



Title	Two male sterility-inducing cytoplasm of beet (<i>Beta vulgaris</i>) are genetically distinct but have closely related mitochondrial genomes: implication of a substoichiometric mitochondrial DNA molecule in their evolution
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1 **Title: Two male sterility-inducing cytoplasms of beet (*Beta vulgaris*) are genetically distinct but have**
2 **closely related mitochondrial genomes: implication of a substoichiometric mitochondrial DNA**
3 **molecule in their evolution**

4
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34

35 Abstract

36

37 I-12CMS(2) and I-12CMS(3) are sugar beet lines with different sources of cytoplasmic male sterility
38 (CMS) derived from wild beets in Turkey and Pakistan, respectively. We established that I-12CMS(2) has a
39 genetically distinct cytoplasm, but its mitochondrial genome is very similar to I-12CMS(3). Male fertility
40 was assessed in F₁ hybrids produced with a common pollen parent. Fertility in the F₁'s carrying the
41 I-12CMS(3) cytoplasm exceeded that of the F₁'s with the I-12CMS(2) cytoplasm. Organization of the
42 I-12CMS(2) and I-12CMS(3) mitochondrial genomes were compared based on their physical maps.
43 Mitochondrial genomes of the two strains were largely collinear, except for a large deletion in the
44 noncoding region of I-12CMS(2). Because a mitochondrial *orf129* in the I-12CMS(3) cytoplasm is
45 associated with a male sterility phenotype and preservation of *orf129* was evident in I-12CMS(2),
46 I-12CMS(2) *orf129* was investigated in detail. I-12CMS(2) plants contained three to five times more
47 ORF129 protein than did I-12CMS(3) plants. A single nucleotide substitution, present in the putative
48 promoter region of *orf129*, appeared to be responsible for the differential accumulation of *orf129* transcript.
49 A long N-terminal extension of *atp6* is a common feature of some beet CMSs and is found in I-12CMS(2),
50 but the amino acid sequence is unique. I-12CMS(3) mitochondria, but not I-12CMS(2) mitochondria, were
51 found to be heteroplasmic. This heteroplasmy is characterized by a substoichiometric DNA molecule(s)
52 that has at least two I-12CMS(2)-type mitochondrial loci, suggesting the possibility that the I-12CMS(2)
53 mitochondrial genome might have evolved from such a substoichiometric DNA molecule in I-12CMS(3)
54 mitochondria.

55

56 Key words: F₁ hybrid, mitochondrial evolution, mitochondrial gene, nuclear-cytoplasmic interaction, plant
57 mitochondria, substoichiometric DNA

58

59

60 Author contribution: TKi, TM, and TKu designed this study; YO, TA, RY-K, MPY, KK, SE, MM, KT, YK,
61 SY, TS and TKu did the experiments; YO, MPY, KK, SE, TKi, TM, and TKu analyzed the data; TM and
62 TKu wrote the manuscript.

63

64

65 Key message

66 We characterized a novel male sterility-inducing cytoplasm by genetic and molecular biological
67 approaches. The mitochondrial genome of this novel cytoplasm appears to have evolved from another male
68 sterility-inducing cytoplasm.

69

70

71 Introduction

72 Cytoplasmic male sterility (CMS) is a maternally inherited character causing pollen abortion (Chen and Liu
73 2014). CMS occurs in more than 140 plant species (Laser and Lersten 1972). Affecting only male
74 reproductive organs, CMS is used in hybrid seed production in which self pollination of the seed parent
75 must be prevented (Budar et al. 2006). These CMS-based hybrid seed production systems, however, result
76 in the cytoplasmic uniformity of the hybrids if the system relies on a single CMS source. This genetic
77 vulnerability is of special concern (Laughnan and Gabay-Laughnan 1983; Levings 1993). Therefore,
78 exploring novel cytoplasm is an important task for securing CMS-based hybrid seed production systems.

79 The expression of CMS is genetically explained by male-sterility (MS) inducing cytoplasm and one or
80 several recessive nuclear genes termed restorer of fertility (*rf*) genes (Hanson and Bentolila 2004).

81 Otherwise, CMS is not expressed. For example, the combination of MS-inducing cytoplasm and dominant
82 alleles of restorer-of-fertility genes (*Rf*) results in the fertility restoration of a CMS plant. The *Rf* is, however,
83 unable to restore non-cognate MS-inducing cytoplasm. Hence, one of the methodologies for distinguishing
84 CMS resources uses test crosses to monitor responses to a specific *Rf* (Duvick 1965).

85 The test cross for cytoplasmic discrimination is, however, laborious. An alternative may be to use a
86 molecular approach to find a characteristic of the MS-inducing cytoplasm. Because CMS is encoded by
87 mitochondria (Schnable and Wise 1998), attempts to find molecular differences between multiple
88 MS-inducing mitochondria have been made. An organizational comparison of multiple mitochondrial
89 genomes of the same species is one such example (L'Homme et al. 1997; Allen et al. 2007; Kawanishi et al.
90 2010; Fujii et al. 2010).

91 Mitochondrial genomes of different MS-inducing cytoplasm, as well as of non-MS-inducing
92 cytoplasm exhibit varying degrees of genomic rearrangements (inversions, translocations, and/or
93 insertions/deletions) (Kubo et al. 2011). The mechanisms for how these rearrangements occurred are still a
94 subject of debate (Kubo and Newton 2008; Christensen 2014). One of the unique features in plant
95 mitochondrial genome evolution is the involvement of substoichiometric DNA molecules that are
96 maintained for successive generations. Such DNA molecules are thought to accumulate mutations and may
97 become predominant mitochondrial DNA molecules either spontaneously or from the effect of a particular
98 nuclear gene (Janska et al. 1998).

99 In sugar beet (*Beta vulgaris* ssp. *vulgaris*), current commercial varieties are hybrids using a single
100 source of CMS, termed CMS-Owen (Owen 1942; Bosemark 2006). This situation represents a significant
101 genetic vulnerability (Bosemark 1979), and other sources of cytoplasm have been explored. I-12CMS(3) is
102 a sugar beet CMS line developed by R. K. Oldemeyer, who introduced MS-inducing cytoplasm from wild
103 beets (*Beta vulgaris* L. ssp. *maritima*) to sugar beets by recurrent backcrossing (Mikami et al. 1985).
104 Molecular studies showed that the I-12CMS(3) cytoplasm differs from CMS-Owen in the electrophoretic

105 pattern of restriction endonuclease-digested mitochondrial (mt) DNA and the identity of polypeptides
106 synthesized in mitochondria (Mikami et al. 1985; Hallden et al. 1992). An ORF termed *orf129* was
107 identified as the CMS-associated ORF (Yamamoto et al. 2008) in I-12CMS(3) mitochondria. A large part
108 of the primary sequence of *orf129* consists of a sequence of unknown origin that is absent from
109 non-MS-inducing cytoplasms or CMS-Owen (Yamamoto et al. 2008). Translation products of *orf129*
110 (ORF129) were detected in vegetative and reproductive organs as proteins loosely associated with the
111 mitochondrial inner membrane. Transgenic tobacco plants expressing a transgene consisting of *orf129*
112 fused with a mitochondrial transit peptide sequence exhibited male sterility (Yamamoto et al. 2008).

113 I-12CMS(3) and CMS-Owen are characterized by a long (389 and 387 amino-acid residues,
114 respectively) N-terminal extension of *atp6* (Yamamoto et al. 2005; Yamamoto et al. 2008), whereas the
115 length of that region in non-MS-inducing cytoplasm is only five amino acids. Although the detailed
116 mechanism is unclear about how the *atp6* N-terminal extension is processed, it is likely that once the
117 precursor protein containing both the N-terminal extension and mature ATP6 is translated, then the
118 precursor is cut into two separate proteins (Yamamoto et al. 2005). The translation product of the
119 N-terminal extension of CMS-Owen was detected in vegetative organs as well as in anthers, whereas that of
120 I-12CMS(3) was detected in vegetative organs only (Yamamoto et al. 2005; Yamamoto et al. 2008).

121 Although the cytoplasm of I-12CMS(3) is derived from a wild Pakistani beet (Mikami et al. 1985), the
122 same cytoplasm, known as CMS-E, is involved in the most common CMS found in European wild beets
123 (Touzet 2012). Additionally, plants with *orf129* have been identified in leaf beet and garden beet accessions
124 (Cheng et al. 2011). Therefore, I-12CMS(3)/CMS-E appears to be widely distributed in *B. vulgaris* in terms
125 of taxonomy and geography.

126 At one time, I-12CMS(3)/CMS-E and CMS-Owen were thought to have emerged independently from
127 non-MS-inducing cytoplasm, a notion based on the genealogy constructed from nucleotide sequence
128 polymorphisms of plastid DNA fragments or mitochondrial DNA fragments (Fenart et al. 2006; Nishizawa
129 et al. 2007). More recently, however, a mitochondrial genome-wide comparison produced a phylogenetic
130 tree in which I-12CMS(3)/CMS-E and CMS-Owen were grouped into the cluster that contained other beet
131 CMS but none of the non-MS-inducing cytoplasms. This result provided the basis for another hypothesis
132 about the monophyletic origin of beet CMSs (Touzet 2012). The monophyletic origin of beet CMSs would
133 be supported if an example could be found of MS-inducing cytoplasm evolving from one type to another.

134 We report here that I-12CMS(2), another Oldemeyer's CMS line whose cytoplasmic donor is a wild
135 beet from Turkey (Mikami et al. 1985), has a novel cytoplasm that may have evolved from
136 I-12CMS(3)/CMS-E. The objective of this study was to identify difference(s) between I-12CMS(3) and
137 I-12CMS(2) at the phenotypic level by a test cross and at molecular level by analyzing mitochondrial
138 genomes. These data are important not only for sugar beet breeding but also for obtaining insight into the
139 evolution of CMS in *B. vulgaris*. Interestingly, a substoichiometric DNA molecule containing two

140 I-12CMS(2)-type loci exists in I-12CMS(3) mitochondria, suggesting that such a DNA molecule might
141 play an important role in the evolution of beet CMS.

142

143 Materials and methods

144

145 Plant materials and fertility scoring

146

147 I-12CMS(R), I-12CMS(2) and I-12CMS(3) possess different sources of cytoplasm, but the nuclear
148 genotype is equivalent to that of a sugar beet inbred maintainer line, I-12 61L (Mikami et al. 1985).
149 I-12CMS(R) carries the CMS-Owen, whereas I-12CMS(2) and I-12CMS(3) have MS cytoplasms derived
150 from wild beets collected in Turkey and Pakistan, respectively. NK-183mm-O (developed by the Hokkaido
151 Agricultural Research Center, National Agriculture and Food Research Organization, Japan) is an inbred
152 maintainer line of CMS-Owen (Moritani et al. 2013), i.e. with normal fertile cytoplasm and is devoid of *Rf*
153 for CMS-Owen. TK-81mm-CMS and TK-81mm-O are a CMS-Owen line and its maintainer line,
154 respectively (Yamamoto et al. 2005). Fertility was determined by the following three criteria: anther color,
155 pollen shedding and pollen stainability. Pollen stainability estimates involved squashing anthers from open
156 flowers in a drop of cotton-blue stain (Hagihara et al. 2005). At least three flowers per plant were examined.
157 Fertile plants bore well-dehiscing, yellow anthers with 20% and more stainable pollen grains. Sterile plants
158 had translucent or brown shrunken anthers. No dehiscence was observed in sterile plants. Partially fertile
159 plants produced less than 20% stainable pollen. Anthers of partially fertile plants were light-yellow or
160 orange in color and usually failed to dehiscence. The statistical significance of data was calculated using
161 Fisher's exact test posted at <http://aoki2.si.gunma-u.ac.jp/exact/fisher/getpar.html> (accessed on
162 10/Feb/2015).

163

164 Nucleic acids isolation

165

166 Mitochondrial (mt) DNA and mtRNA were isolated according to the methods of Mikami et al. (1985) and
167 Kubo et al. (2000), respectively. Total cellular DNA was isolated according to the protocol of Doyle and
168 Doyle (1990). Total cellular RNA was isolated from green leaves using an RNeasy Plant Mini Kit (Qiagen,
169 Valencia, CA).

170

171 Library construction and chromosome walking

172 MtDNAs from I-12CMS(2) and I-12CMS(3) were partially digested with *MboI* (Takara Bio, Ohtsu, Japan)
173 and fractionated on continuous sucrose density gradients (Sambrook et al. 1989). Fragments of 15 to 20 kbp
174 were collected and ligated into the *BamHI*-digested lambda DASH vector (Stratagene, La Jolla, CA). The

175 DNA ligation mixture was packaged *in vitro* using Gigapack Gold (Stratagene). A total of 384 recombinant
176 phages were randomly chosen from each of the resulting mtDNA libraries and used for a physical mapping
177 study. DNA fragments located every 5 kbp on the TK-81mm-O (normal cytoplasm) mtDNA map (Kubo et
178 al. 1995), and several known mitochondrial gene sequences were used as probes for plaque hybridization.
179 DNA fragments at the extremities of the clone contigs were used to ‘walk’ the mitochondrial genome. All
180 overlapping clones selected were tested by hybridization with several mitochondrial gene probes as well as
181 with DNA fragments on the TK-81mm-O mtDNA map (Kubo et al. 1995), to ensure that each clone was a
182 true representation of the mtDNA sequences.

183

184 RNA gel blot hybridization

185

186 MtRNA was fractionated by electrophoresis in a 1.4% agarose gel containing 0.66 M formaldehyde and
187 then transferred to Hybond N+ membrane (GE Healthcare, UK, Amersham Place, England). Specific
188 probes for *orf129*- and *atp9* mRNA detection were PCR amplified from cloned DNA with the primers
189 described in Cheng et al. (2011) and Matsunaga et al. (2011), respectively. Hybridization probes were
190 labeled with ³²P using a Megaprime DNA labeling kit (GE Healthcare) or with alkaline phosphatase using
191 the AlkPhos Direct DNA labeling system (GE Healthcare).

192

193 DNA sequencing

194

195 The DNA fragments of interest were cloned into the pBluescript vector (Stratagene) and then sequenced
196 using a Li-COR4000L DNA sequencer (Li-COR, Lincoln, NE) or an ABI3130 genetic analyzer (Applied
197 Biosystems, Foster City, CA). Nucleotide sequences were assembled using Sequencher 4.0 (Gene Codes
198 Corporation, Ann Arbor, MI). A homology search (BLAST) was conducted on the NCBI web site
199 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequence data were deposited with DDBJ/EMBL/GenBank under
200 accession nos. LC032133 (I-12CMS(2) *atp1-rrn26*), AB355937 (I-12CMS(3) *orf129*), LC032134
201 (I-12CMS(2) *atp6*), and AB490412 (I-12CMS(2) *orf129*).

202

203 Polymerase chain reaction (PCR)

204

205 Primers used in this study are listed in Table S1. For quantitative reverse-transcription (qRT) PCR, RNA
206 samples (4 µg) were treated with RNase-free DNase I and reverse transcribed with SuperScript III
207 First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA) using random hexamer
208 primers. Reagents for qRT-PCR contain SYBR GreenER qPCR SuperMix Universal (Life Technologies)
209 and primers (0.2 µM each). PCR was monitored using a Chromo 4, Opticon Monitor (ver 3.1) with a

210 DNA-Engine PTC-200 (Bio-Rad Laboratories, Hercules, CA). The PCR protocol was 95°C for 10 min,
211 then 40 cycles of 95°C for 15 sec and 60.8°C for 1min. A series of dilutions of I-12CMS(2) cDNA were
212 used as the standard samples for quantification. Using this series, qRT-PCR generated a standard curve (X
213 axis, factor of dilution; Y axis, threshold cycle number) for mRNAs of *orf129* (primers orf129-Fw and
214 orf129-Rv) and an actin gene (*Act*) (primers actin-Fw and actin-Rv). The ratio of *orf129:Act* in each sample
215 was calculated based on the results of qRT-PCR analysis (repeated three times for each sample). Student's
216 *t*-test was done using Microsoft Excel (Microsoft Office 2008 for Mac; Microsoft Japan, Tokyo, Japan). For
217 circularized RNA RT-PCR (Kuhn and Binder 2002), DNase I-treated RNA samples were treated with
218 tobacco acid pyrophosphatase as described in Muller and Storchova (2013). The RNA was circularized as
219 described previously (Matsunaga et al. 2011). Complementary DNA was synthesized with SuperScript III
220 First-Strand Synthesis System for RT-PCR and primer orf129-162R. PCR amplification of *orf129* cDNA
221 used primers orf129-54R and orf129-339F (protocol: 94°C, 3min; 35 cycles of 94°C, 30sec, 55°C, 30sec,
222 and 72°C, 1min; 72°C, 4min). PCR products were cloned using a TOPO TA Cloning kit (Life Technologies).
223 For PCR amplification of genomic DNA, about 10 ng of total cellular DNA was mixed with 10 µl of 1 x
224 Quick Taq HS DyeMix (Toyobo, Osaka, Japan) or 1 x GoTaq Green Master Mix (Promega, Madison, WI).
225 For PCR amplification of PCR products, the template PCR products were treated with Exonuclease I
226 (Takara Bio) (1 unit, 37°C, 1h) to degrade residual primers before the PCR amplification. PCR protocols are
227 described in the figure legends. PCR products were electrophoresed in 2% agarose gels. Gels were stained
228 with ethidium bromide solution (Sambrook et al. 1989).

229

230 Immunoblotting

231

232 Anti-ORF129 antiserum was raised against the carboxyl half of ORF129 (D50-Q129) as described
233 previously (Yamamoto et al. 2008). Anti-preSATP6 was raised against an oligopeptide corresponding to the
234 C-terminal sequence of preSATP6 (Yamamoto et al. 2005). Total cellular proteins were isolated from green
235 leaves as described in Cheng et al. (2009). Proteins were separated by SDS-PAGE and electroblotted onto
236 Hybond ECL membrane (GE Healthcare). The concentration of primary antisera used for immunoblots was
237 42.5 ng/mL. Signal bands were detected as described previously (Yamamoto et al. 2005, 2008).

238

239

240 Results

241

242 I-12CMS(2), I-12CMS(3) and I-12CMS(R) differ in their ability to restore fertility

243

244 Three CMS lines, I-12CMS(2), I-12CMS(3) and I-12CMS(R) (CMS-Owen), all of which bear the same

245 nuclear background, were crossed individually with three plants (#1 to #3) randomly chosen from
246 NK-183mm-O, a line that is devoid of the *Rf* for CMS-Owen but male fertile owing to a normal fertile
247 cytoplasm (i.e. a maintainer for CMS-Owen). As shown in Table 1, NK-183mm-O served as a perfect
248 sterility-maintainer when used as the pollen parent on I-12CMS(R). This same pollen parent line, however,
249 failed to maintain male sterility in most of its hybrids with either I-12CMS(2) or I-12CMS(3) (Table 1).
250 Interestingly, fertility in the F₁ hybrids carrying the I-12CMS(3) cytoplasm apparently exceeded that of the
251 F₁s with the I-12CMS(2) cytoplasm (Fisher's exact test: $p=1.5 \times 10^{-23}$ for NK-183mm-O#1, $p=5.1 \times 10^{-12}$
252 for NK-183mm-O#2, and $p=2.2 \times 10^{-17}$ for NK-183mm-O#3). The cross of I-12CMS(3) x NK-183mm-O
253 yielded fully fertile and partially fertile progeny, whereas the F₁ progeny of I-12CMS(2) with
254 NK-183mm-O tended to group around the partially fertile class (0-20% stainable pollen with cotton-blue
255 stain).

256 A fraction of the F₁ hybrids (e.g. five F₁s of I-12CMS(2) x NK-183mm-O#2 and four F₁s of I-12CMS(3)
257 x NK-183mm-O#2) had white shriveled anthers and were classified as fully sterile. Because the frequency
258 of the fully sterile plants in F₁ progeny appeared to be different depending on the pollen parents (i.e.
259 NK-183mm-O#1 to NK-183mm-O#3), we tested a null hypothesis that fully sterile F₁ plants occurred at the
260 same frequency irrespective of the pollen parents for each of the I-12CMS(2) and I-12CMS(3) by Fisher's
261 exact test. The results rejected the null hypothesis [$p=0.0007$ for I-12CMS(2) and $p=5.6 \times 10^{-5}$ for
262 I-12CMS(3)]. A possible explanation for these results is that, although NK-183mm-O has *Rfs* for
263 I-12CMS(2), as well as for I-12CMS(3), the genetic composition of these *Rfs* differs among the pollen
264 parents. For example, some of the *Rfs* were heterozygous in one pollen parent but homozygous in the others.
265 Also, it is possible that fertility restoration conditioned by NK-183mm-O is highly sensitive to the
266 environment, hence unstable, although the restoration was more efficient for I-12CMS(3).

267

268

269 Organizational differences between the I-12CMS(2) and I-12CMS(3) mitochondrial genomes

270

271 To gain insight into the molecular basis of cytoplasmic differences, we focused our analysis on the
272 organizational differences between the I-12CMS(2) and I-12CMS(3) mitochondrial genomes.
273 Chromosome walking using phage-cloned ~18 kbp DNA fragments that were generated by random
274 shearing of I-12CMS(3) mitochondrial (mt) DNA predicted the existence of two circular chromosomes of
275 274 kbp and 234 kbp, sharing a 103 kbp sequence (Figs. 1 and S1). The I-12CMS(3) genome can also be
276 interpreted as a 508 kbp master chromosome with 103 kbp repeated sequences (Figs. 1 and S1). We also
277 identified two families of repeated sequences that are involved in inter- and/or intra-molecular
278 recombination (shown as R1 [6.4 kbp] and R2 [5.7 kbp] in Figs. 1 and S1). Hence, the molecular
279 organization of the mitochondrial genome of I-12CMS(3) appears to be very complex.

280 During our study, Darracq et al. (2011) reported the shotgun-sequencing-based prediction of the
281 mitochondrial genome organization of CMS-E that is probably equivalent to the I-12CMS(3) cytoplasm.
282 Although their model, one circular molecule (269 kbp) and one linear molecule (110 kbp), is different from
283 ours, a comparison of gene order and restriction site arrangement indicated that the net sequence
284 complexity is nearly the same between the two models. Differences in the configuration are due to the
285 introduction of repeated sequences and the arrangement of unique sequences between repeats. It is likely
286 that differences in the mapping strategy (chromosome walking using ~18 kbp-clone library vs. shotgun
287 sequencing using 5 kbp-clone library) are associated with the different results.

288 An I-12CMS(2) mtDNA physical map was also constructed by identifying a set of overlapping
289 I-12CMS(2) phage clones as was done for the I-12CMS(3) genome. The resultant map was identical to that
290 of I-12CMS(3) except for missing a 13-kbp segment from a region between *atp1* and *rrn26* of I-12CMS(2)
291 (shown as the stippled region in Fig. 1). In our physical maps, one of the repeated sequences named R1
292 partially overlapped with the 13-kbp region in I-12CMS(3), and this R1 copy is truncated in I-12CMS(2)
293 (Fig. 1); however, another intact R1 copy is preserved in I-12CMS(2).

294 Inspection of the restriction site map confirmed the conserved organization of the *atp1-rrn26* region
295 between I-12CMS(2) and TK-81mm-O. The latter is a normal, fertile sugar beet line whose entire
296 mitochondrial DNA sequence is known (Kubo et al. 2000). Comparison of the *atp1-rrn26* regions between
297 CMS-E (DDBJ/EMBL/GenBank accession number FQ014226) and TK-81mm-O (BA000009) revealed a
298 12860-bp sequence that is absent from the TK-81mm-O *atp1-rrn26* region (Fig. 2). In TK-81mm-O, the
299 12860-bp sequence is replaced with a 561-bp sequence that has little homology to the 12860-bp sequence.
300 We confirmed that the 561-bp sequence is conserved in I-12CMS(2) by nucleotide sequencing (see below).
301 No known mitochondrial genes were found in the 12860-bp sequence or the 561-bp sequence. Eight ORFs
302 that can specify 105-265 amino acid residues are annotated in the 12860-bp sequence (FQ014226), but
303 there was not an ORF identified in the 561-bp sequence (BA000009).

304

305

306 A larger amount of ORF129 protein is present in I-12CMS(2) than in I-12CMS(3)

307

308 We sought other molecular differences potentially associated with the cytoplasmic difference. We focused
309 our analysis on *orf129*, an MS-associated mitochondrial gene in I-12CMS(3) (Yamamoto et al. 2008). Our
310 physical mapping data indicated the preservation of *orf129* in I-12CMS(2) (Fig. 1). In order to confirm the
311 presence of ORF129 polypeptide, immunoblot analysis using ORF129-specific antiserum (Yamamoto et al.
312 2008) was done. Total cellular proteins were isolated from leaves of I-12CMS(R), I-12CMS(2), and
313 I-12CMS(3). ORF129 was detected from the preparations of I-12CMS(2) and I-12CMS(3), whereas no
314 detectable signal was seen in I-12CMS(R) (Fig. 3a). What does merit attention is that the signal was more

315 intense in I-12CMS(2) than in I-12CMS(3) (Fig. 3a). Dilutions of the total protein preparations from
316 I-12CMS(2) and I-12CMS(3) were used to compare the relative amounts of ORF129. Relative signal
317 intensities obtained by immunoblot analysis indicated that I-12CMS(2) contains approximately three to
318 five times more ORF129 than does I-12CMS(3) (Fig. 3b).

319 RNA gel blot analysis of total mitochondrial RNA isolated from leaves showed a stronger *orf129*
320 mRNA signal for a 1.1-kb band in I-12CMS(2) than that of I-12CMS(3) (Fig. 3c). On the other hand, the
321 smaller size transcripts are fairly more intense in I-12CMS(3). Accumulation of *orf129* transcripts was
322 compared by quantitative real time RT-PCR (qRT-PCR). Total cellular RNA of leaves was isolated from
323 two I-12CMS(3) plants and two I-12CMS(2) plants. Each of the four samples was subjected to qRT-PCR in
324 which a nuclear actin gene (*Act*) was used as the reference gene. The results showed that accumulation of
325 I-12CMS(2) *orf129* mRNA is about 2.7 times that of I-12CMS(3) *orf129* mRNA, a statistically significant
326 difference (Table 2).

327 No apparent difference at the *orf129* locus was seen on the physical maps between I-12CMS(3) and
328 I-12CMS(2), but it is possible that the difference in *orf129*-mRNA accumulation is associated with a
329 genomic alteration that is difficult to detect by physical mapping. We determined the nucleotide sequence
330 of a 2.2-kbp segment (1.1-kbp *Bam*HI-*Xho*I and 1.1-kbp *Xho*I sub-fragments of a phage clone) of the
331 I-12CMS(2) mtDNA containing *orf129* (Fig. S2). The nucleotide sequence of the corresponding region in
332 I-12CMS(3) was also determined from subclones of a phage clone. The I-12CMS(3) sequence was
333 identical to that of CMS-E.

334 The sequences of the *orf129* coding region (387 bp in length) from the two beet lines, I-12CMS(2) and
335 I-12CMS(3), were perfectly identical and, thus, can encode identical proteins. The sequence identity
336 extended downstream for at least 714 bp, from nucleotide +388 (the first nucleotide residue of the *orf129*
337 initiation codon is defined as +1) to the 3'-end of the sequenced region (nucleotide +1104). The 5' flanking
338 regions (from the 5'-end nucleotide -1138 of the sequenced region to nucleotide -1) of the two genes were
339 virtually identical. The only difference was a single nucleotide substitution identified at nucleotide -490; a
340 G residue in the I-12CMS(3) *orf129* versus a T in the I-12CMS(2) *orf129* (Fig. S2).

341 To find an association between the G-T polymorphism and *orf129* expression, cDNAs containing
342 transcript termini of *orf129* mRNA were PCR amplified by circularized RNA reverse transcription PCR
343 (CR-RT-PCR), in which RNAs were reverse transcribed after their 5' and 3' ends were ligated by T4 RNA
344 ligase (Kuhn and Binder 2002). The resultant cDNAs were PCR amplified, cloned into plasmid vectors and
345 sequenced. Some of our cDNA clones have additional nucleotides that were not encoded in genomic DNA
346 (Table S2). These additional nucleotides appeared to be inserted between transcript termini in the cDNA. It
347 is likely that post-transcriptional modification, such as polyadenylation (Lange et al. 2009), occurred in
348 some *orf129* mRNAs. The mapped termini are detailed in Fig. S2 and Table S2, and are summarized in Fig.
349 4a.

350 The mapped 3' termini were almost always confined within the same ~12 bp region in I-12CMS(3) and
 351 I-12CMS(2) (Fig. 4a and Table S2). On the other hand, the 5' termini were mapped to the region from -479
 352 to -114. In I-12CMS(2), eight of the eleven cDNA clones were derived from transcripts whose 5' termini
 353 were mapped within a region of -479 to -472 (Table S2). Considering the size of these transcripts deduced
 354 from mapped transcript termini (~1050 bases), they likely represent the 1.1 kb-signal band on the RNA gel
 355 blot (Fig. 3c). In I-12CMS(3), the number of cDNA clones representing 1.1-kb signal band is three out of
 356 eleven (Table S2). The other cDNA clones enabled us to map the 5' termini from -278 to -153 (Table S2 and
 357 Fig. 4a), suggesting that they represent the smaller size transcripts on the RNA gel blot (Fig. 3c).

358 The G-T polymorphism at -490 was close to the 5' termini of the 1.1-kb transcripts (Fig. 4b). The DNA
 359 sequences surrounding these termini are similar to the promoter consensus sequence (5'-CRTAAGAGA-3'
 360 or 5'-CGTATATAA-3') that has been proposed for mitochondrial genes in dicot plants (Gagliardi and Binder,
 361 2007) (Fig. 4b). This observation suggests that the 1.1-kb *orf129* mRNA is a primary transcript. The -490
 362 polymorphic site is located only 5 bp upstream of this promoter consensus sequence.

363

364

365 Differences in the amino acid sequence of the N-terminal extension of *atp6*

366

367 In I-12CMS(3)/CMS-E mitochondria, there is another expressed ORF that is absent from normal fertile
 368 mitochondria. This ORF is fused with *atp6* as an N-terminal extension (Yamamoto et al. 2008; Darracq et al.
 369 2011). On our physical map, no apparent structural alteration is seen between the *atp6* loci of I-12CMS(2)
 370 and I-12CMS(3) (Fig. 1), indicating that I-12CMS(2) *atp6* also has an homologous N-terminal extension.
 371 To compare the nucleotide sequence of *atp6*, we determined the sequence of I-12CMS(2) *atp6* that is on a
 372 subclone of a phage clone (Fig. S3).

373 I-12CMS(2) *atp6* is 1956 bp in length with the potential to encode a 652 amino acid residue polypeptide.
 374 We identified the N-terminal extension of I-12CMS(2) ATP6 polypeptide by comparison with I-12CMS(3)
 375 ATP6 (Fig. S4). In both I-12CMS(2) and I-12CMS(3) ATP6, the length of the N-terminal extension is 389
 376 amino acid residues. Sequence homology between the two N-terminal extensions is 94.1%. Besides the
 377 N-terminal extension, I-12CMS(2) *atp6* and I-12CMS(3) *atp6* are identical at the nucleotide and amino
 378 acid sequence levels.

379 To see the expression of the N-terminal extension of I-12CMS(2) *atp6*, immunoblot analysis was done
 380 using antiserum against preSATP6, an N-terminal extension of *atp6* in CMS-Owen mitochondria
 381 (Yamamoto et al. 2005). Because the amino acid sequence of the antigen used for raising anti-preSATP6
 382 antiserum is conserved in I-12CMS(2) ATP6 (Fig. S4), and because anti-preSATP6 antiserum reacted with
 383 I-12CMS(3) protein (Yamamoto et al. 2008), this antiserum is expected to react with the translation product
 384 of I-12CMS(2) N-terminal extension, if any. As shown in Fig. 5, we detected signal bands of low intensity

385 from total cellular proteins of I-12CMS(2) and I-12CMS(3) leaves. The apparent molecular mass of the two
386 signal bands was indistinguishable.

387

388

389 A substoichiometric mitochondrial DNA molecule in I-12CMS(3)

390

391 Our next task was to develop a molecular marker that enables one to discriminate I-12CMS(2) and
392 I-12CMS(3) cytoplasms. We thought that the *atp1-rrn26* region was a suitable target for designing
393 oligonucleotide primers that could be used for specific PCR amplification of I-12CMS(2) or I-12CMS(3)
394 mtDNA. Two primers were designed that correspond to specific regions of I-12CMS(2) or I-12CMS(3)
395 (Fig. 6a). The I-12CMS(2)-specific primer has no significant homology to nucleotide sequences of
396 FQ014226 or FQ014231, entries that constitute the complete mtDNA sequence of CMS-E. A common
397 primer was designed outside of the polymorphic region (Fig. 6a).

398 PCR amplification targeting the I-12CMS(3)-specific region gave us amplicons of the expected size of
399 0.6 kbp from two I-12CMS(3) plants (Fig. 6b). Nucleotide sequences of these amplicons matched with
400 those from CMS-E mitochondria. No amplification was observed from four I-12CMS(2) plants (Fig. 6b),
401 indicating that the I-12CMS(3)-specific sequence is absent from I-12CMS(2) mitochondria.

402 We tested another primer combination that is expected to PCR amplify the I-12CMS(2)-specific region.
403 From four I-12CMS(2) plants, PCR products of the expected size were obtained (Fig. 6b). Nucleotide
404 sequences of these amplicons were matched with TK-81mm-O mitochondria, confirming the
405 abovementioned notion that the I-12CMS(2)-specific region is conserved in TK-81mm-O. On the other
406 hand, PCR products of the same size were amplified from two I-12CMS(3) plants (Fig. 6b). The nucleotide
407 sequences of these PCR products matched perfectly with that of the I-12CMS(2) PCR products. We think
408 that contamination of reagents or utensils with I-12CMS(2) genomic DNA [or the I-12CMS(2) DNA clone]
409 is unlikely as indicated by the control experiment (lane 7 in Fig. 6b). Each of the DNA samples shown in
410 Fig. 6b was isolated from a piece of green leaf excised with a clean razor, making contamination of DNA
411 samples unlikely. Because of the absence of the I-12CMS(2)-specific region from the physical map of
412 I-12CMS(3) (or from FQ014226 or FQ014231), from the draft sequence of the sugar beet nuclear genome
413 (Dohm et al. 2014) or the plastid genome sequence (DDBJ/EMBL/GenBank accession number EF534108),
414 a possible explanation is heteroplasmy regarding the *atp1-rrn26* region in I-12CMS(3) in which a
415 I-12CMS(2)-like DNA molecule exists in substoichiometric levels.

416 The heteroplasmic state of the *orf129* locus was tested. We designed other primer sets for PCR that can
417 detect single nucleotide polymorphisms (SNPs) in the upstream region of *orf129* (Fig. 7a). Specific primers
418 have G or T residues at their 3' ends that correspond to the -490 SNP of *orf129*, by which PCR amplification
419 proceeds only when the 3' end of the primer is complementary to the template DNA (mutant allele-specific

420 PCR amplification, MASA). A common primer corresponds to the upstream region of *orf129* that is
421 conserved between I-12CMS(2) and I-12CMS(3). As shown in Fig. 7b, PCR analysis using the common
422 and I-12CMS(3) specific primers detected the expected signal bands from I-12CMS(3) samples but not
423 from I-12CMS(2). On the other hand, when the I-12CMS(2) specific primer was used, we observed signal
424 bands from I-12CMS(2) samples as well as I-12CMS(3) samples, suggesting heteroplasmy in I-12CMS(3),
425 but PCR amplification from I-12CMS(3) *orf129* with mismatch primers could also be possible. If the
426 observed amplification from I-12CMS(3) samples was due to the mismatch-primer PCR, the I-12CMS(2)
427 specific primer could generate the same PCR product from I-12CMS(3) *orf129*. To test this possibility,
428 PCR products of the upstream region of I-12CMS(3) *orf129* were subjected to PCR amplification with the
429 I-12CMS(2)-specific primers, and we observed no amplification (lane 7 in Fig. 7b).

430 Finally, we tested the heteroplasmic state of the *atp6* locus. Of the several nucleotide substitutions and
431 indels, SNP in +177 was selected as a target for MASA (Fig. 8a). Specific primers have C or A residues at
432 their 3' ends that correspond to the +177 SNP of *atp6*. In this MASA, we saw no trace of heteroplasmy in the
433 *atp6* loci of I-12CMS(2) or I-12CMS(3) (Fig. 8b).

434

435

436 Discussion

437

438 In our test cross presented here, fully restored F₁ plants from I-12CMS(3) and partially restored F₁ plants
439 from I-12CMS(2) seed parents emerged. Because the pollen parent, NK-183mm-O, is devoid of the *Rf* for
440 CMS-Owen, the I-12CMS(3) and I-12CMS(2) CMSs are considered as different cytoplasms from
441 CMS-Owen at the genetic level. Darracq et al. (2011) reported the differences in mitochondrial genome
442 organization between CMS-E and CMS-Owen, a result that included our data. We also observed differential
443 fertility restoration reactions between I-12CMS(3) and I-12CMS(2) CMSs. I-12CMS(2) apparently tended
444 to resist fertility restoration more than I-12CMS(3) when crossed with the pollen parent NK-183mm-O. We
445 assume that I-12CMS(3) and I-12CMS(2) cytoplasms may differentially respond to the same *Rf*(s) even if
446 the molecular data indicate a close relationship between these two cytoplasms. Mitochondrial genome
447 organization is very similar between I-12CMS(3) and I-12CMS(2); both I-12CMS(3) and I-12CMS(2)
448 possess *orf129*, whereas *orf129* is absent from CMS-Owen (Yamamoto et al. 2008); and both I-12CMS(3)
449 and I-12CMS(2) possess a long N-terminal extension of *atp6*. An *Rf* can differentially restore pollen
450 fertility to plants with different sources of CMS that share some features at the molecular level. For example,
451 in rice, CMS-BT and CMS-LD are considered to be different CMSs, but both are associated with
452 mitochondrial gene *orf79* (Chen and Liu 2014). Plants with these two CMSs can restore male fertility by
453 *Rf2*, but the degree of restored fertility is different between CMS-BT and CMS-LD plants (Itabashi et al.
454 2009).

455 The observed difference in the degree of fertility restoration between I-12CMS(3) and I-12CMS(2) can
456 be discussed in relationship to the molecular data. One of the molecular differences is seen in the
457 *atp1-rrn26* region; however, we think it is unlikely that this difference affects MS expression for the
458 following reasons: the altered region in *atp1-rrn26* contains no known mitochondrial gene in I-12CMS(3)
459 or I-12CMS(2) and no function is known for the eight ORFs in the 12860-bp region in I-12CMS(3); and
460 mitochondrial genes close to the altered region are *atp1* and *rrn26*, but they are 6.3 kbp and 2.0 kbp away
461 from the altered region, respectively, which appears to be too remote to affect plant mitochondrial gene
462 expression. Moreover, genetic association of the I-12CMS(2)- or the I-12CMS(3)-type *atp1-rrn26* with MS
463 is improbable, because the former is seen in the normal, fertile line TK-81mm-O, and we found that the
464 I-12CMS(3)-type *atp1-rrn26* is very similar to that of the mitochondrial genome organization of wild beet
465 accession BGRC56777 that has another non-MS-inducing cytoplasm (Nishizawa et al. 2007).

466 On the other hand, the differential fertility restoration between I-12CMS(3) and I-12CMS(2) may be
467 associated with quantitative differences in the accumulation of ORF129 polypeptide. ORF129 polypeptide
468 is more abundant in I-12CMS(2), whose fertility restoration by NK-183mm-O is insufficient. This is an
469 interesting correlation that should be analyzed in the future.

470 The relative abundance of ORF129 protein correlates with the difference in steady-state *orf129*
471 transcript levels. In this connection, of particular interest is the single nucleotide substitution near the 5'
472 terminus of the *orf129* transcript. We hypothesize that this substitution may affect the expression of *orf129*.
473 Mitochondrial promoters have been analyzed in several plant species by identifying transcription initiation
474 sites and aligning the surrounding sequences, thereby revealing conserved promoter motifs (Gagliardi and
475 Binder 2007). In *Arabidopsis thaliana*, for example, half of the transcriptional starts identified are located
476 within the conserved nonanucleotide core motif (CRTAAGAGA or CGTATATAA; transcription initiation
477 nucleotide underlined) (Gagliardi and Binder 2007). Purines appear immediately downstream from the
478 transcription initiation site. Preceding the core motif is usually a sequence rich in A and T nucleotides
479 (AT-box) that has been proven essential for the full function of some mitochondrial promoters *in vitro*
480 (Gagliardi and Binder 2007). DNA sequences around the 5' terminus of the 1.1-kb *orf129* transcripts were
481 found to be very similar to the consensus promoter motif. The single base change mentioned above
482 occurred within the putative AT-box. The promoter of the I-12CMS(2) *orf129* contained a conserved T
483 nucleotide at the position in question, whereas in the I-12CMS(3) *orf129* promoter, a T was replaced by a G.
484 It is probable that this T to G transversion exerted a substantial negative influence on the activity of the
485 I-12CMS(3) *orf129* promoter, given that the AT-box is actually required for full-promoter function.

486 In both I-12CMS(3) and I-12CMS(2), a long N-terminal extension of *atp6* is expressed and the protein
487 product is detected in leaves. However, because the protein product is undetectable in anthers of
488 I-12CMS(3) (Yamamoto et al. 2008), there are no data associating the *atp6* loci with MS expression of
489 I-12CMS(3). On the other hand, the N-terminal extension of CMS-Owen accumulated in anthers as a

490 membrane protein; hence, it may be associated with MS expression (Yamamoto et al. 2005). If very small
491 amounts of the N-terminal extension of *atp6* can reduce the male fertility of I-12CMS(3) and I-12CMS(2),
492 differences in the amino acid sequences of the N-terminal extension may be associated with differential
493 fertility restoration, but this proposal needs further study.

494 The long N-terminal extension is a characteristic of I-12CMS(2), I-12CMS(3) and CMS-Owen
495 (Onodera et al. 1999; Yamamoto et al. 2005; this study). The amino acid sequences of the N-terminal
496 extensions are similar among I-12CMS(2), I-12CMS(3) and CMS-Owen, suggesting a monophyletic origin
497 of these *atp6* genes. This result is consistent with the notion of Darracq et al. (2011), who proposed a
498 monophyletic origin of MS-inducing mitochondria in beet.

499 We found that I-12CMS(3) is heteroplasmic at two mitochondrial loci (*atp1-rrn26* and *orf129*), a
500 finding that provides insight into the evolutionary relationship between I-12CMS(3) and I-12CMS(2)
501 mitochondria. In I-12CMS(3) mitochondria, we postulate the presence of a substoichiometric DNA
502 molecule that has the 561-bp sequence at *atp1-rrn26* and a T residue at -490 in *orf129* (Fig. 9). This
503 substoichiometric molecule resembles the I-12CMS(2) mitochondrial genome but differs at least at
504 nucleotide +177 in *atp6*. On the other hand, the genomes of I-12CMS(2) mitochondria are homoplasmic
505 having the 561-bp sequence at *atp1-rrn26*, a T residue at nucleotide -490 of *orf129* and an A residue at
506 nucleotide +177 of *atp6*. Assuming an intermediate that has heteroplasmic mitochondria similar to
507 I-12CMS(3) but whose substoichiometric DNA molecule encodes A at nucleotide +177 in *atp6* (Fig. 9), we
508 propose a model to explain the evolution of I-12CMS(3) mitochondria to I-12CMS(2) mitochondria. First,
509 the substoichiometric DNA molecule in the intermediate mitochondria evolved to have A residues at
510 nucleotide +177 in *atp6*. In this regard, Small et al. (1987) proposed that mtDNA molecules accumulating
511 mutations could be maintained as substoichiometric DNA molecules that play an important role in
512 mitochondrial genome evolution. In the next step, the resultant substoichiometric DNA molecule
513 predominated in mitochondria in a manner similar to that shown in common bean (Janska et al. 1998),
514 whereas the other DNA molecule was lost, resulting in I-12CMS(2) in Fig. 9. We cannot exclude the
515 possibility that evolution occurred in the opposite direction (i.e. from the right to the left in Fig. 9). However,
516 evidence would be needed for a preexisting substoichiometric molecule that has the 12860-bp sequence at
517 *atp1-rrn26*, and none was detected in this study. Such a substoichiometric DNA molecule may be found in
518 other beet accessions.

519 We should point out the possibility that there might be an unidentified substoichiometric DNA molecule
520 in I-12CMS(3) or other CMS beets, and such DNA molecules could serve as materials to evolve other
521 MS-inducing mitochondria. Hence, variation in beet CMS might be wider than ever thought.

522

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526

527 References

528 Allen JO, Fauron CM, Minx P, Roark L, Oddiraju S, et al. (2007) Comparisons among two fertile and three
529 male-sterile mitochondrial genomes of maize. *Genetics* 177: 1173-1192

530 Bosemark NO (1979) Genetic poverty of the sugarbeet in Europe. In: A.C. Zeven & A.M. Van Harten
531 (Eds.). *Proc Conf Broadening Genet Base Crops*, Wageningen, the Netherlands. Pudoc Wageningen,
532 pp. 29-35

533 Bosemark NO (2006) Genetics and Breeding. In: Draycott AP (ed) *Sugar Beet*. Blackwell Publishing,
534 Oxford, UK, pp 50-88

535 Budar F, Touzet P, Pelletier G (2006) Cytoplasmic male sterility. In: Ainsworth C (ed) *Flowering and its*
536 *manipulation*. Blackwell, Oxford, pp 147-180

537 Chen L, Liu Y-G (2014) Male sterility and fertility restoration in crops. *Annu Rev Plant Biol* 65: 579-606

538 Cheng D, Kitazaki K, Xu D, Mikami T, Kubo T (2009) The distribution of normal and male-sterile
539 cytoplasm in Chinese sugar-beet germplasm. *Euphytica* 165: 345-351

540 Cheng D, Yoshida Y, Kitazaki K, Negoro S, Takahashi H, et al. (2011) Mitochondrial genome diversity in
541 *Beta vulgaris* L. ssp. *vulgaris* (Leaf and Garden Beet Groups) and its implications concerning the
542 dissemination of the crop. *Genet Res Crop Evol* 58: 553-560

543 Christensen CA (2014) Genes and junk in plant mitochondria--repair mechanisms and selection. *Genome*
544 *Biol Evol* 6: 1448-1453

545 Darracq A, Varre JS, Marechal-Drouard L, Courseaux A, Castric V, et al. (2011) Structural and content
546 diversity of mitochondrial genome in beet: a comparative genomic analysis. *Genome Biol Evol* 3:
547 723-736

548 Dohm JC, Minoche AE, Holtgrawe D, Capella-Gutierrez S, Zakrzewski F, et al. (2014) The genome of the
549 recently domesticated crop plant sugar beet (*Beta vulgaris*). *Nature* 505: 546-549

550 Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15

551 Duvick DN (1965) Cytoplasmic pollen sterility in corn. *Adv Genet* 13: 1-56

552 Fenart S, Touzet P, Arnaud J-F, Cuguen J (2006) Emergence of gynodioecy in wild beet (*Beta vulgaris*
553 *maritima* L.): a genealogical approach using chloroplastic nucleotide sequences. *Proc Royal Soc*
554 *London B* 273: 1391-1398

555 Fujii S, Kazama T, Yamada M, Toriyama K (2010) Discovery of global genomic re-organization based on
556 comparison of two newly sequenced rice mitochondrial genomes with cytoplasmic male
557 sterility-related genes. *BMC Genomics* 11: 209

558 Gagliardi D, Binder S (2007) Expression of the plant mitochondrial genome. In: Logan DC (ed) *Plant*
559 *Mitochondria*, Blackwell, Oxford, pp 50-96

- 560 Hagihara E, Itchoda N, Habu Y, Iida S, Mikami T, et al. (2005) Molecular mapping of a fertility restorer
561 gene for Owen cytoplasmic male sterility in sugar beet. *Theor Appl Genet* 111: 250-255
- 562 Hallden C, Lind C, Moller IM (1992) Variation in mitochondrial translation products in fertile and
563 cytoplasmic male-sterile sugar beets. *Theor Appl Genet* 85: 139-145
- 564 Hanson MR, Bentolila S (2004) Interactions of mitochondrial and nuclear genes that affect male
565 gametophyte development. *Plant Cell* 16: S154-S169
- 566 Itabashi E, Kazama T, Toriyama K (2009) Characterization of cytoplasmic male sterility of rice with Lead
567 Rice cytoplasm in comparison with that with Chinsurah Boro II cytoplasm. *Plant Cell Rep* 28:
568 233-239
- 569 Janska H, Sarria R, Woloszynska M, Arrieta-Montiel M, Mackenzie SA (1998) Stoichiometric shifts in the
570 common bean mitochondrial genome leading to male sterility and spontaneous reversion to fertility.
571 *Plant Cell* 10: 1163-1180
- 572 Kawanishi Y, Shinada H, Matsunaga M, Masaki Y, Mikami T, et al. (2010) A new source of cytoplasmic
573 male sterility found in wild beet and its relationship to other CMS types. *Genome* 53: 251-256
- 574 Kubo T, Kitazaki K, Matsunaga M, Kagami H, Mikami T (2011) Male sterility-inducing mitochondrial
575 genomes: how do they differ? *Crit Rev Plant Sci* 30: 378-400
- 576 Kubo T, Newton KJ (2008) Angiosperm mitochondrial genomes and mutations. *Mitochondrion* 8: 5-14
- 577 Kubo T, Nishizawa S, Sugawara A, Itchoda N, Estiati A, et al. (2000) The complete nucleotide sequence of
578 the mitochondrial genome of sugar beet (*Beta vulgaris* L.) reveals a novel gene for tRNA^{Cys}(GCA).
579 *Nucleic Acids Res* 28: 2571-2576
- 580 Kubo T, Satoh Y, Muro T, Kinoshita T, Mikami T (1995) Physical and gene organization of mitochondrial
581 DNA from the fertile cytoplasm of sugarbeet (*Beta vulgaris* L.). *Curr Genet* 28: 235-241
- 582 Kuhn J, Binder S (2002) RT-PCR analysis of 5' and 3'-end-ligated mRNAs identifies the extremities of *cox2*
583 transcripts in pea mitochondria. *Nucleic Acids Res* 30: 439-446
- 584 Lange H, Sement FM, Canaday J, Gagliardi D (2009) Polyadenylation-assisted RNA degradation processes
585 in plants. *Trends Plant Sci* 14: 497-504
- 586 Laser KD, Lersten NR (1972) Anatomy and cytology of microsporogenesis in cytoplasmic male sterile
587 angiosperms. *Bot Rev* 3: 425-454
- 588 Laughnan JR, Gabay-Laughnan S (1983) Cytoplasmic male sterility in maize. *Ann Rev Genet* 17: 27-48
- 589 Levings CS III (1993) Thoughts on cytoplasmic male sterility in *cms-T* maize. *Plant Cell* 5: 1285-1290
- 590 L'Homme Y, Stahl RJ, Li X-Q, Hameed A, Brown GG (1997) *Brassica nap* cytoplasmic male sterility is
591 associated with expression of a mtDNA region containing a chimeric gene similar to the *pol*
592 CMS-associated *orf224* gene. *Curr Genet* 31: 325-335
- 593 Matsunaga M, Nagano H, Mikami T, Kubo T (2011) Large 3' UTR of sugar beet *rps3* is truncated in
594 cytoplasmic male-sterile mitochondria. *Plant Cell Rep* 30: 231-238

- 595 Mikami T, Kishima Y, Sugiura M, Kinoshita T (1985) Organelle genome diversity in sugar-beet with
596 normal and different sources of male sterile cytoplasms. *Theor Appl Genet* 71:166-171
- 597 Moritani M, Taguchi K, Kitazaki K, Matsuhira H, Katsuyama K, et al. (2013) Identification of the
598 predominant nonrestoring allele for Owen-type cytoplasmic male sterility in sugar beet (*Beta vulgaris*
599 L.): development of molecular markers for the maintainer genotype. *Mol Breed* 32: 91-100
- 600 Muller K, Storchova H (2013) Transcription of *atp1* is influenced by both genomic configuration and
601 nuclear background in the highly rearranged mitochondrial genomes of *Silene vulgaris*. *Plant Mol*
602 *Biol* 81: 495-505
- 603 Nishizawa S, Mikami T, Kubo T (2007) Mitochondrial DNA phylogeny of cultivated and wild beets:
604 relationships among cytoplasmic male-sterility-inducing and nonsterilizing cytoplasms. *Genetics*
605 177: 1703-1712
- 606 Onodera Y, Yamamoto MP, Kubo T, Mikami T (1999) Heterogeneity of the *atp6* presequences in normal
607 and different sources of male-sterile cytoplasms of sugar beet. *J Plant Physiol* 155: 656-660
- 608 Owen FV (1942) Male sterility in sugar beets produced by complementary effects of cytoplasmic and
609 mendelian inheritance. *Am J Bot* 29:692
- 610 Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, Second edition. Cold
611 Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- 612 Schnable PS, Wise RP (1998) The molecular basis of cytoplasmic male sterility and fertility restoration.
613 *Trends Plant Sci* 3: 175-180
- 614 Small ID, Isaac PG, Leaver CJ (1987) Stoichiometric differences in DNA molecules containing the *atpA*
615 gene suggest mechanisms for the generation of mitochondrial genome diversity in maize. *EMBO J* 6:
616 865-869
- 617 Touzet P (2012) Mitochondrial genome evolution and gynodioecy. In: L. Marechal-Drouard (ed.).
618 *Mitochondrial Genome Evolution*, Academic Press, Oxford, UK, pp. 71-98
- 619 Yamamoto MP, Kubo T, Mikami T (2005) The 5'-leader sequence of sugar beet mitochondrial *atp6*
620 encodes a novel polypeptide that is characteristic of Owen cytoplasmic male sterility. *Mol Genet*
621 *Genomics* 273: 342-349
- 622 Yamamoto MP, Shinada H, Onodera Y, Komaki C, Mikami T, et al. (2008) A male sterility-associated
623 mitochondrial protein in wild beets causes pollen disruption in transgenic plants. *Plant J* 54:
624 1027-1036
- 625
- 626

627 **Table 1** Male fertility in the F₁ progeny of three CMS sources with a common pollen parent

Cross combination	Number of plants			Total
	Fertile	Partial fertile	Sterile	
I-12CMS(R) x NK-183mm-O#1	0	0	34	34
I-12CMS(2) x NK-183mm-O#1	0	30	8	38
I-12CMS(3) x NK-183mm-O#1	49	1	1	51
I-12CMS(R) x NK-183mm-O#2	0	0	89	89
I-12CMS(2) x NK-183mm-O#2	0	57	5	62
I-12CMS(3) x NK-183mm-O#2	22	15	4	41
I-12CMS(R) x NK-183mm-O#3	0	0	19	19
I-12CMS(2) x NK-183mm-O#3	0	55	0	55
I-12CMS(3) x NK-183mm-O#3	19	4	6	29

628

629

630 **Table 2** Relative accumulation of *orf129* mRNA in I-12CMS(2) and I-12CMS(3) measured by qRT-PCR

Reference gene	Sugar beet line	Relative accumulation of <i>orf129</i> mRNA (\pm SD)	p^1
<i>Act</i>	I-12CMS(2)	1.061 \pm 0.273	0.00029
	I-12CMS(3)	0.390 \pm 0.130	

631 ¹Student's *t*-test.

632

633

634 Figure legends

635 **Fig 1** Physical map of the master chromosome of the I-12CMS(3) mitochondrial genome. The 103
 636 kbp-repeated sequences are the regions marked from 1 to 2 and from 3 to 4. Regions from 2 to 3 and from 4
 637 to 1 do not have features in common. Two recombinogenic sequence-families are indicated by solid lines
 638 (R1) and dashed horizontal lines (R2), respectively. Two of the R1 copies share additional regions of
 639 homology shown by brackets, and another copy of the additional region exists. The stippled region is
 640 deleted in the I-12CMS(2) mitochondrial genome (see Fig. 2). Locations of some mitochondrial genes are
 641 shown. Restriction enzymes are *XhoI* (Xh), *SalI* (Sa), *XbaI* (Xb), and *SmaI* (Sm). The scale bar is shown
 642 below (kbp)

643

644 **Fig. 2** Comparison of the mitochondrial *atp1-rrn26* intergenic region between CMS-E and TK-81mm-O.
 645 Nucleotide residues are numbered according to the DDBJ/EMBL/GenBank accession numbers of
 646 FQ014226 (CMS-E) and BA000009 (TK-81mm-O). Boxed regions with lower case letters indicate
 647 conserved sequences between CMS-E and TK-81mm-O. Regions with upper case letters indicate regions
 648 unique to CMS-E or TK-81mm-O. Internal sequences are omitted, and the length of the unique region is
 649 shown. The repeated sequences in CMS-E (Darracq et al. 2011) are underlined

650

651 **Fig. 3** Expression analysis of *orf129*. Panel a. Immunoblot analysis of total cellular proteins from green
 652 leaves of I-12CMS(R) (R), I-12CMS(2) (2), and I-12CMS(3) (3). Protein samples were electrophoresed in
 653 a 15% SDS-polyacrylamide gel. The blot was probed with anti-ORF129 antiserum or stained with Ponceau
 654 S. The size of the signal bands is shown on the left. Panel b. Immunoblot analysis of total cellular proteins
 655 from I-12CMS(2) and I-12CMS(3) green leaves. The total protein from I-12CMS(2) was diluted as
 656 indicated. The blot was probed with anti-ORF129 antiserum. Panel c. RNA gel blot analysis of total
 657 mitochondrial RNA from I-12CMS(2) and I-12CMS(3) leaves. The blots were probed with *orf129*- or
 658 *atp9*-specific probes. Sizes of the signal bands are shown on the left

659

660 **Fig. 4** Mapping transcript termini onto the *orf129* locus. Panel a. Organization of the *orf129* locus. The
 661 protein coding region is indicated by a box. The direction of transcription is from left to right. Vertical
 662 arrows indicate mapped transcript termini. Those above the horizontal line are from I-12CMS(3) and below
 663 are from I-12CMS(2). Numbers in parentheses indicate the number of cDNA clones representing the
 664 termini if the number of clones is more than one (see Table S2). Panel b. Alignment of nucleotide sequences
 665 in the region from -490 to -472 in I-12CMS(3) and I-12CMS(2). Lower case letters indicate alterations in
 666 nucleotide residues. Nonanucleotide motifs that resemble typical mitochondrial promoters are underlined.
 667 Transcript termini are indicated as in panel a

668

669 **Fig. 5** Expression analysis of N-terminal extension of *atp6*. Total cellular proteins from TK-81mm-O (lane
 670 1), TK-81mm-CMS (lane 2), I-12CMS(2) (lane 3), I-12CMS(3) (lane 4) and I-12CMS(R) (lane 5) green
 671 leaves were electrophoresed in a 12% SDS polyacrylamide gel. The blot was probed with anti-preSATP6
 672 antiserum (a and b) or stained with Ponceau S (c). Exposure time to X-ray film was 1min (a) and 30min (b).
 673 Size markers are shown on the left (kDa)

674

675 **Fig. 6** Heteroplasmy in the mitochondrial *atp1-rrn26* intergenic region of I-12CMS(3). Panel a.
 676 Organization of the *atp1-rrn26* intergenic region in I-12CMS(3) and I-12CMS(2). Boxes indicate
 677 conserved regions between the two sugar beet lines. Solid and dashed lines indicate regions unique to
 678 I-12CMS(3) and I-12CMS(2), respectively. See also Fig. 2. Positions of PCR primers are shown by
 679 triangles. Panel b. Negative images of gels after electrophoresis of PCR products. Total cellular DNAs of
 680 four I-12CMS(2) plants (lanes 1 to 4) and two I-12CMS(3) plants (lanes 5 and 6) were subjected to PCR
 681 analysis using two primer combinations (6-1 and 6-3, and 6-2 and 6-3). Lane 7 is a mock reaction in which
 682 template DNA was omitted. The PCR protocol was: 94°C for 2min; and 35 cycles of 94°C for 30sec, 55°C
 683 for 30sec, and 68°C for 30sec. The sizes of signal bands are shown on the left (kbp)

684

685 **Fig. 7** Heteroplasmy at the mitochondrial *orf129* locus of I-12CMS(3). Panel a. Organization of the *orf129*
 686 locus. The open box indicates the *orf129*-coding region. The direction of transcription is shown by an arrow.
 687 A single nucleotide polymorphism in the upstream of *orf129* is guanine (G) or thymine (T) in I-12CMS(3)
 688 or I-12CMS(2), respectively. Positions of PCR primers are shown by triangles. Note that the 3' termini of
 689 primer 7-1 and primer 7-2 are T and G, respectively. Panel b. Negative images of gels after electrophoresis
 690 of PCR products. Total cellular DNAs of four I-12CMS(2) plants (lanes 1 to 4) and two I-12CMS(3) plants
 691 (lanes 5 and 6) were subjected to PCR analysis using two primer combinations (7-1 and 7-3, and 7-2 and
 692 7-3). Lane 7 is a control experiment in which PCR product from I-12CMS(3) total cellular DNA with
 693 primers 7-2 and 7-4 was used as template. The PCR protocol was: 95°C for 3min; and 30 cycles of 95°C for
 694 30sec, 64°C for 30sec, and 72°C for 30sec. The sizes of signal bands are shown on the left (kbp)

695

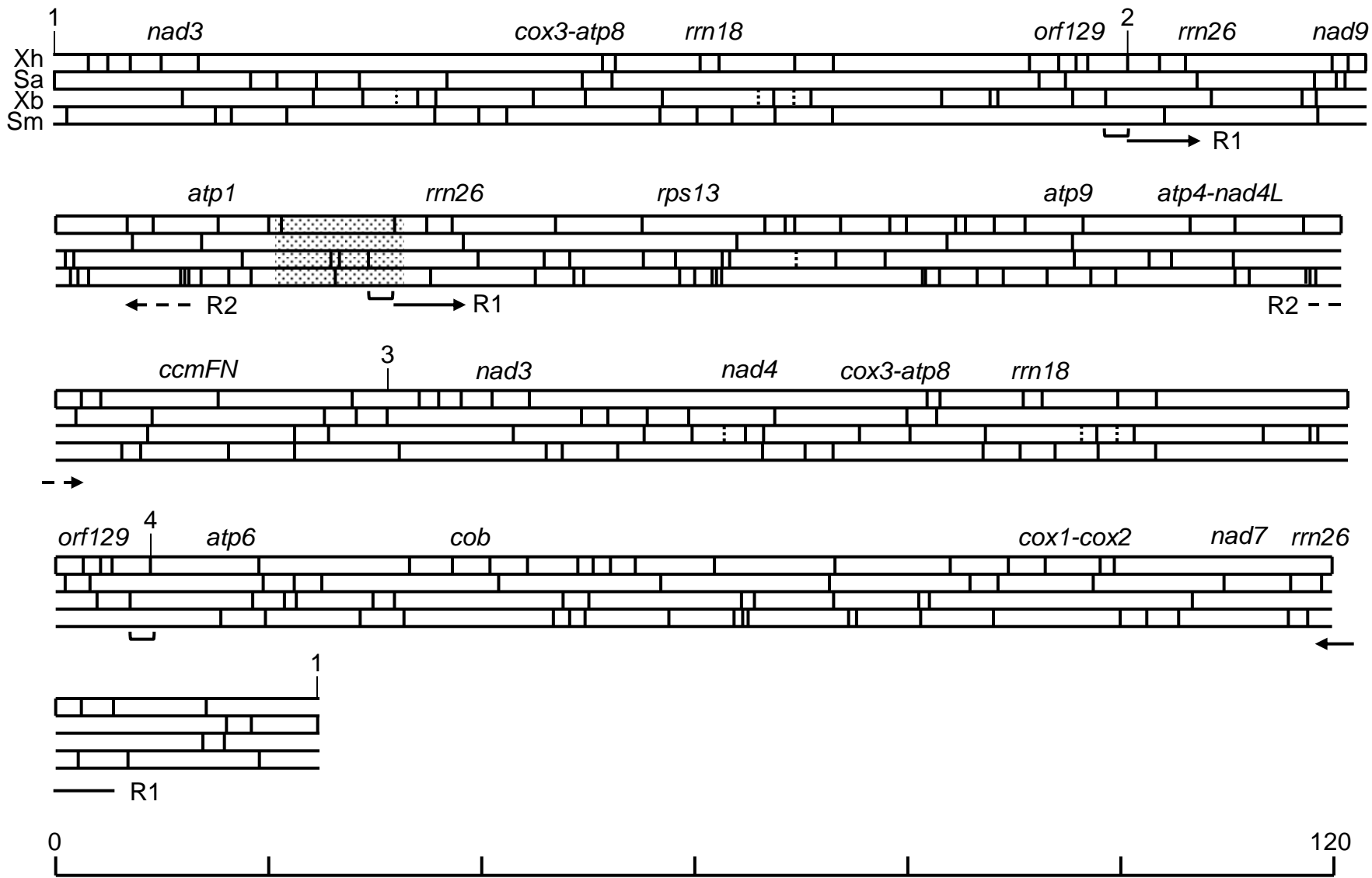
696 **Fig. 8** Mutant allele-specific PCR amplification of the *atp6* locus. Panel a. Organization of the *atp6* locus.
 697 The open box indicates the *atp6*-coding region. The direction of transcription is shown by an arrow. A
 698 single nucleotide polymorphism at nucleotide +177 (the first coding nucleotide is defined as +1) is cytosine
 699 (C) or adenine (A) in I-12CMS(3) or I-12CMS(2), respectively. Positions of PCR primers are shown by
 700 triangles. Note that the 3' termini of primer 8-1 and primer 8-2 are C and A, respectively. Panel b. Negative
 701 images of gels after electrophoresis of PCR products. Total cellular DNAs of four I-12CMS(2) plants (lanes
 702 1 to 4) and two I-12CMS(3) plants (lanes 5 and 6) were subjected to PCR analysis using two primer
 703 combinations (8-1 and 8-3, and 8-2 and 8-3). Lane 7 is a mock reaction in which template DNA was omitted.

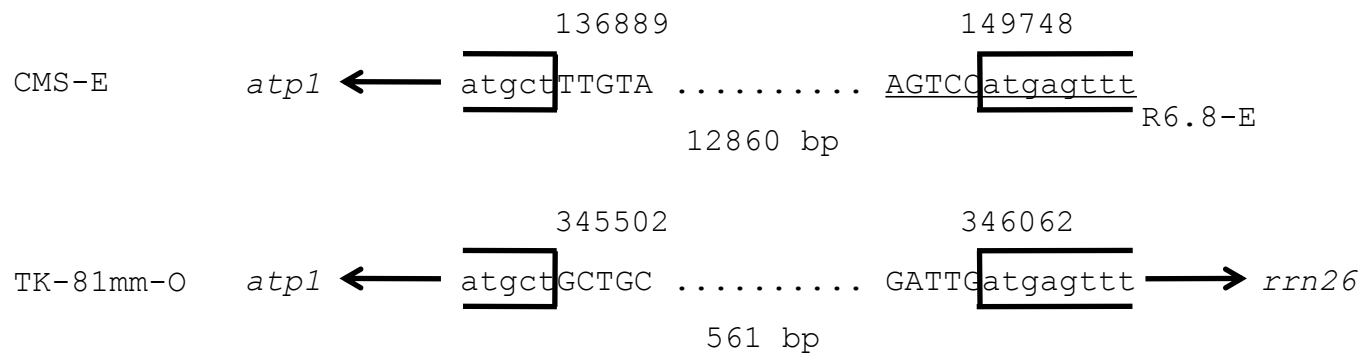
704 The PCR protocol was: 95°C for 3min; and 30 cycles of 95°C for 30sec, 60°C for 30sec, and 72°C for 30sec.

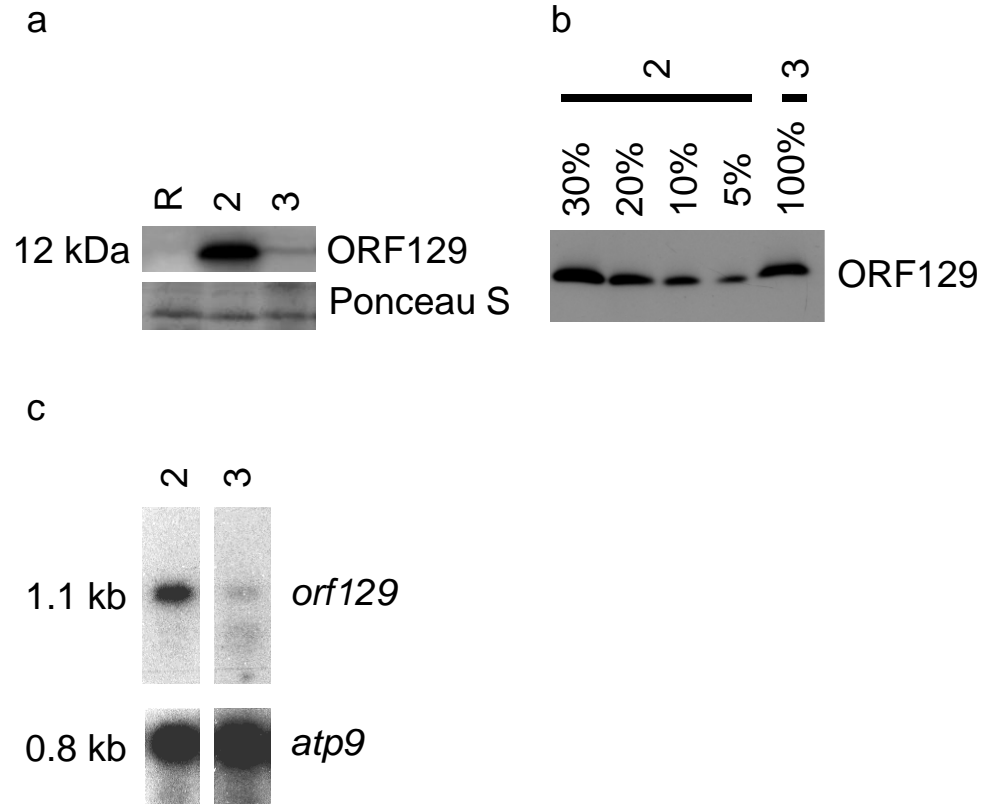
705 The sizes of signal bands are shown on the left (kbp)

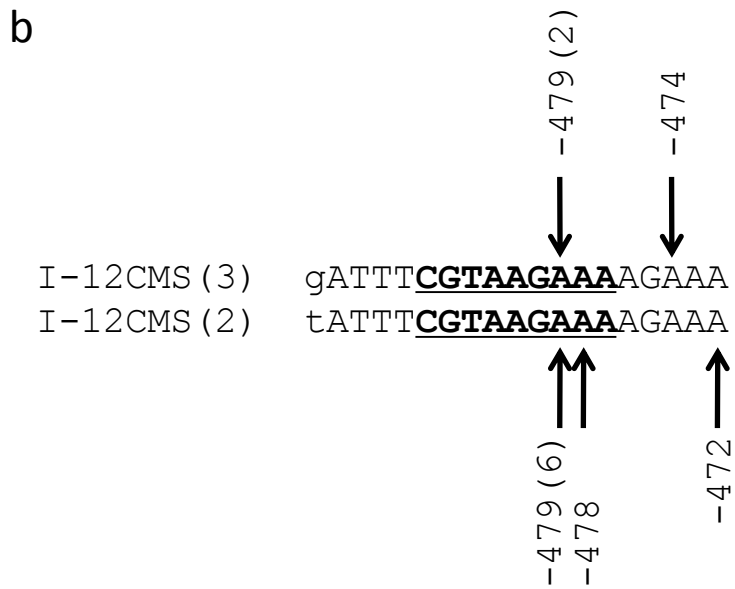
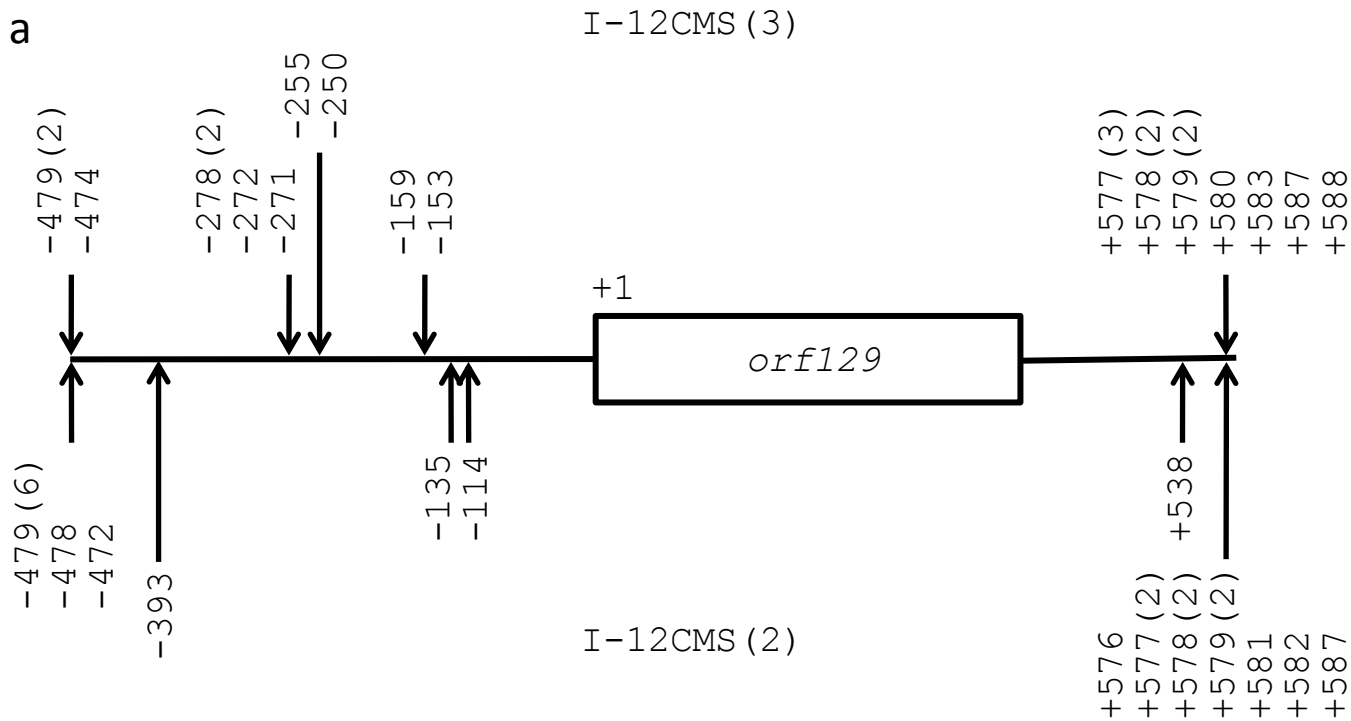
706

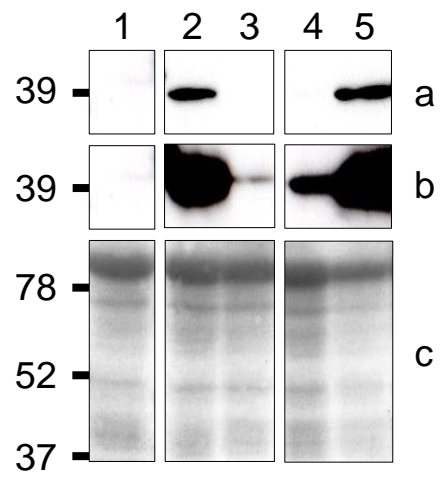
707 Fig. 9 Evolutionary model for the emergence of I-12CMS(2) mitochondria from I-12CMS(3) mitochondria
708 through a possible intermediate. Circles represent mitochondrial genomes: those present as predominant
709 DNA molecules and those present as substoichiometric DNA molecules are shown by solid lines and dotted
710 lines, respectively. Three loci are shown on the circles but the position and the copy number of the loci does
711 not exactly correspond to the organization of the mitochondrial master chromosome shown in Fig. 1. At the
712 *atp6-rrn26* loci, large and small rectangles denote the 12860-bp sequence and the 561-bp sequence,
713 respectively. At the *orf129* loci, the nucleotide sequence polymorphism at nucleotide -490 (G or T) is
714 shown. At the *atp6* loci, the nucleotide sequence polymorphism at nucleotide +177 (C or A) is shown. In
715 I-12CMS(3) mitochondria, two types of mitochondrial DNA molecules coexist, one being at
716 substoichiometric levels. Note that the *atp6* allele of this substoichiometric DNA molecule is unknown
717 (shown by '?'). This substoichiometric DNA molecule evolved into an intermediate molecule with an A
718 residue at nucleotide +177 in *atp6* (this intermediate molecule has not been found and is still an assumption).
719 The evolved intermediate (substoichiometric) DNA molecule eventually predominated in I-12CMS(2).
720 Because no substoichiometric DNA molecule with the 12860-bp sequence at *atp1-rrn26*, G at nucleotide
721 -490 of *orf129*, or C at nucleotide +177 of *atp6* was found in I-12CMS(2), such substoichiometric DNA
722 molecules are missing from I-12CMS(2) mitochondria in this model

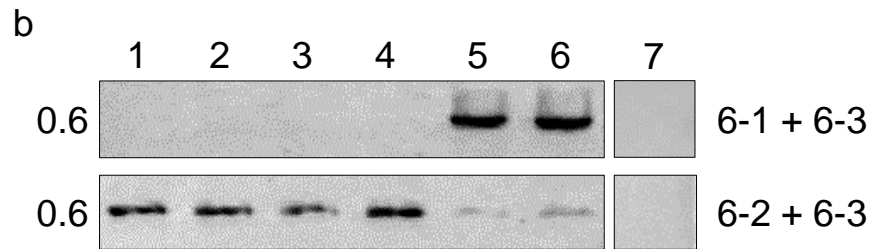
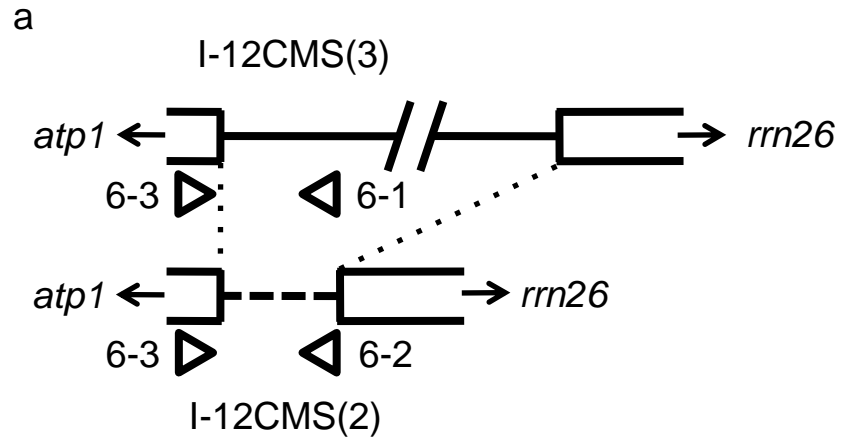


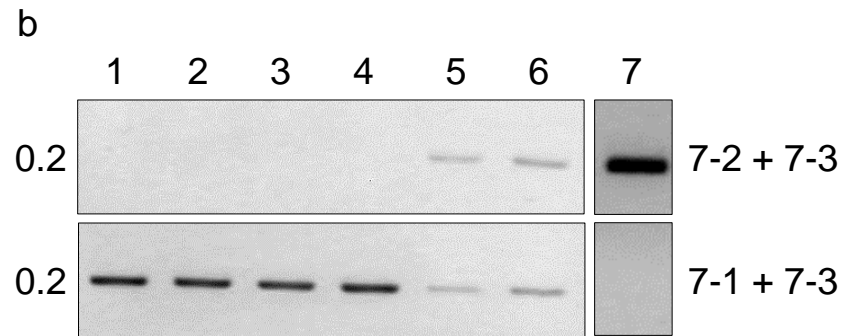
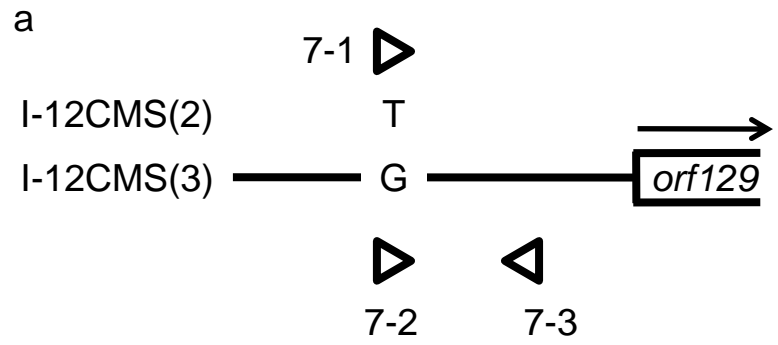








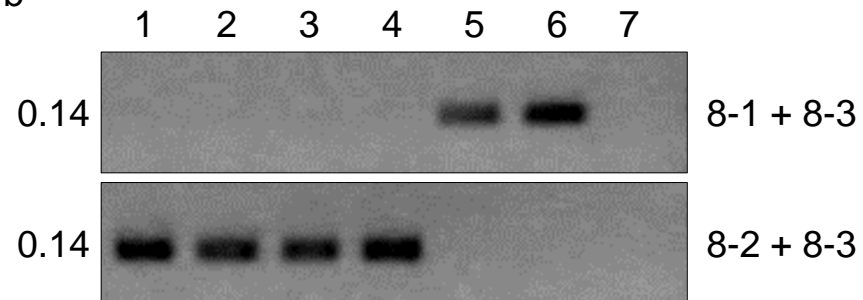


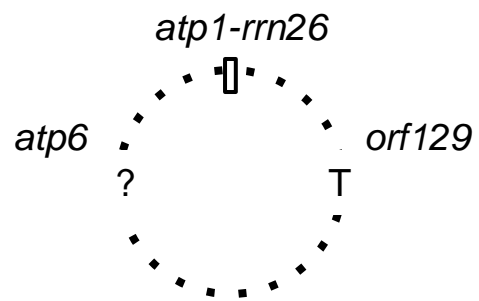
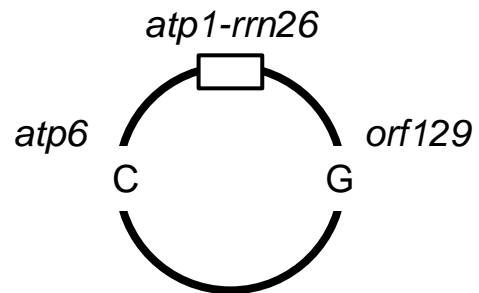


a

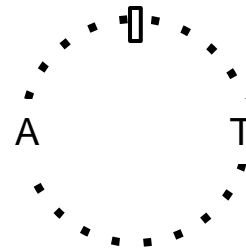
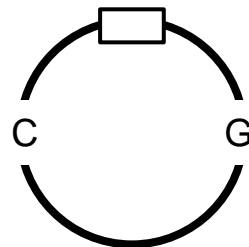


b

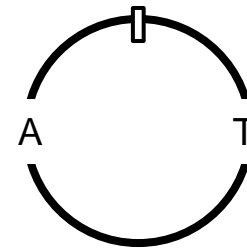




I-12CMS(3)



Intermediate



I-12CMS(2)

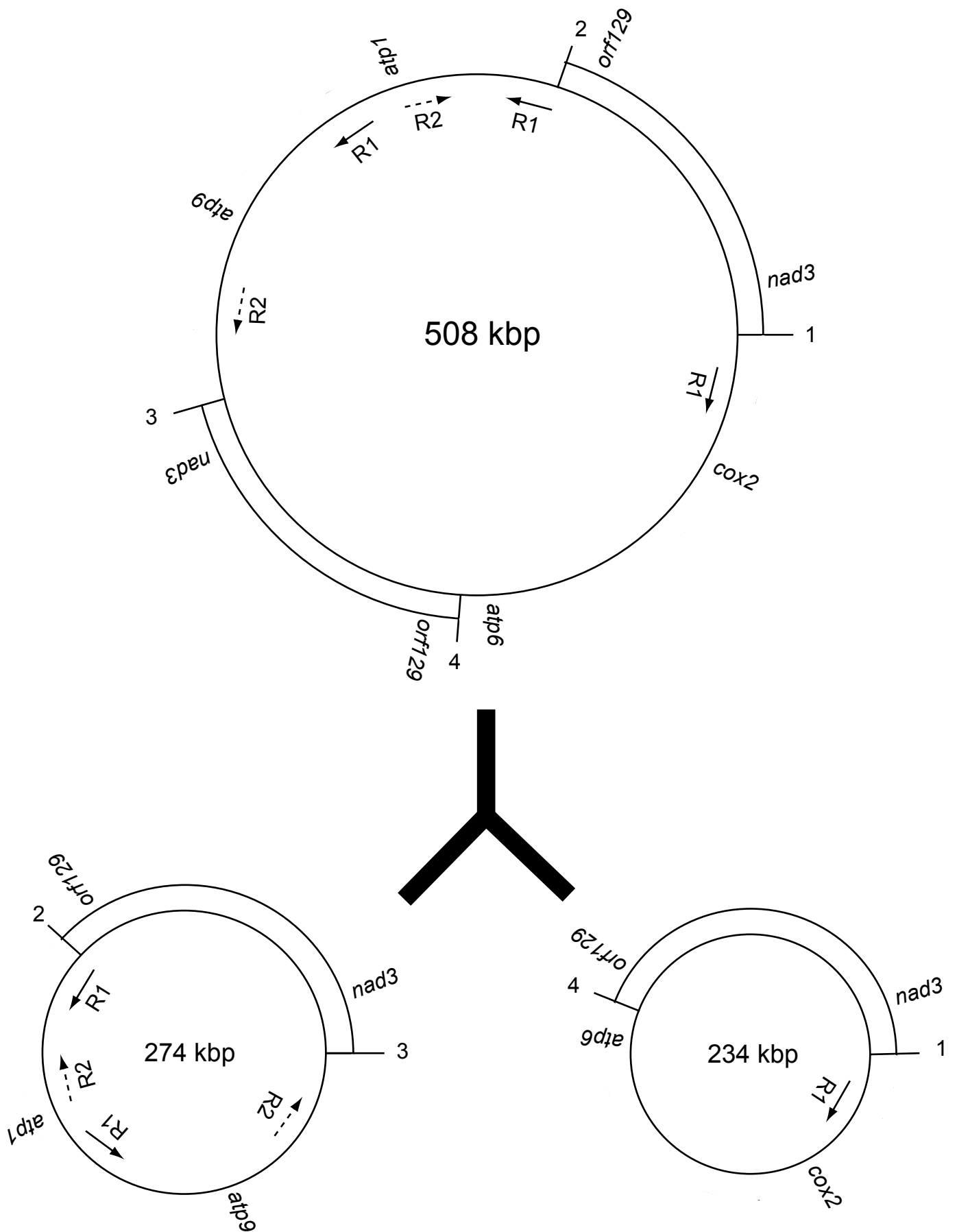


Fig. S1 Interconversion of the 508-kbp master chromosome of I-12CMS(3) mitochondria into two circular molecules of 274- and 234-kbp. Points indicated by 1 to 4 correspond to those in Fig. 1. The 103-kbp repeated sequences are boxed. Two recombinogenic sequence families are indicated by solid lines (R1) and dashed lines (R2), respectively. The locations of some mitochondrial genes are indicated.

GGATCCCGCAAACGGAGACATGAACCAAGCAGGAAGAGGAATTGGAGCAGCTCTTTGCCT -1079
TATGTTATGATCTCATAACACCCGAAAGATTAGACCCCTTTTTTTTTCCTTTCTTTTCAGGATG -1019
AAAACACAGGGCAGGAGGGTATAGAAAGTCTCTATATGCCGTCCATAGTCCATCGACGC -959
CTCGTAGTACCACTTACAGTACTCGCAGAGTGATTGAGTCCAAGATTTTAACCTCAATCTT -899
CTCAACGAGGAAAGAAGCATCGTCTGTCGAGCTGGCGAGAGAAATCCCTCTATAAGTCTCTC -839
TACGTAACCCCAATCGGGCTTACAGCTCTCAACTAACATCATAACCAATCTTATAACAGCT -779
TACCAGAAAACCTCTGGAAAACCTAACCCAGACTGAGAAAAGGCATAGGACGGATACCACC -719
TGGAGAGAGGGGCGACAAGATGTGCCGAAACCCGTGACGATTGTAATTTGGGTTAATGTT -659
ACAAGGCTTACAGGAAGACCTCCTAACCCCTCTTAATCTCAGAGAAGTCTGAATGTTATT -599
ACACCCATTTGCTTTGCTTCATCACCGGCATTTTAAAGCCACAGCGAACCAAGCTGACC -539
-490
G
TACCTCTATCACTAAACTCTACCCCTGGGCTCTTTTACCTCTAAAAAGATATTTTCGTAAGA -479
AAAGAAAGTGCTTTTTTCTAGGGTAGATGAAAGGAATGCCGAAAGGCTATCCGAGTTCAC -419
AGGCGTAGTTGCGTGTACTTCATTTCAATTGAAATTGAGTTCGTTACCGCACCGTGGGTAG -359
AAGATCCTTCTCTTTCTCTGTGAAGATAGGCTGTGCTTTGCATGTCAAGTGCGAAATTGG -299
ATCGAAAAATTGCGTATGATATGAAAAGTTTATGGTCTATCTAAGACTCTCTTTTGATATC -239
AATCACTAAACTAAAGAAAAGTAAAATTTGAAAAATCATAAGATAAGAGAAGAAAGAAGAG -179
TATCTTTTCGTTGTAGCCATAAGAAGCTCGACCCAAGCAATTCCTATGCCCCAAAACCTCCA -119
TGTTTCCGGATCATTTGGTTAAGAACCAACCGGCAATTTATGTCTTCCCTGAATTTGGGAGA -59
GCAAGAATCAGTCTTCTTCATTTCTCGAGAGAGCAGAGCAGTCAAAGAATGAACCAAT +2
M
GAATCCTTATATTTCTGCAGTTTTTTGGCCGACATGGCCACAGCAATTTTAACGATTGCAGG +62
N P Y I L Q F L A D M A T A I L T I A G
AGTTGCATATCTACCACTTATTGTGTTAATTGTATTTAGGGCCGGCGGCCTTCGAGGGTT +122
V A Y L P L I V L I V F R A G G L R G L
AAATGAAGAGAATGCAAGCGAAAGACTCTTAGACCTTTGTTGTGATACGCTGAAGGAACA +182
N E E N A S E R L L D L C C D T L K E Q
GATCAAAAACAAAATGAAGAGTTACTCCAGTTTTATTATAATAATTCGTACCTTTGCC +242
I K N K I E E L L Q V Y Y N N S V P L P
GTCTGGTCCGAGGATTCGAAGACGCAGCAGCCTTCCCTCCATCAGGATTCAGAATCCTTGGA +302
S G R R I Q D A A A F L H Q D S E S L E
ACAACGTCTAATGATTTTGAAAAATATGACCGAATTTGGGCGTTCAAAGTCAAGAATTTTT +362
Q L L M I L K N M T E L G V Q S Q E F L
ACAAGTTTTACTCTATCTCTCACAGTGAAGATAGATGGAGTCTTTGCACTACAATGGATT +422
Q V L L Y L S Q *
TGGCGGGAGTACTTCTTTATTATAAGCGAGGACCGCCTTTTATGTTACTCGTCGTTTGGC +482
TTGAATCTCGTTCCTTAGGAGGCTCGCAAGTTTTTAAGAAGTTACATTGGAAAAAGGCT +542
CCTCCCCGAACATGCCTTCGGTGAAGGTGGGGTTCGTC AAT AAG A ACGAGGCCCGCTCTT +602
TGGGCGGGGAAAGGAAGTGGGTCCCTTTTCGCTTTATTTTCAGTTTAGGTCGATCAGAAGG +662
CGCTGGAACCTGCTTGTTCACAGTTGATAATCGGATATAAGCGGATAGCTAATCCCTCTT +722
TCAACGAGAGTTGCGGACCTCTCTTACTTACTCTTAGTGCTGTGCGAGCCTCGGTTTCA +782
TTTCTTCTTCTAAGGCAGCTAGCTAGGCCTCTTGAGAGCTCAGGATTGGACGGAACCTT +842
ACGGTTGGACTATCGAACAGCTGTTGGTGGGGAATAATCTCCTTTCCCTTGATTGCTGCG +902
GGCCTTCATTGCCGAAACTGGACTGGCTAGGATCACTAGAGGGGACAGACTACCAGGACT +962
AGTCCCGCTTCCCAATAGGAAGAGCAAACAGCAACTATTAGTTTCAGCTTTCCGGGCTCTCA +1022
TAACAGCCCTGGTTTTGAGCATCTCCCTAGCCTACCTACTTTTCAGAAGGTCAAGTCTAATA +1082
GAGTAGAGGCAATCCACTCGAG +1104

Fig. S2 Nucleotide sequence of *orf129* from I-12CMS(2). Nucleotide residues are numbered from the first residue of the putative initiation codon of *orf129*. The deduced amino acid sequence of the translation product is shown below the nucleotide sequence. The only nucleotide alteration in the corresponding region of I-12CMS(3) is shown above the nucleotide sequence. Mapped transcript termini are highlighted in red.

I-12CMS-2 AAGCTTGTTTTCAACTTTTCTATAAATTGCTGCTTTTGGCTTCTAGCTAGTTC -282
 I-12CMS-3 AAGCTTGTTTTCAACTTTTCTATAAATTGCTGCTTTTGGCTTCTAGCTAGTTC -290

I-12CMS-2 AGTGGATAAGATGGCTGCGCTCCAGCTCACTCCTGACTCTTAAAAAGGGA -232
 I-12CMS-3 AGTGGATAAGATGGCTGCGCTCCAGCTCACTCCTGACTCTTAAAAAGGGA -240

I-12CMS-2 GGATTCACGGGTTTCAATAGATAATATCGTAAGTAAGAAAGAACGTTTTTC -182
 I-12CMS-3 GGATTCACGGGTTTCAATAGATAATATCGTAAGTAAGAAAGAACGTTTTTC -190

I-12CMS-2 ACTAATTACTTTTCATAGAGAGAGACCCTCCCCACCCAGTATAATGTCCT -132
 I-12CMS-3 ACTAATTACTTTTCATAGAGAGAGACCCTCCCCACCCAGTATAATGTCCT -140

I-12CMS-2 CTCGAAAATCGGATACCTTTTTCGTTTTTCATTTCTCCATTTCTTTCTTGG -82
 I-12CMS-3 CTCGAAAATCGGATACCTTTTTCGTTTTTCATTTCTCCATTTCTTTCTTGG -90

I-12CMS-2 TTGGATCAACCCAACCGGTGATTTCCGACA-----AGTCTTTCTTCA -40
 I-12CMS-3 TTGGATCAACCCAACCGGTGATTTCCGACATTCGACAAGTCTTTCTTCA -40

>atp6

I-12CMS-2 TTTTTGAGCGGACAGCAGAAAGAAAAAAGAAAAAATTTGATGATGACTCA 11
 I-12CMS-3 TTTTTGAGCGGACAGCAGAAAGAAAAAAGATAAATTTTGATGATGACTCA 11

I-12CMS-2 TTGGAAGAAGGAGATAACTGCTTTGGCACAACAGATAATTACTAATGTCC 61
 I-12CMS-3 TTGGAAGAAGGAGATAACTGCTTTGGCAAACCAGATAATTACTAATGTCC 61

I-12CMS-2 CTCGACCTACACGGCGAGTCTTATTCCGACTCGGCGTGCTGTTTCGCCCTC 111
 I-12CMS-3 CTCGACCTACACGGCGAGTCTTATTCCGACTCGGCGTGCTGTTTCGCCCTC 111

I-12CMS-2 TTTTGGGTCGGGAGACGCGGGCTGTTGTTGTTTGAACGGAAATATTCT 161
 I-12CMS-3 TTTGAGGTCGGGAGACACGGGCTGTTGTT---TGAAACGGAAATATTCT 158
 *** *****

I-12CMS-2 ACAAGCCCTCTTTGGAATAGGAACAGCGGCTTCTATTTTGTTTTTTTGTT 211
 I-12CMS-3 ACAAGCCCTCTTTGGCATAGGAACAGCGGCTTCTATTTTGTTTTTTTGTA 208

I-12CMS-2 ATTTCCGCACCCGAGAGCACTTTCTCTATACATTTCTACTAACGATTTAT 261
 I-12CMS-3 ATTTCCGCACCCGAGAGCACTTTCTCTATACATTTCTACTAACGATTTAT 258

I-12CMS-2 TTGGCTTTATTCTTCCCTAAAGAGGGCTTTCCCTCTTTTGGAAATTCGCCTT 311
 I-12CMS-3 TTGGCTTTATTCTTCCCTAAAGAGGGCTTTCCCCCTTTTGGAAATTCGCCTT 308

I-12CMS-2 TTTGGCTATATTGATCTTTTCGGTTTGCTTCTTTCCGAAAAATGGACGAT 361
 I-12CMS-3 TTTGGCTATATTGATCTTTTCGGTTTGCTTCTTTCCCTAAAAATGGACGAT 358

I-12CMS-2 TACGGCATTTCGCTAGAGAACTGTTCTTTTATTTTACTACTCCTTTGGGGG 411
 I-12CMS-3 TACGGCATTTCGCTAGAGAACTGTTCTTTTATTTTCTACTCCTTTGGGGG 408

I-12CMS-2 AGTTACCGGCTTTTGGAGACTCCGTGGCACGAATGGCCACTCTATGTTTG 461
 I-12CMS-3 AGTTACCGGCTTTTGGAGACTCCGTGGCACGAATGGCCACTCTATGTTTG 458

I-12CMS-2 TGGTCTCTTAATGTTATTTCACGCTTCTTCAAGCGCTGGTCCATCGAAAGG 511
 I-12CMS-3 TGGTCTCTTAATGTTATTTCACGCTTCTTCAAGCGCTGGTCCATCGAAAGG 508

I-12CMS-2 ACGAAGGGGTCGGCAAGTTTATTCTGCCGGCTACCTTCCTAATTTTAATG 561
I-12CMS-3 ACGAAGGGGTCGGCAAGTTTATTCTGCCGGCTACCTTCCTAATTTTAATG 558

I-12CMS-2 AGTGCCCTCGTGCTCCTTGGGCTTCACTCGGGGCGATTACATAATCTGCC 611
I-12CMS-3 AGTGCCCTCGTGCTCCTTGGGCTTCACTCAGGGGCGATTACATAATCTACC 608

I-12CMS-2 GGAAAAGGCGGCCCTAACGGGCGCCGAGTCTTAATACTCTTTAGCCTAG 661
I-12CMS-3 GGAAAAGGCGGCCCTAACGGGCGCCGAGTCTTAATAATCTTTAGCCAAG 658

I-12CMS-2 CGGCCGCCGAGTCCACACGGGAACGGTGGCTTGCATATGGGGCGTATCTC 711
I-12CMS-3 CGGCCGCCGAGTCCACAAGGGAGCGGTGGCTTGAATTGGGGCGTATCTC 708

I-12CMS-2 ATGTGTATCCCGTGTATACTATATCTCTAGGAGGGCCACGGATTGGCC 761
I-12CMS-3 ATGTGTATCCCGTGTATACTATATCTCTAGGAGGGCCACGGATTGGCC 758

I-12CMS-2 CCTATTCGTACAACAAATCGGCTTTTTTTTTATTATCCTCGTCCTAGTAT 811
I-12CMS-3 CCTATTCGTACAACAAATCCGCCTTTTTTTTTTATCCTCGGCCTAGTAT 808

I-12CMS-2 TCG---TTTTTTGGGTTGTTACTTATGAGGACGTAAAAGAGCAGAAGAAT 858
I-12CMS-3 TCGGTTTTTTTTGGGTTGTTACTTATGAGGACGTAAAAGAGCAGAAGGAT 858
*** *****

I-12CMS-2 CTGCTCCCAGATCCTATCTGCGATCTTCTCCGCTTTGGCGTCGGCGAGGTG 908
I-12CMS-3 CTGCTCCCAGATCCTATCTGCGATCTTCTCCACTTTGGCGTCGGCGAGGTG 908

I-12CMS-2 TGGCCCCGGCGTCTGGGTAAAAGAGTGGCTCGCCGCCTTTTCCTTCACCA 958
I-12CMS-3 TGGCCCCGGCGTCTGGGTAAAAGAGTGGCTCGCCGCCTTTTCCTTCACCA 958

I-12CMS-2 TTGCGGCGTATTTGTTATTTGAGAAGGTAGGCTACCGCAAAGATACCCTT 1008
I-12CMS-3 TTGGGGCGTATTTTTTATTTGAGAAGGTAGGCTACCGCAAAGATACCCTT 1008
*** *****

I-12CMS-2 CAAGAAACGTCCCGTACTTGGGAAGCTTGGTTTTGCTTTTTTGCTGTTCT 1058
I-12CMS-3 CAAGAAACGTCCCGTACTTGGGAAGCTTGGTTTTGCTTTTTTGCTGTTCT 1058

I-12CMS-2 TTCTTTTGGTGTGCACTTAGTTTTTTTCATGCATCACCCGCCCCAGGGG 1108
I-12CMS-3 TTCTTTTGGTGTGCACTTAGTTTTTTTCATGCATCACCCGCCCCAGGGG 1108

I-12CMS-2 TCGGAAC TGAAGTGAAGCTTTTCACGATGCGATTAATCCAGCAGCGGGA 1158
I-12CMS-3 TCGGAAC TGAAGTGAAGCTTTTCACGATGCGATTAATCCAGCAGCGGGA 1158

I-12CMS-2 CGGATCCCCAGCCCCTAGAGCAATTTTCCATTCTCCATTGATTCCCTAT 1208
I-12CMS-3 CGGATCCCCAGCCCCTAGAGCAATTTTCCATTCTCCATTGATTCCCTAT 1208

I-12CMS-2 GAAAATAGGAAACTTGTATTTCTCATTACAAATCCATCTTTGTTTATGC 1258
I-12CMS-3 GAAAATAGGAAACTTGTATTTCTCATTACAAATCCATCTTTGTTTATGC 1258

I-12CMS-2 TGCTAACTCTCAGTTTGGTCCTACTTCTGCTTCATTTTGTACTAAAAAG 1308
I-12CMS-3 TGCTAACTCTCAGTTTGGTCCTACTTCTGCTTCATTTTGTACTAAAAAG 1308

I-12CMS-2 GGAGGAGGAAACTCAGTACCAAATGTTTGGCAATCCTTGGTAGAGCTTAT 1358
I-12CMS-3 GGAGGAGGAAACTCAGTACCAAATGTTTGGCAATCCTTGGTAGAGCTTAT 1358

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I-12CMS-2 TTATGATTTTCGTGCTGAACCTGGTAAACGAACAAATAGGTGGTCTTTCCG 1408
I-12CMS-3 TTATGATTTTCGTGCTGAACCTGGTAAACGAACAAATAGGTGGTCTTTCCG 1408
*****

I-12CMS-2 GAAATGTGAAACAAAAGTTTTTCCCTTGCATCTTGGTCACTTTTACTTTT 1458
I-12CMS-3 GAAATGTGAAACAAAAGTTTTTCCCTTGCATCTTGGTCACTTTTACTTTT 1458
*****

I-12CMS-2 TTGTTATTTTCGTAATCTCCAGGGTATGATACCCTATAGCTTTACAGTTAC 1508
I-12CMS-3 TTGTTATTTTCGTAATCTCCAGGGTATGATACCCTATAGCTTTACAGTTAC 1508
*****

I-12CMS-2 AAGTCATTTTCTCATTACTTTGGGTCTTTCATTTTCCATTTTATTGGCA 1558
I-12CMS-3 AAGTCATTTTCTCATTACTTTGGGTCTTTCATTTTCCATTTTATTGGCA 1558
*****

I-12CMS-2 TTACTATAGTGGGATTTCAAAGAAATGGGCTTCATTTTTTAAGCTTCTCA 1608
I-12CMS-3 TTACTATAGTGGGATTTCAAAGAAATGGGCTTCATTTTTTAAGCTTCTCA 1608
*****

I-12CMS-2 TTACCTGCAGGAGTCCCGCTGCCGTTAGCACCTTTTTTAGTACTCCTTGA 1658
I-12CMS-3 TTACCTGCAGGAGTCCCGCTGCCGTTAGCACCTTTTTTAGTACTCCTTGA 1658
*****

I-12CMS-2 GCTAATCCCTCATTGTTTTTCGCGCATTAAAGCTCAGGAATACGTTTATTTG 1708
I-12CMS-3 GCTAATCCCTCATTGTTTTTCGCGCATTAAAGCTCAGGAATACGTTTATTTG 1708
*****

I-12CMS-2 CTAATATGATGGCCGGTCATAGTTCAGTAAAGATTTTAAGTGGGTTCGCT 1758
I-12CMS-3 CTAATATGATGGCCGGTCATAGTTCAGTAAAGATTTTAAGTGGGTTCGCT 1758
*****

I-12CMS-2 TGGACTATGCTATGTATGAATGATCTTTTATATTTTCATAGGAGATCTTGG 1808
I-12CMS-3 TGGACTATGCTATGTATGAATGATCTTTTATATTTTCATAGGAGATCTTGG 1808
*****

I-12CMS-2 TCCTTTATTTATAGTTCTTGCATTAACCGGTCTTGAATTAGGTGTAGCTA 1858
I-12CMS-3 TCCTTTATTTATAGTTCTTGCATTAACCGGTCTTGAATTAGGTGTAGCTA 1858
*****

I-12CMS-2 TATTACAAGCTCATGTTTTTACGATCTTAATCTGTATTTACTTGAATGAT 1908
I-12CMS-3 TATTACAAGCTCATGTTTTTACGATCTTAATCTGTATTTACTTGAATGAT 1908
*****

I-12CMS-2 GCTACAAATCTCCATCAAATTCCTTTTTCTTTTTATTAGAATTTTATA 1958
I-12CMS-3 GCTACAAATCTCCATCAAATTCCTTTTTCTTTTTATTAGAATTTTATA 1958
*****

I-12CMS-2 ATT 1961
I-12CMS-3 ATT 1961
***

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Fig. S3 Comparison of nucleotide sequences derived from I-12CMS(2) and I-12CMS(3) *atp6* loci. Dashes are introduced for maximum matching. Matched residues are marked by asterisks. Nucleotide residues are numbered from the underlined translation initiation codons. The termination codons are double-underlined. Sources of sequence data are DDBJ/EMBL/GenBank accession number AB015177 and this study. Nucleotide sequences were aligned using ClustalW (<http://clustalw.ddbj.nig.ac.jp/index.php?lang=ja>).

```

I-12CMS-2 MMTHWKKEITALAQIIITNVPRPTRRVLFGLGVLFALFWVGRRLLLFETKIILQALFGI 60
I-12CMS-3 MMTHWKKEITALANQIIITNVPRPTRRVLFGLGVLFTLFEVGRHG-LLFETEIILQALFGI 59
*****

I-12CMS-2 GTAASILFFCYFRTREHFLYTFLLTIYLALFFLKRAFPLLEFAFLAILIFSVCFFPKNGR 120
I-12CMS-3 GTAASILFFCNFRTREHFLYTFLLTIYLALFFLKRAFPLLEFAFLAILIFSVCFFPKNGR 119
*****

I-12CMS-2 LRHSLENCSFILLLLWGSYRLLETPWHEWPLYVCGLLMLFTLLQALVHRKDEGVGKFILP 180
I-12CMS-3 LRHSLENCSFIFLLLWGSYRLLETPWHEWPLYVCGLLMLFTLLQALVHRKDEGVGKFILP 179
*****

I-12CMS-2 ATFLILMSALVLLGLHSGAIHNLPEKAALTGAAVLILFSLAAAESTRERWLAYGAYLMCI 240
I-12CMS-3 ATFLILMSALVLLGLHSGAIHNLPEKAALTGAAVLIIFSQAAAESTRERWLAIGAYLMCI 239
*****

I-12CMS-2 PCILYLLGGATDWPLFVQQIGFFFIILVLVF-VFVWVVTYEDVKEQKNLLPILSAIFSALA 299
I-12CMS-3 PCILYILGGATDWPLFVQQIRLFFFILGLVFGFFVWVVTYEDVKEQKDLLPILSAIFSTLA 299
*****

I-12CMS-2 SARCGPGVWVKEWLAAFSFTIAAYLLFEKVGYRKDTLQETSRTWEAWFCFFAVLSSFGVAL 359
I-12CMS-3 SARCGPGVWVKEWLAGFSFTIGAYFLFEKVGYRKDTLQETSRTWEAWFCFFAVLSSFGVAL 359
*****

presequence/core region
I-12CMS-2 SFFHASPAPGVGTESEAFHDAINPAAGRIPSPLEQFSILPLIPMKIGNLYFSFTNPSLFM 419
I-12CMS-3 SFFHASPAPGVGTESEAFHDAINPAAGRIPSPLEQFSILPLIPMKIGNLYFSFTNPSLFM 419
*****

I-12CMS-2 LLTSLVLLLLHFVTKKGGGNSVPNVQSLVELIYDFVLNLVNEQIGGLSGNVKQKFFPC 479
I-12CMS-3 LLTSLVLLLLHFVTKKGGGNSVPNVQSLVELIYDFVLNLVNEQIGGLSGNVKQKFFPC 479
*****

I-12CMS-2 ILVTFTFLLFRNLQGMIPYSFTVTSHFLITLGLSFSIFIGITIVGFQRNGLHFLSSLPA 539
I-12CMS-3 ILVTFTFLLFRNLQGMIPYSFTVTSHFLITLGLSFSIFIGITIVGFQRNGLHFLSSLPA 539
*****

I-12CMS-2 GVPLPLAPFLVLLELIPHCFRALSSGIRLFANMMAGHSSVKILSGFAWTMLCMNDLLYFI 599
I-12CMS-3 GVPLPLAPFLVLLELIPHCFRALSSGIRLFANMMAGHSSVKILSGFAWTMLCMNDLLYFI 599
*****

I-12CMS-2 GDLGPLFIVLALTGLELGVAILQAHVFTILICIYLNDATNLHQNSFFFLLEFL 652
I-12CMS-3 GDLGPLFIVLALTGLELGVAILQAHVFTILICIYLNDATNLHQNSFFFLLEFL 652
*****

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Fig. S4 Alignment of deduced amino acid sequences of I-12CMS(2) and I-12CMS(3) ATP6. Dashes are introduced for maximum matching. Matched residues are marked by asterisks. The junction between the N-terminal extension and the core region is shown by a slashed line. Antigenic regions of α preS_{ATP6} that are conserved in I-12CMS(2) and I-12CMS(3) are underlined.

Table S1 Nucleotide sequences of primers used in this study

Name of primer	Nucleotide sequence
orf129-Fw	5'-TTGTTGTGATACGCTGAAGGAA-3'
orf129-Rv	5'-CCAGACGGCAAAGGTACAGA-3'
actin-Fw	5'-AGACCTTCAATGTGCCTGCT-3'
actin-Rv	5'-ACGACCAGCAAGATCCAAAC-3'
orf129-162R	5'-ACA AAGGTCTAAGAGTCTTTCGCTTGCA-3'
orf129-54R	5'-CGTTAAAATTGCTGTGGCCATGTC-3'
orf129-339F	5'-GGGCGTTCAAAGTCAAGAATTTTTACAAG-3'
6-1	5'-TATCACCACCAACAAAGTATAAGTCAT-3'
6-2	5'-CAATCACATCTAGCTTCTTCTGAGATAT-3'
6-3	5'-GACTTGAAAGCCTTCTTCCCGAAA-3'
7-1	5'-GGCTCTTTTACCTCTAAAAGAT-3'
7-2	5'-TCTACCCTGGGCTCTTTTACCTCTAAAAGAG-3'
7-3	5'-GACATGCAAAGCACAGCCTA-3'
8-1	5'-TCTACAAGCCCTCTTTGGC-3'
8-2	5'-TCTACAAGCCCTCTTTGGA-3'
8-3	5'-CAAAAAGGCGAATTCCAAAA-3'

Table S2 Summary of *orf129*-transcript termini in I-12CMS(2) and I-12CMS(3) by cDNA sequencing.

Sugar beet line	Plasmid clone ID	5' terminus ¹	3' terminus ¹	Additional nucleotides ²
I-12CMS(2)	2-1	-114	+579	
	2-2	-135	+577	
	2-3	-393	+577	
	2-4	-472	+576	
	2-5	-478	+578	AAAAAAAAA
	2-6	-479	+538	
	2-7	-479	+582	TAAA
	2-8	-479	+579	AA
	2-9	-479	+578	CTAA
	2-10	-479	+587	
	2-11	-479	+581	
I-12CMS(3)	3-1	-153	+577	
	3-2	-159	+580	
	3-3	-250	+587	
	3-4	-255	+583	
	3-5	-271	+579	
	3-6	-272	+579	
	3-7	-278	+588	
	3-8	-278	+577	
	3-9	-474	+578	A
	3-10	-479	+578	AA
	3-11	-479	+577	AAA

¹Number corresponds to Fig. S2.

²Nucleotides not encoded by genomic DNA.