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Title: **Large 3´ UTR of sugar beet *rps3* is truncated in cytoplasmic male-sterile mitochondria**

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Abstract

Genomic alteration near or within mitochondrial gene is often associated with cytoplasmic male sterility (CMS). Its influence on the expression of the mitochondrial gene was proposed as one of the possible causes of CMS. In sugar-beet mitochondrial *rps3*, whose downstream 1056-bp region contains *Norf246*, an apparently non-functional open reading frame (ORF), was deleted in CMS mitochondria. In our previous study, normal *rps3* (3.8 kb), CMS *rps3* (2.7 kb), and *Norf246* (3.8 kb and 0.9 kb) were shown to be transcribed. The present study was conducted to determine whether the deletion affected gene expression. Reverse transcription (RT)-PCR analysis revealed the co-transcription of *rps3* and *Norf246*. By circularized RNA (CR) RT-PCR analysis, the 5′ and 3′ termini of the 3.8-kb and the 0.9-kb transcripts were determined. The results suggested that the 3.8-kb transcripts were the *rps3* mRNA bearing an ~464-base 5′ untranslated region (UTR) and ~1508-base 3′ UTR, whereas no functional ORF was observed in the 0.9-kb transcripts. CR-RT-PCR revealed that the 3′ UTR of the 2.7-kb transcripts was reduced to ~460 bases. However, no difference in the accumulation of RPS3 polypeptide and RNA editing was detected by protein gel blot analysis and cDNA sequencing. Although the deleted region encoded the truncated-*atp9* that was edited, no influence on the pattern and frequency of RNA editing of genuine *atp9* was evident. The results eliminated *rps3* as a candidate for the CMS gene, making *preSatp6*, a unique ORF fused with CMS *atp6*, the sole CMS-associated region in sugar beet.

Key words- plant mitochondria, untranslated region, gene expression, RNA editing, chimeric gene, pseudo gene

Introduction

Mitochondrial transcripts maintain an untranslated region (UTR) in their 5′ and 3′ extremities with the exception of metazoans in which a UTR is rarely found (Scheffler 1999). Examples of involvement of mitochondrial UTR with post-transcriptional gene expression processes are known. In yeast, 5′-UTR plays an important role in the initiation of translation (Dunstan et al. 1997; Mittelmeier and Dieckmann 1995). However, it is unclear whether plant mitochondrial 5′ or 3′ UTR is involved in gene expression. The degree of conservation of nucleotide sequences preceding protein-coding genes is low among plant species, especially in genes encoding ATPase subunits (Hazle and Bonen 2007). A very large 5′ UTR was reported

in wheat *ccmC*, where a 5´ UTR occupied more than 70% of the transcript region (Bonnard and Grienenberger 1995). On the other hand, 3´ UTR was missing in the major transcripts of cauliflower *nad6*, *Arabidopsis nad6*, and *Arabidopsis ccmC* (Raczynska et al. 2006; Forner et al. 2007).

Some of alterations in UTR have been associated with cytoplasmic male sterility (CMS), which is a mitochondrial mutation in higher plants that causes pollens to be aborted or be non-functional but otherwise normal (Schnable and Wise 1998; Chase 2007; Fujii and Toriyama 2008). For example, *urf13-T*, which is associated with T-type CMS in maize, is a unique open reading frame (ORF) occurring in the 5´ UTR of genuine *atp4* (formerly referred to as *orf25*) as a result of mitochondrial genome rearrangement (Dewey et al. 1986). Another unique ORF termed *pcf* is associated with petunia CMS, occurring in the 5´ UTR of *nad3* (Young and Hanson 1987). The identified unique ORFs are most likely causal agents of CMS, but the mechanism by which they cause the phenotype is currently unknown. A principal hypothesis is that the unique ORF has a direct effect on mitochondrial function. In anther tissues, translation products of such unique ORFs have been detected (Budar and Berthome 2007), which may cause mitochondrial impairment and lead to male sterility. Another hypothesis is that the unique ORFs themselves may have no direct effect on mitochondrial function, but rather the occurrence of a unique ORF in the UTR may impair expression of the linked mitochondrial gene (Hanson, 1991; Hanson and Bentolila 2004). Therefore, the question inevitably arises whether sequence alteration in the UTR could affect mitochondrial gene expression.

As CMS is the important breeding character for the hybrid seed production of sugar beet, we conducted genome-wide comparative studies on nucleotide sequence and gene expression between normal and CMS sugar beets (Satoh et al. 2004; Yamamoto et al. 2005), and found an alteration of the transcription profile in six regions. Because the nuclear *restorer-of-fertility* gene, which often alters the transcription pattern of a CMS-associated mitochondrial gene (Hanson and Bentolila 2004), did not affect the transcription of any sugar beet mitochondrial gene, our approach was to characterize all the six regions to identify the CMS-associated region in sugar beet. Of the six regions, five were associated with unique ORF to CMS mitochondria, whereas one unique ORF was transcribed in normal mitochondria (Yamamoto et al. 2005). The ORF, termed *Norf246*, exhibited chimeric organization that shared the 5´ part of 167 bp with *rps3* while the remaining part showed no homology to any sequence in the public data base (Kubo et al. 2000; Satoh et al. 2004) (Fig. 1). *Norf246* was located downstream of *rps3*; therefore, genuine and truncated *rps3* copies are linked in the normal mitochondrial genome in a head-to-tail manner at a distance of 190 bp. In the CMS mitochondria, a 1056-bp region including *Norf246* was deleted (Fig. 1) and its related sequence was missing. In the normal mitochondria, a signal band of 3.8 kb was detected on the RNA-gel blot when the *rps3* ORF was used as a probe [Fig. 6 in Kubo et al. (1999)]. On the other hand, when the downstream *Norf246* was used as a probe, the same 3.8-kb band and an additional 0.9-kb band were detected [Fig. 4 in

Satoh et al. (2004)]. No polypeptide corresponding to *Norf246* was found among *in organello* translation products of normal mitochondria (Yamamoto et al. 2005), making it unlikely that *Norf246* encodes a functional polypeptide. In the CMS mitochondria, transcripts of *rps3* were detected rather as broad signal bands of ~2.7 kb [Fig. 6 in Kubo et al. (1999)]. In the context mentioned previously, the possibility that deletion of the 1056-bp region affects the expression of upstream *rps3* may not be ruled out. It should be noted that aberrant expression of a ribosomal protein gene disturbs mitochondrial function in yeast (Wiltshire et al. 1999).

Sugar beet *rps3* is also an interesting gene because it is organizationally distinct from typical plant *rps3*, which is interrupted by a single group II intron and is often linked to upstream *rps19* and downstream *rpl16* (Hunt and Newton 1991; Sutton et al. 1993). None of these features are preserved in sugar beet *rps3*, raising the possibility that sugar beet *rps3* is a pseudo copy. Although evolutionary conservation of the *rps19-rps3-rpl16* cluster seems to be a remnant of an ancestral S10-like operon that can be seen in many bacteria (Ye et al. 1993), the question remains whether clustering is important for gene expression in the plant mitochondria.

In this study, we investigated sugar beet *rps3* using a combination of techniques with the objective of determining whether or not the *rps3* gene was a candidate for the CMS gene.

Materials and methods

Plant Materials

The sugar beet line TK81-O is fertile with normal cytoplasm (Kubo et al. 2000) and TK81-MS is a male sterile line with the Owen-type cytoplasmic male sterile cytoplasm (Satoh et al. 2004). The two lines were isonuclear lines that differ in their cytoplasms, but the nuclear genotype is nearly identical. The sugar beet seeds were a gift from the National Agricultural Research Center for Hokkaido Region.

Isolation of mitochondria

Isolation of the mitochondria was done according to the method of Lind et al. (1991) with some modifications. Basically, 150-500 g of tap roots were homogenized in ice-cold buffer R [0.5 M mannitol, 2 mM ethylene glycol bis (β-aminoethylether)-N, N, N´, N´-tetraacetic acid (EGTA), 0.1% w/v bovine serum

albumin (BSA), 5 mM β-mercaptoethanol, and 25 mM MOPS-KOH pH 7.5] using a Waring blender (Waring, Torrington, CT). The homogenate was filtered through four layers of cheesecloth and two layers of Miracloth, followed by centrifugation at 2500 x *g* (4˚C) for 5 min. The supernatant was centrifuged at 12000 x *g* (4˚C) for 15 min. The resultant pellet was dissolved in wash buffer [0.5 M mannitol, 1 mM EGTA, 0.1% w/v BSA, and 5 mM MOPS-KOH pH 7.5] and centrifuged again (12000 x *g*, 4˚C, 15 min). The last step was repeated once more.

Isolation of nucleic acids from mitochondria

Mitochondrial (mt) DNA was isolated according to the method of Mikami et al. (1985). Mitochondrial RNA was extracted from the mitochondria using the RNeasy Plant Mini kit (QIAGEN, Hilden, Germany) according to the instructions´ manual.

RNA gel blot analysis

Five μg of mt RNA was electrophoresed in a 1.4% agarose gel (1 x SSPE) containing 0.66 M formaldehyde, then capillary transferred to Hybond N+ (GE Healthcare UK, Amersham Place, England) according to Sambook et al. (1989). DNA fragment was labeled with ^{32}P using Megaprime DNA labeling kit (GE Healthcare). Hybridization was carried out as described in Sambrook et al. (1989). Signal bands were detected by using BAS2000 Image Analyzer (Fuji Photo Film, Tokyo, Japan).

Circularized RNA (CR) reverse transcription (RT) PCR and RT-PCR

CR-RT-PCR was carried out according to the method of Forner et al. (2007) with some modifications. Basically, 50 μg of mt RNA was treated with DNase I, then dissolved in a buffer [0.5 U/μL T4 RNA ligase (Ambion, Austin, TX) and 1 x RNA Ligase buffer (supplied by manufacturer)], and incubated at 37˚C for 1h. RT was performed using Superscript III (Invitrogen, Carlsbad, CA) according to the instructions´ manual. The subsequent PCR reaction was carried out using BlendTaq (Toyobo Life Science, Osaka, Japan). RT-PCR was performed according to the method of Singer-Sam et al. (1990).

Sequences of oligonucleotide primers

Primer 1, 5´- TGGGTAAGGATAAGGTAA-3´; primer 2, 5´-GGTGCCGGTGGATTATTCAAGTCGAA-3´; primer 3, 5´-GTGATCGGTCATGGTATCC-3´; primer 4, 5´-GGGGGGTAGAGGGGATCCGTATATGTTG-3´; primer 5, 5´-CCGCCCGGAATCAGGGCCTCCCCTTTCCT-3´; primer 6, 5´-GGGGGCATCCCCGGATGCTCCGACCA-3´; primer 7, 5´-CTACAGCTCCCGGAAGCAGCGGGAG-3´; primer 8, 5´-GAGGAAAGGAATTGATAGAGTTTTC-3´; primer 9, 5´-GACCCGAAGTGATTTAGAAAGATTC-3´; primer 10, 5´-ATGGATACCATGACCGATCACCATC-3´; primer 11, 5´-TCCAACATTTTATCCTTAATTGACC-3´; primer 12, 5´-GATGGAGTTAGAGTTTATAATAACC-3´; primer 13, 5´-TACCACTCTGAATACCTTCTTGAAC-3´; primer 14, 5´-TTAGGCAAATGATCTACTCTG-3´; primer 15, 5´-TAGCAGGAACCTCACAAACC-3´; primer 16, 5´-TTTCGCCAATGACGGATTTC-3´; primer 17, 5-´CGCATTGTTTGCCCTAATGATGGCCTTT-3´; primer 18, 5´-CCAATACCGATAGCAGCTCCCGCTAAAG-3´.

Molecular cloning and sequencing

The PCR products were excised from agarose gels and ligated into pCR-Blunt vectors (Invitrogen, Carlsbad, CA). Nucleotide sequences were determined using Li-COR4200L (Li-COR, Lincoln NE, USA) or ABI3130 (Applied Biosystems, Foster City, CA). Sequence analysis was conducted using GENETYX (GENETYX CORPORATION, Tokyo, Japan) or Sequencher (Hitachi Software Engineering Co., Ltd., Tokyo, Japan). Sequence alignment was carried out using CLUSTALW (http://clustalw.ddbj.nig.ac.jp/top-j.html) and then modified manually.

Protein gel blot analysis

An oligopeptide (NH_2-KCLAEAKKRLTHFIRQAND-COOH) corresponding to a region from K302 to D320 of sugar beet RPS3 was used as antigen; the antiserum was prepared by immunization of rabbits, as

described by Yamamoto et al. (2005). The antiserum was affinity-purified against the oligopeptide-coupled FMP-activated cellulofine (Seikagakukogyo, Tokyo, Japan). The mitochondrial extracts were electrophoresed in a 10% SDS polyacryl amide gel and transferred onto Hybond P (GE Healthcare). The membrane was soaked in a blocking solution (5% low-fat dried milk powder, 25 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.05% Tween20) for 1 h at room temperature or for 16 h at 4 ˚C,then soaked in the Solution 1 of Can Get Signal System (Toyobo Life Science, Osaka, Japan) containing the primary antibody (1.0-1.5 mg/mL). The membrane was washed three times with TBS-T (25 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.05% Tween20) and soaked in Solution 2 of Can Get Signal System containing HRP-conjugated goat anti-rabbit IgG (1:10000 dilution; GE Healthcare). After washing three times with TBS-T, the membrane was soaked in ECL-plus (GE Healthcare), then exposed to X-ray films.

Results and Discussion

Expression of *rps3* in normal mitochondria

The size of the *rps3* transcripts in the sample used in this study was confirmed by RNA gel blot analysis. As shown in Fig. 2, 3.8-kb transcripts were detected from TK81-O, whereas 2.7- and 2.5-kb transcripts were detected from TK81-MS, which is consistent with what had been obtained previously [Fig. 6 in Kubo et al. (1999)], assuming that the broad band contained multiple-sized transcripts. No other transcripts was detected in contrast to Fig. 6 of Kubo et al. (1999), which suggests that some transcripts appear under certain physiological and/or developmental condition, as seen in wheat mitochondria (Li-Pook-Than et al. 2004).

Together with previous data (see Introduction), we inferred that the 3.8-kb transcripts were dicistronic RNA containing *rps3* and *Norf246*, and that the 0.9-kb transcripts were monocistronic RNA containing only *Norf246*. We first examined the co-transcription of *rps3* and *Norf246.* Primers 1 and 2 corresponded to the coding regions of *rps3* and *Norf246*, respectively. RT PCR analysis using these primers resulted in the detection of the expected 1250-bp fragment (Fig. S1a), indicating the co-transcription of *rps3* and *Norf246.* To determine whether all the ORFs of *rps3* and *Norf246* were covered with these transcripts we conducted CR-RT-PCR analysis (Kuhn and Binder 2002) to map the termini of the transcripts. This analysis included the circularization of mt RNA by the T4 RNA ligase. The junction of the 5´ and 3´ termini was amplified by RT-PCR, and the resultant DNA fragments were cloned into the plasmid vector and their nucleotide

sequences were determined. Forner et al. (2007) pointed out that both 5′ termini of primary transcripts and processed transcripts could be detected because primary transcripts that were originally triphosphated in their 5′ termini could become monophosphated because the di or triphosphates are unstable. The circularized 3.8-kb transcripts were reverse transcribed with primer 3 and PCR amplified with primers 4 and 5 to amplify the junction of the 5′ and 3′ termini (Fig. 1). We obtained the PCR products of the 1850 bp fragment (Fig. S1b). The nucleotide sequence of the cloned PCR products enabled us to map the precise 5′ and 3′ termini of the 3.8-kb transcripts (see summary in Table 1). No non-encoded nucleotides such as poly A were observed in any of the clones analyzed by CR-RT-PCR in this study. The 5′ termini were mapped from –463 to –449 (all positions are given relative to the initiation codon of *rps3* at +1) (Fig. 1 and Table 1). Because no consensus motif of the mitochondrial promoter in dicots (5′-CRTAAGAGA-3′ or 5′-CGTATATAA-3′; Gagliardi and Binder 2007) was found in the vicinity of the mapped 5′ termini, the 3.8-kb transcripts were either transcribed from non-consensus promoter or processed from a primary transcript that could not be detected by our experimental methods. In *Arabidopsis*, both those cases were reported (Forner et al. 2007; Kuhn et al. 2005). The 3′ termini were mapped around +2992 or +3165, of which the latter was dominant (Fig. 1 and Table 1). The transcript size (3452-3629 bases, of which majority was >3600 bases) deduced from the 5′ and 3′ termini was very close to that found in the RNA gel blot analysis. We failed to find any stem-loop structure that may contribute to the formation of the detected 3′ termini.

The 5′ and 3′ termini of the 0.9-kb transcript were mapped using primer 2 for RT, and primers 6 and 7 for PCR amplification (Fig. S1c). In contrast to the case of the 3.8-kb transcript, both 5′ and 3′ termini of the 0.9 kb transcript were scatteringly mapped (Fig. 1 and Table 1). The 5′ termini were mapped in the region from +1846 to +2266 and the 3′ termini from +2669 to +2985. The deduced transcript size was 661 to 1064 bases, but the majority of the transcripts were ~0.8 kb, which is consistent with the previous data [Fig. 4 in Satoh et al. (2004)]. Minor transcripts that were under the detectable level of RNA gel blot analysis could be amplified by CR-RT-PCR. Besides the 710-bp PCR product (Fig. S1c), minor PCR products were recovered from the agarose gel.

It is likely that the 0.9-kb transcripts were products of the 5′- and 3′-trimmed 3.8-kb transcripts. Alternatively, it might be possible that the transcript with the 5′ terminal at +1846 was a primary transcript, because the two overlapping motifs 5′-CATATCATA-3′ and 5′-CATATATGG-3′ from +1837 to +1845 and +1842 to +1850, respectively, resembled the consensus motif of mitochondrial promoter of dicots (Gagliardi and Binder 2007). However, no 5′ terminal was mapped to -1, which is the homologous position of +1864 (Fig. 1). Further analysis is necessary to see the reason why the 0.9-kb transcripts are accumulated.

These results revealed that the 3.8 kb transcripts covered the entire *rps3* ORF and *Norf246*, but the majority of 0.9-kb transcripts were too short to cover the entire *Norf246* or, in one RNA species, the 5´ UTR was only 1 bp. In our previous study, no polypeptide corresponding to *Norf246* was found (Yamamoto et al. 2005). The 0.9 kb transcripts seemed incapable of translation. The *Norf246* could be translated from the 3.8-kb transcript, but translation did not occur because of insufficient *cis*-acting regulatory elements, or because translation products did not accumulate as a result of rapid degradation of the nascent polypeptide. We next desired to detect the translation product of *rps3*. Antiserum against the 19-mer oligopeptide corresponding to a region from the 302nd to 320th amino acid residues reacted with a polypeptide whose apparent molecular weight was 68 kDa, as obtained from total mitochondrial protein (Fig. 3). The predicted molecular weight of RPS3 from genomic sequencing was 64.1 kDa, which was close to that of the observed signal band.

In plant mitochondria, mRNAs encoding protein products were subjected to RNA editing, which is the post-transcriptional substitution of specific cytidine residues into uridine residues (Takenaka et al. 2008). RNA editing of sugar beet *rps3* was determined by sequencing cDNA clones, which were reverse transcribed from mt RNA and amplified with three primer combinations, primers 8 and 9, primers 10 and 11, and primers 12 and 13. All 30 clones, 10 from each primer combination, were sequenced and compared with the genomic sequence. We found 5 C-to-U editing sites in sugar beet *rps3*, which converted the genomically encoded serine or proline codon into the leucine codon on transcripts (Fig. S2). No partially edited transcripts were observed in the 30 clones. The amino acid sequence of sugar beet RPS3 after RNA editing exhibited improved conservation with other plant RPS3 proteins (Fig. S2). These results led to the conclusion that sugar beet *rps3* is a functional copy in the mitochondrial genome with a 5´ UTR of ~464 bases and 3´ UTR of ~1508 bases.

Expression of *rps3* in the CMS mitochondria

The mt RNA of TK81-MS was circularized and reverse transcribed with primer 3. The junction of 5´ and 3´ termini was PCR amplified with primers 4 and 5, which resulted in 950-bp and 750-bp PCR products (Fig. S1d). Each PCR product was cloned into a plasmid vector and 27 clones, 13-14 clones of each PCR product, were sequenced. The 5´ termini were mapped in a region from –463 to –440, whereas the 3´ termini were mapped in two regions +1922 to +1948 and +2113 to +2116 (Fig. 1 and Table 1). Therefore, *rps3* transcripts in CMS sugar beet covered the entire ORF with 5´ UTR of ~464 bases and 3´ UTR of ~284 bases or ~460 bases. The deduced size of transcripts was ~2400 and ~2580 bases, which is consistent with Fig. 2.

The 5´ termini of TK81-MS *rps3* were mapped to almost identical positions to TK81-O (Fig. 1 and Table 1). The 3´ termini of TK81-MS *rps3* and those of TK81-O were close: the mapped position +2113 to +2116 in the TK81-MS mitochondria corresponded closely to the 3´ termini of the 3.8-kb transcripts in TK81-O (+3163 to +3164) and those mapped to +1922 to +1948 in TK81-MS were near +2992 to +2993 of TK81-O. However, the inner 3´ termini were more active in TK81-MS than TK81-O according to the distribution of the number of cDNA clones and RNA gel blot analysis. A possibility is that deletion of the 1056-bp region modified the mode of interaction between transcripts and the nuclear encoded factors that stabilize the transcripts.

The accumulation of RPS3 was investigated by protein gel blot analysis. The size and signal intensity was indistinguishable from that of TK81-O (Fig. 3). RNA editing of *rps3* in TK81-MS was examined by the same procedure as in TK81-O (total number of cDNA clones was 30). Five cytidine residues were found to be edited as in TK81-O (Fig. S2). All the clones sequenced were completely edited, indicating that TK81-MS *rps3* was expressed as in TK81-O.

Comparison of RNA editing between genuine *atp9* and truncated *atp9*

From the CR-RT-PCR analysis of TK81-O, we determined unexpected C-to-U editing at +2808 and +2819, which were downstream of *Norf246* (Fig. 1) in all cDNA clones covering these two sites. Further analysis indicated that there was no other editing in the 3´ UTR of *rps3* (data not shown). A homology search using the edited region as a query revealed that the two edited sites were included in a 150-bp region that is identical to the 3´ half of the *atp9* gene (Fig. S3). This means that an edited pseudo *atp9* copy was transcribed in normal mitochondrial but was absent in CMS mitochondria. In plastids, where RNA editing of C-to-U substitution type occurs as in the mitochondria, frequency of editing could be changed if an extra editable sequence was transcribed from a transgene, perhaps due to its competitive effect on editing factors (Chaudhuri et al. 1995). With this in mind, we examined the sites and frequency of RNA editing in genuine *atp9* of TK81-MS to determine whether RNA editing was deregulated, for example, if it may show excessive or other abnormal editing. cDNA of genuine *atp9* was reverse transcribed from mt RNA and PCR amplified with primers 14 and 15. The nucleotide sequence of 10 cDNA clones revealed that five cytidine residues were edited to uridine on the transcripts (Fig. S3). All 10 clones exhibited complete editing. Of these, one created a stop codon, three changed the amino acid identity of the codon, and one introduced a synonymous change. We simultaneously conducted the editing analysis of TK81-O *atp9*, but no difference was found between the two sugar beet lines (Fig. S3). The degree of conservation in amino acid sequences

of other plant ATP9 was improved after editing (data not shown). These results suggest that the RNA editing of *atp9* was stable irrespective of the copy number of target sites in the mitochondria. To see whether there are any editings in 5′ or 3′ UTR, transcript termini of *atp9* were determined by CR-RT-PCR. Primer 16 was used for RT, and primers 17 and 18 for PCR amplification. The results indicate that the length of 5′ and 3′ UTR is ~282 and ~187 bases (Fig. 1 and Table S1), respectively, but none of the editing sites was found (Fig. S3 and Table S1).

We compared the editing sites between the pseudo *atp9* and genuine *atp9*, and found that the two editings in the pseudo *atp9* also occurred in the genuine *atp9* (Fig. S3). On the other hand, a cytidine residue (+2692) was not edited at all in the pseudo *atp9* whereas it was edited in the genuine *atp9* (Fig. S3). The cytidine residue of +2692 shared upstream 18 bases with genuine *atp9* but differed in the further upstream region. It is known that factors specifying editing sites in plant mitochondria recognize 5′ upstream of the editing site (Takenaka, et al. 2008). For the occurrence of editing in +2692, the homology of 18 bases might be insufficient.

Conclusion

We examined the effect of deletion in the 3′ UTR of sugar beet *rps3*. We also examined RNA editing of *atp9*, which was assumed to be affected by presence or absence of the potential competitor in the deleted region. However, there was no evidence that the accumulation of RPS3 polypeptide and the sites and frequency of RNA editing of *rps3* or *atp9* were changed. Therefore, it seems unlikely that abnormal expression of *rps3* or *atp9* was involved in sugar beet CMS. Our preliminary data also suggest that the activity of complex V in the mitochondrial electron transport chain was indistinguishable between normal and CMS sugar beets, supporting the hypothesis that the expression of *atp9* was unaffected (M. Matsunaga, T. Mikami and T. Kubo, unpublished data). However, since two amino acid residues differed between TK81-O *rps3* and TK81-MS *rps3* (Satoh et al. 2004), further study may be necessary.

In the mitochondrial genome of the CMS sugar beet, six regions were found to generate altered transcripts through comparison of mitochondrial genome sequences and genome-wide transcriptional analysis, namely, *cox2*, *cox1*, *atp1*, *atp6*, *orf324-rps13*, and *rps3* (Senda et al. 1991; Senda et al. 1993; Kubo and Mikami 1996; Onodera et al. 1999; Kubo et al. 1999; Satoh et al. 2004; Yamamoto et al. 2005). Thus far, no translational consequence has been determined in these transcriptionally altered regions with the exception of *atp6*. In CMS *atp6*, a unique ORF termed *preSatp6* was fused with the 5′ end of the conserved *atp6* region and translated as a 39-kDa polypeptide, which may be a product proteolytically cleaved from

the fused preSATP6-ATP6 precursor (Yamamoto et al. 2005). The preSATP6 polypeptide was present as an oligomeric form in the mitochondrial membrane, which may be harmful for the mitochondria, while expression of the downstream *atp6* seemed unaffected (Onodera et al. 1999; Yamamoto et al. 2005).

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Figure legends

Fig. 1 Schematic organization of *rps3* and *atp9* gene loci of TK81-O and TK81-MS based on DDBJ/EMBL/GenBank accession numbers BA000009 and BA000024 and this study. The *rps3* ORF is colored in black. In the *Norf246* ORF, the sequence homologous to the *rps3* ORF is black color, whereas the unique sequence is uncolored. One-hundred-fifty-four bp sequences preceding the *rps3* ORF are indicated by stripes and the *atp9* ORF and pseudo *atp9* copy areas are dotted. The positions of cytidine residues that are subjected to RNA editing on the transcript are indicated by vertical lines. Arrowheads point to the position and orientation of primers. 5′ and 3′ termini of transcripts containing *rps3* ORF are indicated by uncolored triangles and diamonds, respectively. 5′ and 3′ termini of 0.9 kb transcripts are indicated by colored triangles and diamonds, respectively. 5′ and 3′ termini of *atp9* transcripts are indicated by striped triangles and diamonds, respectively. Dashed lines indicate the 1056-bp region that is deleted in the CMS mitochondria. The scale bar is shown below the diagram

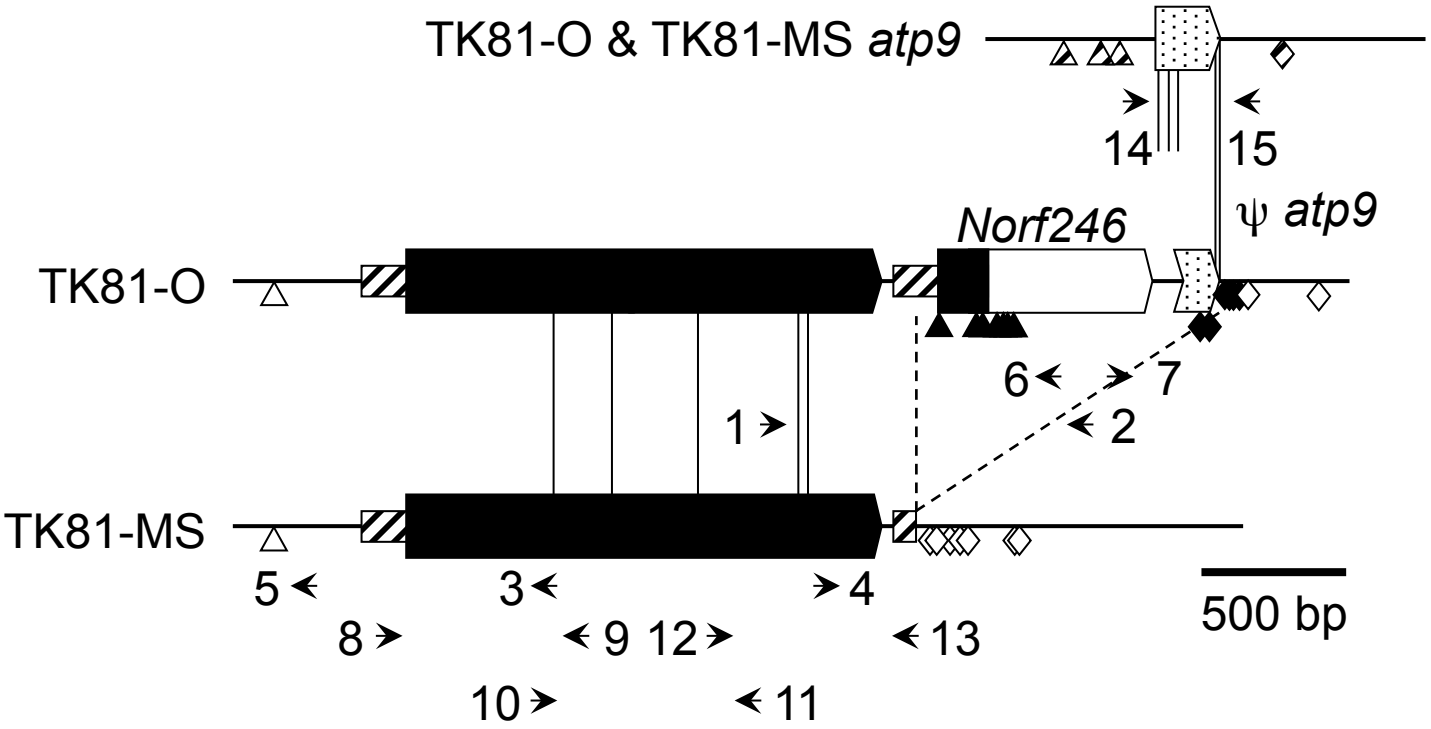
Fig. 2 RNA gel blot analysis of mitochondrial RNA isolated from tap roots of TK81-O and TK81-MS. The probe was PCR amplified with primers 12 and 13. Size of the transcripts are shown (kb)

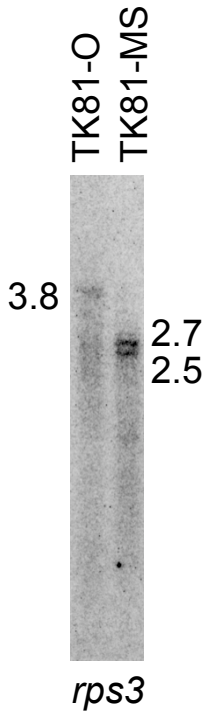
Fig. 3 Protein gel blot analysis of total mitochondrial protein from TK81-O and TK81-MS probed with anti-RPS3 antiserum (αRPS3) and anti-COXI (αCOXI), as control. αRPS3 antiserum was raised against the 19-mer oligopeptide (see Materials and Methods). Details of the αCOXI antiserum were described by Yamamoto et al. (2005). The size of the signal bands (kDa) is indicated to their left

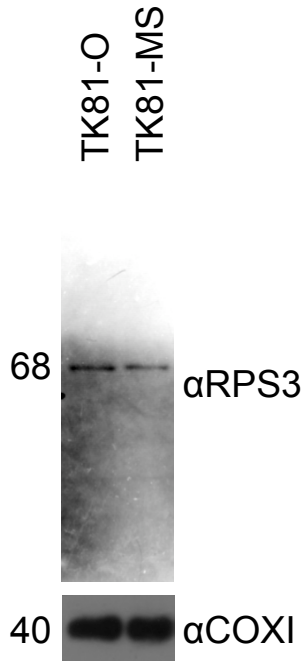
Table 1 Mapped 5´ and 3´ termini of transcripts of sugar beet *rps3* locus by CR-RT-PCR

TK81-O						TK81-MS		
Primers 4+5			Primers 6+7			Primers 4+5		
5´ termini*	3´ termini*	No. of clones	5´ termini*	3´ termini*	No. of clones	5´ termini*	3´ termini*	No. of clones
–463	+3163	6	+1846	+2753	1	–463	+2113	3
–463	+3166	1	+1857	+2889	1	–462	+1948	1
–463	+3165	1	+1920	+2983	1	–462	+1945	1
–463	+3164	4	+1933	+2809	1	–462	+1936	1
–463	+2993	1	+1959	+2669	1	–461	+1947	1
–461	+3166	1	+1969	+2866	1	–460	+2116	1
–461	+3003	1	+2005	+2887	1	–460	+2115	2
–460	+2992	1	+2060	+2868	1	–460	+2114	3
–460	+2993	1	+2063	+2887	1	–460	+2113	2
–460	+3165	1	+2095	+2985	1	–460	+1945	2
–460	+3167	2	+2179	+2924	1	–460	+1940	1
–457	+3166	1	+2179	+2982	1	–460	+1936	2
–449	+3163	1	+2179	+2983	3	–460	+1922	1
–449	+3164	1	+2179	+2985	1	–459	+1940	1
			+2192	+2983	1	–458	+1940	1
			+2253	+2983	1	–456	+1940	1
			+2256	+2983	1	–453	+1940	1
			+2266	+2983	1	–449	+1940	1
			+2266	+2920	1	–440	+1940	1
			+2266	+2926	1			
			+2266	+2985	1			
Total number of clones		23			23			27

*, numbers are started from the initiation codon of *rps3* ORF


TK81-O & TK81-MS atp9
14
15
ψ atp9
Norf246
TK81-O
6
7
1
2
TK81-MS
5
3
4
8
9
12
13
10
11
500 bp


TK81-O
TK81-MS
3.8
2.7
2.5
rps3


TK81-O
TK81-MS
68
αRPS3
40
αCOXI