The complete nucleotide sequence of the mitochondrial genome of sugar beet (*Beta vulgaris* L.) reveals a novel gene for tRNA^Cys(GCA)

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ABSTRACT

We determined the complete nucleotide sequence of the mitochondrial genome of an angiosperm, sugar beet (*Beta vulgaris* cv TK81-O). The 368 799 bp genome contains 29 protein, five rRNA and 25 tRNA genes, most of which are also shared by the mitochondrial genome of *Arabidopsis thaliana*, the only other completely sequenced angiosperm mitochondrial genome. However, four genes identified here (namely *rps13*, *trnF*-GAA, *ccb577* and *trnC2*-GCA) are missing in *Arabidopsis* mitochondria. In addition, four genes found in *Arabidopsis* (*ccb228*, *rpl2*, *rpl16* and *trnY2*-GUA) are entirely absent in sugar beet or present only in severely truncated form. Introns, duplicated sequences, additional reading frames and inserted foreign sequences (chloroplast, nuclear and plasmid DNA sequences) contribute significantly to the overall size of the sugar beet mitochondrial genome. Nevertheless, 55.6% of the genome has no obvious features of information. We identified a novel tRNA^Cys gene (*trnC2*-GCA) which shows no sequence homology with any tRNA^Cys genes reported so far in higher plants. Intriguingly, this tRNA gene is actually transcribed into a mature tRNA, whereas the native tRNA^Cys gene (*trnC1*-GCA) is most likely a pseudogene.

INTRODUCTION

Over the past decade much effort has been directed towards determining the entire genomic sequence of the mitochondrial DNAs (mtDNAs) from a wide array of organisms (1). However, the studied organisms are taxonomically highly biased; most of the complete mtDNA sequences published are from animals, protists and ascomycete fungi, whereas the available sequences for plants are limited to the liverwort *Marchantia polymorpha* (2) and the angiosperm *Arabidopsis thaliana* (3).

Despite the fact that mitochondria have essentially the same metabolic function in all eukaryotes which contain them,

higher plant mtDNAs exhibit a number of unique structural features (e.g. large size, rapid evolutionary rearrangements and chloroplast DNA insertions) and specific modes of expression (e.g. *cis*- and *trans*-splicing, RNA editing and universal genetic code usage) (4). Moreover, they encode significantly more genes than do their fungal and animal counterparts. Ribosomal protein genes as well as the genes involved in cytochrome *c* biogenesis comprise a significant fraction of such additional genes identified so far in plant mitochondria (4). The complete sequence of the *Arabidopsis* mtDNA reveals approximately 85 open reading frames (ORFs) of unknown function, some of which may encode plant-specific mitochondrial proteins (3).

It is also interesting to note that the study of different plant lineages has demonstrated the recent evolutionary transfer of certain mitochondrial genes to the nucleus, and therefore the gene content of mitochondria may vary in different species (4). The determination of the complete mtDNA sequence of another angiosperm would thus allow us to examine the entire coding potential of plant mitochondrial genomes and to ascertain whether or not the plant mitochondrial gene content and organization have been conserved during flowering plant evolution. In addition, it should greatly facilitate the identification of new genes and provide insights into specific mechanisms underlying mitochondrial genome divergence.

In this article we describe the complete sequence of the mitochondrial genome of sugar beet (*Beta vulgaris* L.). We found an intriguing diversity of gene complements between sugar beet and *Arabidopsis* mitochondrial genomes, although most of the genes reported in *Arabidopsis* are shared by sugar beet. Of particular interest is the occurrence in sugar beet mtDNA of a gene for tRNA^Cys which shows no significant similarity to the plant mitochondrial, chloroplast or nuclear tRNA^Cys genes identified to date. This novel gene is actually transcribed in sugar beet mitochondria whereas the standard (native) tRNA^Cys gene seems not to be functional.

MATERIALS AND METHODS

Sequencing procedures

The clone bank covering the entire mitochondrial genome of normal fertile sugar beet (cv TK81-O) was previously constructed using λ-DASH (Stratagene) and cosmid pHC79

(Boehringer) (5). We selected three cosmid clones for sequencing the *rrn26*-carrying repeats and 21 λ clones for sequencing the remaining region of the genome. mtDNA in each λ clone was amplified by long PCR using an LA-PCR kit (Takara Shuzo) and then physically sheared. The resultant fragments were blunt-ended using T4 DNA polymerase and Klenow fragment and subcloned into pBluescript (Stratagene). The purified cosmid DNA was sonicated and used for subcloning. Shotgun sequencing was continued until a minimum of 4-fold sequence coverage was achieved for each clone. The DNA sequencer used was a Li-COR 4200L (Li-COR). The PCR products obtained by amplification of the sugar beet mtDNA were used for gap filling and confirmation of the sequences. Sequence alignments were generated using Sequencher 3.0 software (Gene Codes). A database search was done with the BLAST network service (http://www.dna.affrc.go.jp/htdocs/Blast/blast2.html) and a tRNA gene search with the tRNAscan-SE service (http://www.genetics.wustl.edu/eddy/tRNAscan-SE/) (6). The nucleotide sequence reported in this paper has been deposited in the DDBJ/GenBank/EMBL databases under accession nos AP000396 and AP000397.

RNA isolation and hybridization

Mitochondria were prepared from green leaves as described (7) and were used for RNA isolation (8). Aliquots of total mtRNA (5 µg) were electrophoresed through 1.4% agarose gels containing 0.66 M formaldehyde. Gels were blotted onto Hybond N+ membranes (Amersham) and hybridized with a ^{32}P-labeled probe.

RESULTS AND DISCUSSION

Conserved genes

Our strategy for analysis of the sugar beet mitochondrial genome was based upon assembling the sequences of partially overlapping clones from λ and cosmid libraries and of long PCR products which have been assigned on the physical map of the genome (5). The entire genome size of the sugar beet mitochondrion is 368 799 bp, with an overall G+C content of 43.9%. These figures are comparable to those of *Arabidopsis* (366 924 bp in size and a G+C content of 44.8%) (3). Homology searches to match the entire genomic sequence with known sequences in the public DNA database detected 29 protein coding genes, five rRNA genes and 25 tRNA genes, which altogether account for 11.3% of the genome (Table 1). The positions of these genes in the sugar beet mitochondrial genome are depicted in Figure 1. Although most of the genes identified here are also found in *Arabidopsis* mitochondria, a few differences are noted in the gene content between sugar beet and *Arabidopsis*. As shown in Table 1, the *rps13*, *ccb577*, *trnF*-GAA and *trnC2*-GCA (see below) genes are present in sugar beet mitochondria but not in the mtDNA of *Arabidopsis*. Conversely, sugar beet mitochondria apparently lack four genes (*ccb228*, *rpl2*, *rpl16* and *trnY2*-GUA) which are encoded by *Arabidopsis* mtDNA.

An alignment of all known mitochondrial RNA editing sites (see for example 9) with the homologous nucleotide sequence of sugar beet mtDNA allowed us to predict at least 370 C→U editing sites in 28 gene/ORF transcripts of sugar beet. This number of editing events in sugar beet is somewhat fewer than in *Arabidopsis* (441 editings). We also experimentally verified the RNA editing-mediated creation of initiation (*nad1*, *nad4L* and *atp6*) and termination codons (*atp6* and *atp9*), which is not documented in the corresponding *Arabidopsis* transcripts (10,11; T.Kubo *et al.*, unpublished data).

Repeated sequences

We previously reported that the sugar beet *rrn26* gene is found exclusively within a three-copy recombining-repeat family (the *rrn26* repeat) (5). The present study indicates that the common sequence unit shared by all three *rrn26* repeat copies is 6222 bp long and contains *trnfM*-CAU as well as *rrn26* (Fig. 1). As can be seen in Figure 1, in two of the three repeat copies sequence identity continues a further 16 517 bp beyond the downstream junction (93 bp downstream from *trnfM*-CAU) of the common sequence unit. The same relationship holds between two of the three upstream *rrn26* repeat flanks, which share an additional 3528 bp sequence beyond the upstream junction of the common sequence unit (Fig. 1). We found perfect identity in the base sequence over the entire length of the repeated sequence elements, suggesting that direct comparison of the repeat copies and correction of any mutational changes that appear in any one of the copies are ongoing processes.

The sugar beet mtDNA also contains 72 short repeated DNA segments, the sizes of which vary from 50 to 626 bp. These short repeats seem not to be active in frequent recombinations and they can be divided into 35 unrelated families. The repeated sequence elements cover 10.3% of the genome.

Promiscuous sequences

It is well documented that plant mtDNAs contain many sequences of foreign origin. Chloroplast DNA (cpDNA) sequences provided the first such example (12) and sequences of nuclear origin are also found in plant mitochondrial genomes (13). Our analysis indicates that cpDNA-derived sequences comprise 2.1% of the mitochondrial genome of sugar beet. The cpDNA insertions vary in size from 25 to 3366 bp. The largest fragment bears *rps7*, the last two exons of *rps12* and *trnV*-GAC. Comparison of this 3.4 kb sequence to the corresponding sequences of sugar beet (accession no. AB032426) and tobacco cpDNAs (Z00044) revealed that the integrated sequence in question is closer to the sugar beet cpDNA than to tobacco cpDNA (details to be published elsewhere). This result supports the suggestion that cpDNA insertion took place after the divergence of sugar beet and tobacco. The cpDNA insertions include six intact and most likely functional tRNA genes which are also shared by *Arabidopsis* mtDNA (Table 1). However, most of the remaining cpDNA-like sequences are not detected in *Arabidopsis*.

We also found nuclear-like sequences covering 3.3% of the mitochondrial genome of sugar beet. We identified 21 retrotransposon-like sequences with lengths of between 25 and 2827 bp, only two of which exhibit similarity to the mitochondrial retrotransposon sequences of *Arabidopsis*. The most conspicuous example is an *orf84–orf100–orf78–orf764* cluster which seems to be related to the *gypsy*-type retrotransposon. This cluster hybridized to sugar beet nuclear DNA (data not shown) but no corresponding sequence was observed in *Arabidopsis* mito-chondria. Another noteworthy sequence of nuclear origin is a

Table 1. Gene content of sugar beet (Bv) mtDNA compared to *Arabidopsis* (At)

Gene (map co-ordinate in kb)	Bv	At	Gene (map co-ordinate in kb)	Bv	At
Complex I			Ribosomal RNAs		
*nad1** (102, 105, 152, 243)	+	+	*rrn5* (308)	+	+
*nad2** (75, 240)	+	+	*rrn18* (309)	+	+
nad3 (263)	+	+	*rrn26* (125, 212, 348)	+[a]	+
nad4 (285)	+	+	Intronic ORF (in *nad1-i4*)		
nad4L (55)	+	+	*mat-r* (244)	+	+
*nad5** (141, 171, 319)	+	+	Sec-independent membrane targeting	+	+
nad6 (193)	+	+	and translocation system		
nad7 (216)	+	+	*tatC* (199)	+	+
nad9 (0)	+	+	Other ORF		
Complex II			*orf25* (54)	+	+
sdh4	–	ψ?	tRNAs		
Complex III			'Native'		
cob (179)	+	+	*trnC1*-GCA (83)	ψ	+
Complex IV			*trnE*-UUC (91)	+	+
cox1 (238)	+	+	*trnF*-GAA (329)	+	–
cox2 (234)	+	+	*trnG*-GCC (315)	+	+
cox3 (297)	+	+	*trnI*-CAU (197)	+	+
Complex V			*trnK*-UUU (163)	+	+
atp1 (337)	+	+	*trnfM*-CAU (125, 153, 216, 352)	+[b]	+
atp6 (201)	+	+	*trnP*-UGG (329)	+	+
atp8 (299)	+	+	*trnQ*-UUG (313)	+	+
atp9 (72)	+	+	*trnS*-GCU (332)	+	+
Cytochrome *c* biogenesis			*trnS*-UGA (154)	+	+
ccb206 (156)	+	+	*trnY1*-GUA (80)	+	+
ccb228 (49)	ψ	+	'Chloroplast-like'		
ccb438 (37)	+	+	*trnD*-GUC (289)	+	+
ccb577 (33)	+	ψ?	*trnH*-GUG (93)	+	+
Ribosomal proteins			*trnN*-GUU (81)	+	+
rpl2	–	+	*trnM*-CAU (89)	+	+
rpl5 (181)	+	+	*trnP*-UGG (1)	ψ	ψ
rpl16	–	+	*trnS*-GGA (250)	+	+
rps3 (271)	+	+	*trnV*-GAC (7)	ψ	–
rps4 (191)	+	+	*trnW*-CCA (1)	+	+
rps7 (231)	+	+	Converted from trnF-GAA		
rps12 (263)	+	+	*trnY2*-GUA	–	+
rps13 (107)	+	–	Origin unknown		
			trnC2-GCA (123, 354)	+[c]	–

*, *trans*-splicing; +, present; ψ, pseudogene; ψ?, likely pseudogene; –, absent; a, three copies; b, four copies; c, two copies.

168 bp segment with homology to (*R*)-mandelonitrile lyase ($P = 8 \times 10^{-21}$).

Introns

As in other higher plants, the sugar beet mitochondrial rRNA and tRNA genes lack introns. Nonetheless, sugar beet mtDNA has at least 20 introns in seven protein coding genes, all of which are categorized as group II introns based on their distinctive secondary structures. The sum of all intron lengths is ~25 800 bp, representing 7.0% of the genome. A total of six *trans*-splicing introns are found in the *nad1*, *nad2* and *nad5* genes and 14 *cis*-splicing introns are found in the seven protein coding genes (*cox2*, *nad1*, *nad2*, *nad4*, *nad5*, *nad7* and *ccb438*). Interestingly, we found sugar beet *rps3* to completely
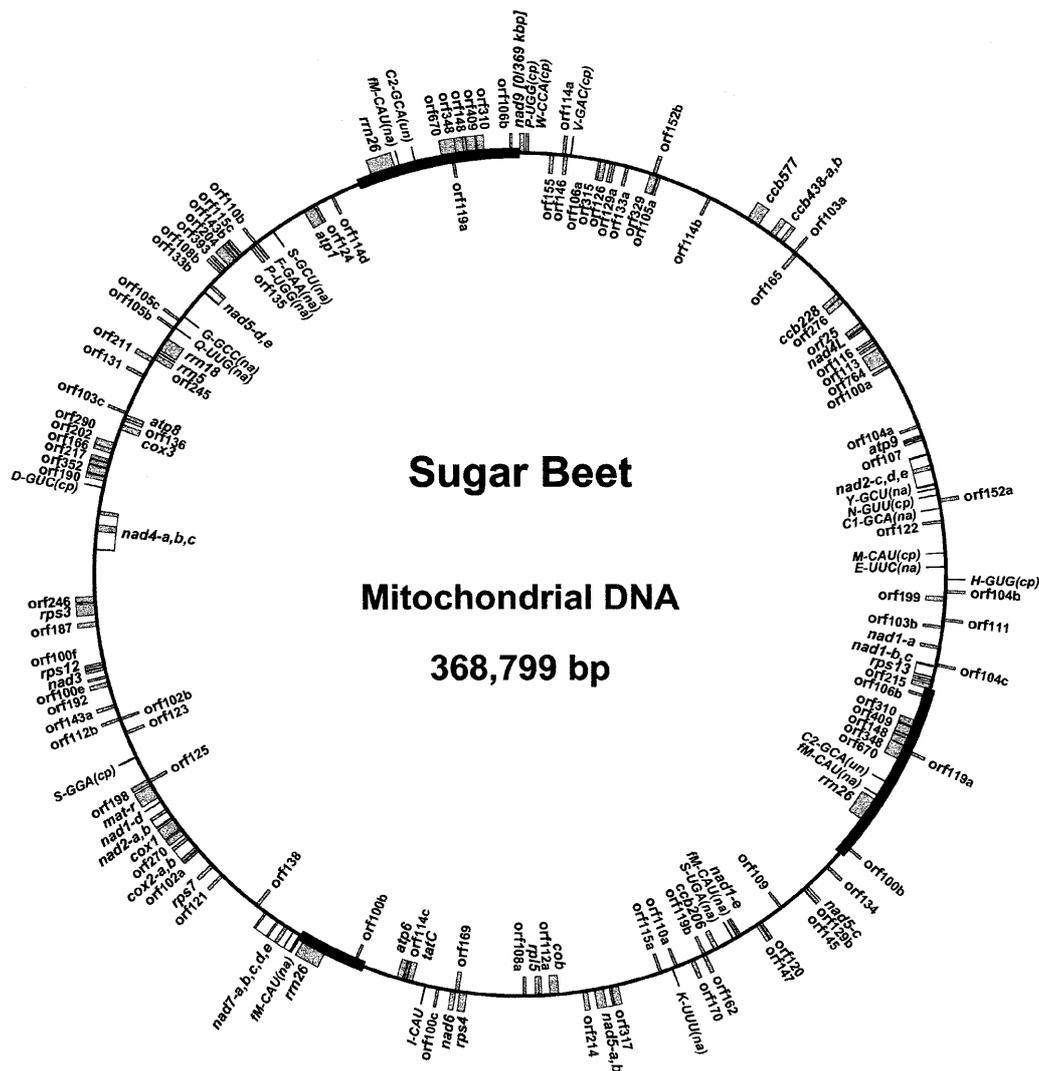
Figure 1. Gene map of the sugar beet mitochondrial genome. Protein, rRNA and tRNA coding genes and ORFs longer than 100 amino acids are indicated by gray boxes. *cis*-splicing introns are shown by thin lines. Orientations are clockwise for the genes shown outside the circle and counterclockwise for the genes shown inside the circle. Abbreviations for the three classes of tRNA genes are: na, native (genuine); cp, chloroplast-like; un, origin unknown. Three-copy repeated sequences which are active in recombination are indicated by bold lines. Map coordinate 0 has been arbitrarily assigned to the first nucleotide of the *nad9* ORF (top of the figure) and numbering proceeds clockwise.

lack introns. This contrasts with the situation reported in other plant species (3,14,15), where the *rps3* coding region is interrupted by a single intron. We also noted that the sugar beet *nad4* gene does not contain the second intron found in several higher plants, including both monocots and dicots (16; N.Itchoda, T.Kubo and T.Mikami, manuscript in preparation).

Residual sequences

We searched for ORFs longer than 60 codons that begin with a methionine codon and end with a classical termination codon. A total of 559 ORFs were identified (93 ORFs are longer than 100 codons in size), none of which, however, exhibited apparent homology to any reported genes (or to any of the *Arabidopsis* ORFs; 3) or carried significant protein motifs. It thus seems unlikely that these ORFs code for additional

important protein information. Of the sugar beet mitochondrial genome sequence 3.6% is homologous to mitochondrial plasmid DNAs. These sequences include segments of DNA and RNA polymerase-related ORFs (accession no. Y10854). We also found sequences with homology to the 5'- and 3'-untranslated regions and the intergenic sequences of mitochondrial genes in other plant species; they do not exceed 6.8% of the sugar beet genome. The major portion (55.6%) of the genome has no similarity to any nucleotide or protein sequences in the public databases.

tRNA genes

Twenty-five tRNA genes were identified by homology to their respective counterparts in *Arabidopsis* and other higher plants and by their predicted cloverleaf structures, which define
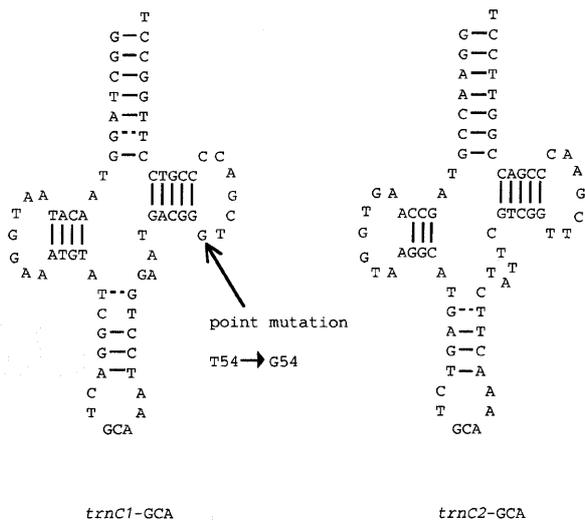
Figure 2. Cloverleaf structure deduced from the *trnC1*-GCA and *trnC2*-GCA gene sequences in sugar beet mitochondria. A mutation (T54→G54) in *trnC1*-GCA is indicated by an arrow.

Figure 3. Northern blot analysis to detect and determine the relative sizes of RNA transcripts of the two sugar beet tRNA^{Cys} genes. Hybridization probes were *trnC2*-GCA-, *trnC1*-GCA-, *trnN*-GUU- and *trnY1*-GUA-specific sequences whose positions are given in Figure 1. The sizes of the transcripts are shown in kilobases.

unambiguous anticodons (Table 1). As shown in Figure 1, *trnfM*-CAU and *trnC2*-GCA are present in four and two copies, respectively, whereas the other tRNA genes occur singly. We previously reported that chloroplast-like *trnP*-UGG is not transcribed in sugar beet mitochondria (17). The same appears to hold true for chloroplast-like *trnV*-GAC (data not shown). As there are no mitochondrially encoded tRNA species for five amino acids (alanine, arginine, leucine, threonine and valine) in sugar beet, tRNA import from the cytosol must be invoked as the mechanism for making up the deficit.

Our analysis also indicates the presence of two *trnC*-GCA genes; the first one is a native *trnC*-GCA (designated *trnC1*-GCA) and the other is a novel one (*trnC2*-GCA). When *trnC1*-GCA was folded into a cloverleaf secondary structure, the invariant T54 was found to be replaced by a G (Fig. 2). T54 is believed to pair with A58 to yield the familiar L-shaped tertiary structure. Hence, a G54-A58 mismatch may have a deleterious effect on expression of the *trnC1*-GCA gene (see below). On the other hand, the tRNA encoded by *trnC2*-GCA retains the invariant and semi-invariant nucleotides characteristic of standard tRNA (18; Fig. 2). Surprisingly, the best match between sugar beet *trnC2*-GCA and sequences in the public databases is with the *trnC*-GCA from a Gram-negative bacterium, *Helicobacter pylori* (accession no. AE000604), although it is too soon to say whether the homology (76%) is of significance. It should also be noted that the *trnC2*-GCA-specific probe failed to hybridize with cpDNA and nuclear DNA from sugar beet (data not shown).

The native *trnC* is likely a pseudogene whereas the novel *trnC* is expressed

Northern blot analysis was carried out to test for the presence in sugar beet mitochondria of RNAs corresponding to the *trnC1*-GCA and *trnC2*-GCA sequences, respectively. tRNA-sized molecules (~80 bp) were detected by hybridization with a *trnC2*-GCA-specific probe, confirming transcription of this
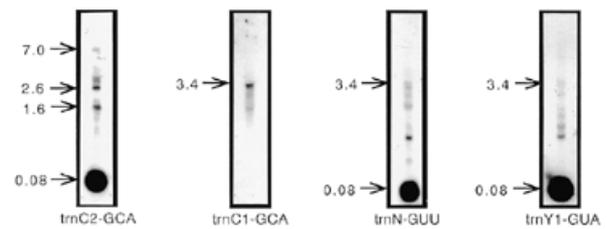
gene (Fig. 3). In addition, a weaker signal (7.0 kb) indicated the presence of larger precursor molecules in the analyzed RNA fraction (Fig. 3). A probe that contains the the 5′-flanking region of the upstream *trnfM*-CAU gene (Fig. 1) hybridized with the 7.0 kb transcript (data not shown), an observation that is consistent with the co-transcription of *trnfM*-CAU and *trnC2*-GCA. In contrast, a *trnC1*-GCA-specific probe hybridized only to an RNA of 3.4 kb, indicating that the *trnC1*-GCA transcript remains linked to a transcript that includes the downstream *trnN*-GUU and *trnY1*-GUA genes (Fig. 1). This was confirmed by hybridization experiments with the *trnN*-GUU and *trnY1*-GUA probes, which identified a 3.4 kb transcript as well as tRNA-sized molecules (Fig. 3). These results lead us to suppose that although the *trnC1*-GCA sequence is transcribed, it is rarely or never processed into a mature molecule. Since no other sequence encoding tRNA^{Cys} is found in the sugar beet mitochondrial genome, it seems reasonable to conclude that the *trnC2*-GCA identified here is the only functional tRNA^{Cys} gene in the sugar beet mitochondrial genome.

CONCLUSIONS

We found that duplicated sequences, introns, additional genes and inserted cpDNA sequences contribute significantly to the overall size of the mitochondrial genome in sugar beet. It was also noted that a large fraction (55.6%) of the sugar beet mitochondrial genomic sequences has no recognizable function and shows no apparent similarity to any sequence in the present databases. Furthermore, when the sugar beet mtDNA sequences are compared to those of *Arabidopsis*, the sequences shared by the two plant species total 78 057 bp, representing 21.2% of the sugar beet genome. Provided that many of the enigmatic sequences in question are of nuclear origin, one can infer that many DNA transfer events have occurred separately in the two evolutionary lineages leading to sugar beet and *Arabidopsis*, respectively.

A further notable finding is that the sugar beet mtDNA contains a novel tRNA^{Cys} gene (*trnC2*-GCA) which shows no sequence homology to either the native or chloroplast-like tRNA^{Cys} genes identified so far in plant mitochondria. The *trnC2*-GCA is most likely the only functional tRNA^{Cys} gene in the sugar beet mitochondrial genome, although it remains to be seen whether the nuclear DNA-encoded tRNA^{Cys} species is imported from the cytoplasm into mitochondria in sugar beet.

We have recently cloned a sugar beet cDNA encoding cysteinyl-tRNA synthase. Interestingly, this protein has been found to be imported into mitochondria (T.Kubo *et al.*, unpublished data). Whether the enzyme efficiently recognizes and aminoacylates the *trnC2*-GCA transcript is currently under investigation. Preliminary PCR surveys of 21 diverse genera of angiosperms indicate that the *trnC2*-GCA homolog is present only in members of Caryophyllales (T.Kubo *et al.*, unpublished data). This raises the possibility that Caryophyllales acquired the *trnC2*-GCA sequence by horizontal transfer events from a distantly related organism. Most recently, Cho *et al.* (19) have described the horizontal transfer of a group I intron from a fungal donor to the mitochondrial genome of vascular plants. An alternative possibility that should be considered is that *trnC2*-GCA resulted from extensive genomic rearrangements, as exemplified in the *trnY*-GUA converted from *trnF*-GAA in *Arabidopsis* mitochondria (20) or chimeric ORFs responsible for cytoplasmic male sterility in various plants (21). Further studies are necessary to fully understand the origin and evolution of *trnC2*-GCA.

ACKNOWLEDGEMENTS

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