparison of the three beet mitochondrial genomes

By phage clone analysis and cross-hybridization experiments, we compared the linear arrangement of the homologous restriction enzyme fragments between three master chromosomes of acc. FR4-31, TK81-O (Kubo et al. 1995) and TK81-MS (Owen cytoplasm) (Kubo et al. 1999). As shown in Fig. 2, the FR4-31 genome could be divided into 12 regions (labeled N1 to N12) whose boundaries or breakpoints were defined by positions where the restriction map identity was lost between the FR4-31 and TK81-O master chromosomes. Likewise, 14 regions (S1 to S14) were delimited by comparing the FR4-31 and TK81-MS master chromosomes. In summary, a comparison of the FR4-31 genome with the TK81-O and TK81-MS genomes identified 27 breakpoints in the FR4-31 genome. Of these, ten represented points where DNA was rearranged in FR4-31 genome, ten represented points where DNA was rearranged in TK81-MS genome, and seven represented points where DNA was rearranged in TK81-O genome. The results are consistent with a view that the Owen and FR4-31 cytoplasms derived independently from an ancestral cytoplasm (see Fenart et al. 2006; Nishizawa et al. 2007).

It should also be noted that the FR4-31 mitochondrial genome contained at least two unique regions not present in the TK81-O and TK81-MS mtDNAs: they are represented as 0.9-kb and 1.6-kb *Xba*I fragments in Fig. 3 (indicated by "1\*" and "2\*", respectively). As seen in Fig. 3, these two *Xba*I fragments hybridized very strongly to the FR4-31 mtDNA, but not at all to the mtDNAs from TK81-O and TK81-MS. Interestingly, the same probes were observed to give hybridization signals to both TK81-O and TK81-MS total DNA. Since the signal intensity appeared too weak to be attributed to high copy number of plastid sequences, the regions unique to the FR4-31 mtDNA are most likely of nuclear origin.

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**Table 1** Test of *Beta vulgaris* ssp. *maritima* acc. FR4-31 as a sterilizing cytoplasm

Cross combination		Number of Plants			
Female (sterile)	Male (fertile)	Fertile*	Partial-fertile**	Sterile***	Total
FR4-31	TK81-O	0	0	3	3
FR4-31	NK198	2	10	1	13
FR4-31 x TK81-O (F1)	FR4-31 x NK198 (F1)	1	9	2	12

\*, yellow and dehiscing anthers with more than 20% stainable pollen with cotton blue.

\*\*, light-yellow or orange, and non-dehiscing anthers with less than 20% stainable pollen.

\*\*\*, white or brown anthers with no pollen.

**Table 2** Restriction fragment length polymorphisms in mtDNA of three different CMS sources in beets \*

Probes	Male-sterile cytoplasm		
	FR4-31	Owen <sup>***</sup>	G <sup>**</sup>
<i>cox1</i>	2.7 <sup>****</sup>	1.6	2.7
<i>atp6</i>	4.0	3.0	4.0
<i>atp9</i>	1.2+2.0	1.2+3.5	1.2+2.0
<i>nad9</i>	5.0 + 0.8	5.6	4.6 + 0.8

<sup>\*</sup>, the *Eco*RI digests of total DNAs from three male-sterile cytoplasm

<sup>\*\*</sup>, data are from Ducos et al. (2001) and Saumitou-Laprade et al. (1993)

<sup>\*\*\*</sup>, the same hybridization patterns were found for a Owen CMS line TK81-MS in this study

<sup>\*\*\*\*</sup>, sizes of signal bands are shown in kb

### Figure captions

Figure 1 Western blot analysis of total cellular protein from leaves using anti-preSATP6 (A) or anti-ORF129 (B) antisera. Samples were electrophoresed through either 15% (A) or 12% (B) SDS polyacrylamide gel. Size markers are shown in the left (kDa). Blots were stained with Ponceau S after electroblotting.

Figure 2 Organizational comparison of TK81-O, FR4-31 and TK81-MS mitochondrial master chromosomes. Physical map of the mitochondrial genome of FR4-31 includes *XhoI*, *SalI* and *XbaI* restriction sites. Locations of representative 15 genes are shown. The orientations and extents of recombination-active repeated sequences are indicated by pink (R1) and green (R2) horizontal arrows. Positions of hybridization probes in Fig. 3 are indicated by the asterisks and numbers. Linkage groups are indicated by sky blue, purple and red horizontal bars for TK81-O and TK81-MS, respectively. Schematic of the rearrangements among the mitochondrial genomes of FR4-31, TK81-O and TK81-MS is shown in the upper panel and lower panel, respectively.

Figure 3 DNA gel blot analyses using the 0.9-kb *XbaI* (indicated by \*1 in Fig. 2) and 1.6-kb *XbaI* (indicated by \*2) as probes. Total cellular DNA (T) and mtDNA (M) of FR4-31, TK81-O and TK81-MS were digested with *HapII* (left panel) or *XbaI* (right panel) and electrophoresed in 1.0% agarose gels. Size markers are shown in the left of each panel (kb).







