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The sugar beet mitochondrial *nad4* gene: an intron loss and its phylogenetic implication in the Caryophyllales

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Abstract The sugar beet mitochondrial gene for subunit IV of NADH dehydrogenase (*nad4*) has been characterized. Unlike the corresponding genes in wheat and turnip, sugar beet *nad4* lacks the second intron (*nad4-i2*). Northern-blot analysis demonstrates transcription of the gene. A total of 19 RNA editing sites were identified in the sugar beet *nad4* transcripts; interestingly, there is no editing in the region which flanks the lost intron. This observation is in favour of intron loss via homologous recombination of an edited RNA intermediate. We also found that the *nad4-i2* intron is absent from the mitochondrial genomes of all examined members of the Caryophyllales, but present in the closely related orders, Polygonales and Plumbaginales, which suggests that the intron was lost in the common ancestor of the Caryophyllales.

Keywords Sugar beet · Mitochondria · *nad4* · Intron · RNA editing · Caryophyllales

Introduction

The loss of mitochondrial genes, or of their introns, has been used to infer phylogenetic relatedness among various plant taxa (Qiu et al. 1998; Adams et al. 2000). We have recently determined the complete mitochondrial DNA (mtDNA) sequence from sugar beet (*Beta vulgaris*, Caryophyllales) (Kubo et al. 2000). The sugar beet mitochondrial genome contains a total of 20 introns dispersed within seven protein-coding genes, all of which are of the group II class. Our attention is now focused on the intron content of the *nad4* (NADH dehydrogenase subunit IV) gene because some of the *nad4* introns are known to be optional among plant species (Gass et al. 1992). The first intron (*nad4-i1*) in *nad4* was reported to be present in all flowering-plant species examined so far. Lamattina and Grienenberger (1991) and Gass et al. (1992) found two additional introns (*nad4-i2* and *nad4-i3*) in the *nad4* locus in a monocot (wheat) and two dicots (turnip and mung bean), whereas the *nad4-i2* intron is absent from lettuce, spinach and sugar beet, and the *nad4-i3* intron is missing in lettuce (Gass et al. 1992; Geiss et al. 1994; Kubo et al. 2000). The *nad4* introns, when present, are located at the same position in all observed examples and are highly conserved in their primary sequence. These observations are most-readily interpreted as indicating that the ancestral gene contained all three introns, which have undergone selective loss in different lineages during evolution.

In this paper we present the characterization of the sugar beet *nad4* gene. We also report that the *nad4-i2* intron was lost in the common ancestor of the Caryophyllales.

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Materials and methods

Plant materials and nucleic acid isolation

The Caryophyllales and the outgroup taxa used in this study are listed in Table 1. The isolation of mtDNA and mtRNA from green leaves was described previously (Mikami et al. 1985; Senda et al. 1993). Total cellular DNA was isolated according to Doyle and Doyle (1990).

Hybridization and PCR

The mtRNA was electrophoresed through a 1.4% agarose gel containing 0.66 M formaldehyde, and then transferred from the gel to

Table 1 Plant specimens used to examine the loss of an *nad4-i2* intron and to investigate the genomic DNA sequences of the three codons of *nad4-ex2* at which RNA editingwas reported to occur in the *nad4-i2*-containing plant species, turnip (Geiss et al. 1994). +, intron present; –, intron absent

Taxon	Taxon
Caryophyllales	Phytolaccaceae
Chenopodiaceae	*Phytolacca acinosa* (–)
Beta vulgaris (sugar beet) (–)	Caryophyllaceae
Spinacea oleracea (spinach) (–)	*Dianthus japonicum* (–)
Chenopodium album (wild spinach) (–)	Polygonales
Kochia scoparia (goosefoot) (–)	Polygonaceae
Solsola komarovii (–)	*Fagopyrum esculentum* (buckwheat) (+)
Amaranthatheae	Plumbaginales
Amaranthus tricolar (tampala) (–)	Plumbaginaceae
Amaranthus retroflexus (–)	*Limonium sinense* (+)
Gomphrena haageana (–)	
Porturacaceae	
Porturaca oleracea (–)	
Porturaca grandiflora (garden portulaca) (–)	
Cactaceae	
Pereskia ssp. (–)	
Basellaceae	
Basella rubra (–)	
Aizoaceae	
Tetragonia tetragonoides (–)	
Nyctaginaceae	
Mirabilis jalapa (four-o'clock) (–)	

a Hybond N+ membrane (Amersham, Little Chalfont, UK). Labelling of the probes with alkali phosphatase and detection of signal bands were done using Gene Images (Amersham, Little Chalfont, UK). The reverse transcribed polymerase chain reaction (RT-PCR) was as described in Kubo et al. (1993). Nucleic acid analyses were performed according to standard protocols (Sambrook et al. 1989). Sequencing of the plasmid DNA was done using Thermo Sequenase (Amersham, Little Chalfont, UK) and the DNA sequencer Li-COR 4000L (Li-COR, Lincoln, Neb., USA) according to the instruction manual. PCR primers used in this study are listed as follows:

primer 1, 5′-GCTGACTTGCACTGATAGACCT-3′,
primer 2, 5′-CCCTATGTTCATTATTATAGGGG-3′,
primer 3, 5′-CCAAACAACTCCAACAAGAAAGGG-3′,
primer 4, 5′-TCTTTTTTTTGCTGATTCCTCT-3′,
primer 5, 5′-GCTTACTCCTCAGTAGCCCATATG-3′,
primer 6, 5′-TATTCTGTGTCCCGTGCTAGG-3′,
primer 7, 5′-AGATCCAAGTAAAGTATAAAGGAAAA-3′,
primer 8, 5′-CAGGATCCGTTATCTTGGCAGG-3′,
primer 9, 5′-GCTGCTAGTACCAGGTAAACTC-3′,
primer 10, 5′-GGTGTTCTATATGACCGACAT-3′,
primer 11, 5′-ATGCATGCGGTCCGGGAACAC-3′,
primer 12, 5′-GCTATTCTGTTGATTCTTCTCCAA-3′, and
primer 13, 5′-TGGGCTACTGAGGAGTAAGCAATG-3′.

Results and discussion

Organization and transcription of the sugar beet *nad4* gene

The entire nucleotide sequence of sugar beet mtDNA was determined in our laboratory (Kubo et al. 2000) and subsequently deposited in the DDBJ/EMBL/GenBank database (AP000396 and AP000397). Its comparison with known sequences in the public DNA database reveals that the sugar beet *nad4* gene is comprised of three exons, which are 154, 313 and 28 codons in length and separated by introns of 1440 and 2465 bp, respectively (Fig. 1). These two introns exhibit high sequence homology with the *nad4-i1* and *nad4-i3* introns in wheat, respectively, and their insertion sites correspond exactly to those in the wheat and turnip *nad4* genes (Lamattina and Grienenberger 1991; Gass et al. 1992). It is thus apparent that the sugar beet *nad4* completely lacks the *nad4-i2* intron. Based on Southern-hybridization data, Gass et al. (1992) previously inferred that *nad4-i2* is missing in spinach, which is assigned to the Chenopodiaceae, together with sugar beet.

Northern-blot analysis of the *nad4* transcripts in sugar beet mitochondria was carried out with exon- and intron-specific probes (Fig. 2). When a probe spanning the first exon and approximately 550 bp of 5′ flanking sequence was used in hybridization experiments, transcripts of 5.8, 3.2 and 1.7 kb were identified. The smallest 1.7-kb transcript was not detected with two intron-specific probes, indicating that this RNA species presumably corresponds to the mature transcript. As shown in Fig. 2, the *nad4-i1* probe gave 5.8- and 3.2-kb signals, whereas the *nad4-i3* probe gave only the 5.8-kb signal. The 3.2-kb RNA most-likely represents a splicing intermediate, with *nad4-i3* being spliced out and *nad4-i1* still present. This is similar to the situation in turnip *nad4* where splicing of the first intron is delayed as compared to that of other introns (Gass et al. 1992).

Distribution of *nad4* introns in the Caryophyllales

We next wished to determine the presence or absence of the *nad4* introns in 15 species representing nine Caryophyllalean families (Rettig et al. 1992; Downie and Palmer 1994). Our analysis also includes buckwheat (Polygonales) and *Limonium sinense* (Plumbaginales), because phylogenetic study of chloroplast *rbcL* sequences (Rettig et al. 1992) indicates a close association of the two orders, Polygonales and Plumbaginales, with the Caryophyllales, and, as a result, these two orders can be

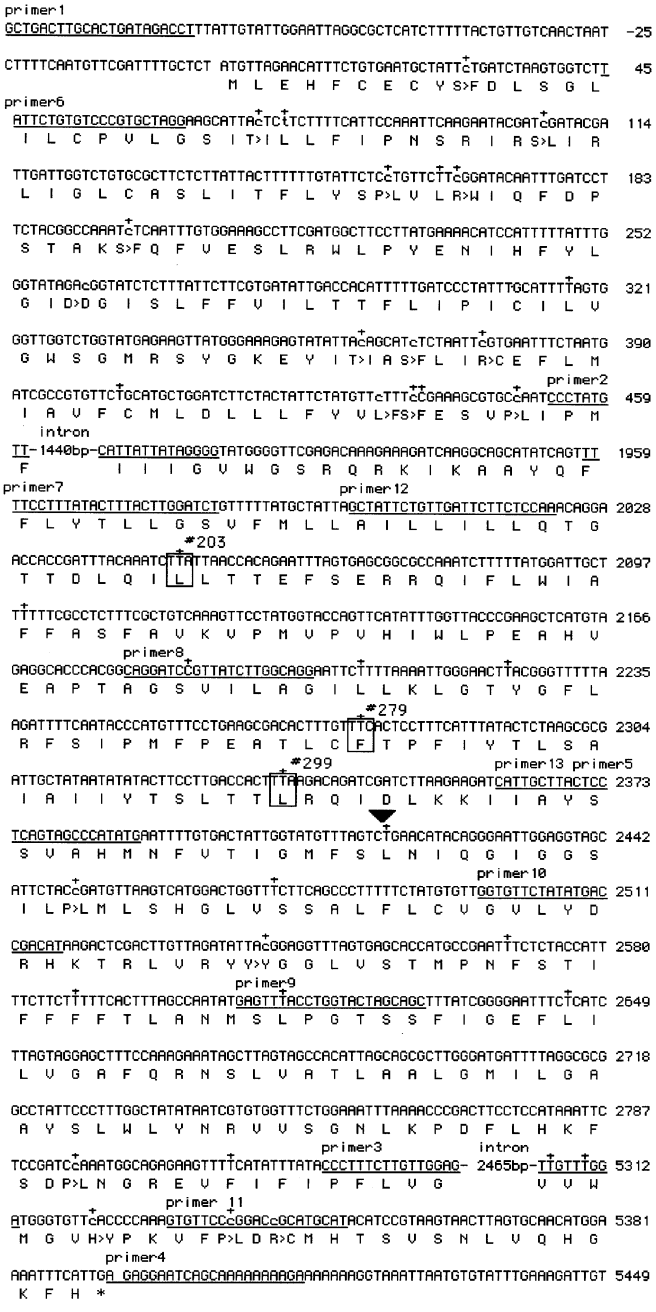

Fig. 1 Nucleotide sequence of the sugar beet *nad4* locus with the corresponding amino-acid sequence of the translation product. Numbering of nucleotides is from the beginning of the *nad4* ORF. The cytidine residues altered by RNA editing are shown by *lower case letters*. The amino-acid residues specified after editing are also shown. Positions of PCR primers are *underlined. The black triangle* indicates the position where *nad4-i2* is inserted wheat and turnip. RNA editing sites in turnip are shown by a *plus sign* (Geiss et al. 1994). The amino-acid residues #203, #279 and #299 are *boxed* (see text)

used as outgroup taxa. Total cellular DNAs prepared from the 18 species (sugar beet also included as a reference) were subjected to PCR analysis using three pairs of intron-specific primers: primers 6 and 7 (*nad4-i1*), primers 8 and 9 (*nad4-i2*) and primers 10 and 11 (*nad4-i3*).

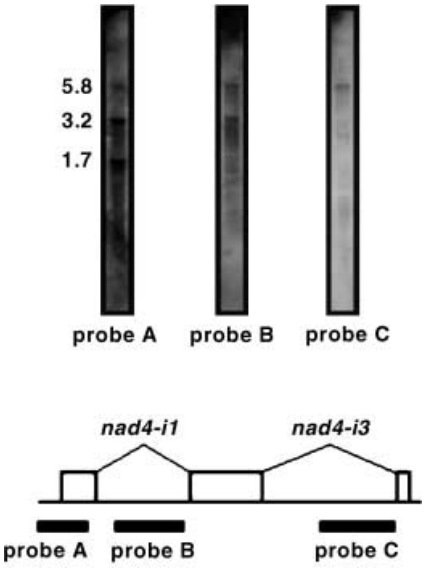

Fig. 2 Northern-blot analysis of the sugar beet *nad4* locus. Total mtRNA was hybridized with exon (*probe A*) and intron (*B and C*) probes. The *probes A*, *B* and C correspond to –550 to 425, 613 to 1428 and 4427 to 5277, respectively, on Fig. 1 (see accession number AB061226). Sizes of the transcripts are shown in kb

As shown in Fig. 3, the primer pair 8/9-directed amplifications resulted in a product of 0.4 kb from templates of all the Caryophyllalean species surveyed, but not from the two outgroup species, where a 3.4-kb amplification was detected instead. The nature of the PCR products was checked by Southern-blot analysis or nucleotide sequencing (data not shown), allowing us to conclude that the difference (approximately 3 kb) in PCR amplicon size is due to the loss of *nad4-i2* in all the Caryophyllalean species under study here. On the other hand, *nad4-i1* and *nad4-i3* are present in all the taxa examined, though the size varies from 1.2 to 1.9 kb for *nad4-i1* and from 2.5 to 2.9 kb for *nad4-i3*.

The presence of all three *nad4* introns in both a monocot (wheat) and several dicots, such as turnip and mung bean, strongly implies that the introns were present in the common ancestor of angiosperms and that their absence in various angiosperms is a derived feature (Lamattina and Grienenberger 1991; Gass et al. 1992). Besides the loss of *nad4-i2* described in the present paper, the same intron loss was reported in lettuce (*Lactuca sativa*, Asterales). The Asterales and Caryophyllales are distantly related to each other both in classification systems, based largely on morphology (Cronquist 1981), and in a phylogeny, based on *rbcL* sequence data (Soltis et al. 1999). We thus consider it much more likely that *nad4-i2* has been lost in at least two separate lineages of dicots than that the *nad4-i2* loss occurred only once.

RNA editing

To analyse the extent of RNA editing in transcrips of the sugar beet *nad4*, four primer combinations (1/3, 1/4, 2/3

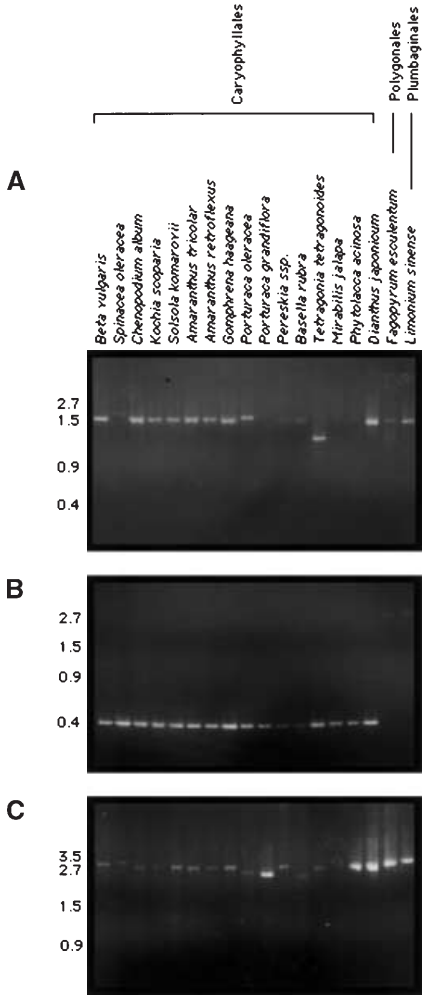

Fig. 3 PCR amplification of the *nad4* introns in 16 species of Caryophyllales as well as two outgroup taxa, *Fagopyrum esculentum* (Polygonales) and *Limonium sinense* (Plumbaginales). *Panels A, B and C* present the results of PCR amplification of *nad4-i1*, *nad4-i2* and *nad4-i3*, respectively. Size markers are shown in kbp

and 4/5) were used to amplify reverse transcripts. In the PCR reactions, no intron-containing PCR product was obtained, indicating that the two introns were correctly spliced out.

As shown in Fig. 1, we found a total of 19 C-to-U editing events, out of which 17 led to a change in the amino-acid specificity of the codon. RNA editing improves the overall sequence conservation of the protein (data not shown). Similar to the wheat and lettuce *nad4*, there is an apparent bias in the distribution of editing sites among exons; the first exon (*nad4-ex1*) is the most-highly edited exon, containing 13 edits; in contrast, no editing site was noted in the sequence (*nad4-ex2*) corresponding to wheat exon 2. Additionally, all seven of the edited bases in the turnip *nad4-ex2* exon (edited much more extensively when compared with wheat and lettuce *nad4-ex2*) are incorporated into the sugar beet genomic sequence (Fig. 1). These observations lead us to infer that the precise loss of the *nad4-i2* intron from sugar beet probably occurred via reverse transcription of an edited RNA intermediate, followed by homologous recombination between the intron-less cDNA and the original gene (Geiss et al. 1994).

With the observations described here in mind, we amplified *nad4-ex2* fragments (using primer pair 12/13) from the 18 taxa, which were subsequently sequenced. Three potential editing sites were found to occur in buckwheat and *Limonium sinense* which contain *nad4-i2*: Ser203 (T*C*A, where the predicted edit is italicized), Ser279 (T*C*C) and Ser299 (T*C*A) (see Fig. 1). Evolutionary conservation of Leu203 (TTA), Phe279 (TTC) and Leu299 (TTA) throughout the NAD4 proteins of angiosperms strongly suggests that the three codons may require RNA editing (Geiss et al. 1994). On the other hand, as might have been expected, Leu203, Phe279 and Leu299 were observed to be genomically encoded in all 16 Caryophyllalean species examined (data not shown). These results strengthen the suggestion that homologous recombination of an edited and spliced cDNA intermediate was involved in the loss of *nad4-i2* from the common ancestor of the Caryophyllales.

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References

Adams KL, Daley DO, Qiu YL, Whelan J, Palmer JD (2000) Repeated, recent and diverse transfers of a mitochondrial gene to the nucleus in flowering plants. Nature 408:354–357

Cronquist A (1981) An integrated system of classification of flowering plants. Columbia University Press, New York

Downie SR, Palmer JD (1994) A chloroplast DNA phylogeny of the Caryophyllales based on structural and inverted repeat restriction-site variation. Systematic Bot 19:236–252

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

Gass DA, Makaroff CA, Palmer JD (1992) Variable intron content of the NADH dehydrogenase subunit-4 gene of plant mitochondria. Curr Genet 21:423–430

Geiss KT, Abbas GM, Makaroff CA (1994) Intron loss from the NADH dehydrogenase subunit 4 gene of lettuce mitochondrial DNA: evidence for homologous recombination of a cDNA intermediate. Mol Gen Genet 243:97–105

Kubo T, Mikami T, Kinoshita T (1993) The sugar beet mitochondrial genome contains an ORF sharing sequence homoloy with the gene for the 30-kDa subunit of bovine mitochondrial complex I. Mol Gen Genet 241:479–482

Kubo T, Nishizawa S, Sugawara A, Itchoda N, Estiati A, Mikami T (2000) The complete nucleotide sequence of the mitochondrial genome of sugar beet (*Beta vulgaris* L.) reveals a novel gene for $tRNA^{Cys}$ (GCA). Nucleic Acids Res 28:2571–2576

Lamattina L, Grienenberger J-M (1991) RNA editing of the transcript coding for subunit 4 of NADH dehydrogenase in wheat mitochondria: uneven distribution of the editing sites among the four exons. Nucleic Acids Res 19:3275–3282

Mikami T, Kishima Y, Sugiura M, Kinoshita T (1985) Organelle genome diversity in sugar beet with normal and different sources of male-sterile cytoplasms. Theor Appl Genet 71:166–171

Qiu Y-L, Cho Y, Cox C, Palmer JD (1998) The gain of three mitochondrial introns identifies liverworts as the earliest land plants. Nature 394:671–674

Rettig JH, Wilson HD, Manhart JR (1992) Phylogeny of the Caryophyllales-gene sequence data. Taxon 41:201–209

Sambrook, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Senda M, Mikami T, Kinoshita T (1993) The sugar beet mitochondrial gene for the ATPase alpha-subunit: sequence, transcription and rearrangements in cytoplasmic male-sterile plants. Curr Genet 24:164–170

Soltis PS, Soltis DE, Chase MW (1999) Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. Nature 402:402–403