Extensive RNA editing and possible double-stranded structures determining editing sites in the atpB transcripts of hornwort chloroplasts

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ABSTRACT

Three nonsense codons and an unusual initiation codon were located within the putative coding region of the atpB gene of chloroplast DNA of the hornwort Anthoceros formosae. Nucleotide sequencing of cDNA prepared from transcripts revealed extensive RNA editing. The unusual initiation codon ACG was changed to AUG and three nonsense codons were converted into sense codons. In total 15 C residues of the genomic DNA were replaced by U residues in the mRNA sequences, while 14 U residues were replaced by C residues. This is the highest number of editing events for a chloroplast mRNA reported so far. Partial editing was also shown in a cDNA clone where 23 sites were edited but six sites remained unedited, representing the existence of premature mRNA. The expected two-dimensional structure of the mRNA shows the existence of a sequence complementary to every editing site, which can produce continuous base pairing longer than 5 bp, suggesting that mispairing in the double strand is the site determinant for RNA editing in Anthoceros chloroplasts. Comparison of the cDNA sequence with other chloroplast genes suggests that the mechanism arose in the first land plants and has been reduced during evolution.

INTRODUCTION

Editing is a post-transcriptional process which changes the primary sequence of RNAs, as compared with that of the corresponding DNA templates, and can therefore be regarded as a step in the regulation of gene expression. RNA editing was originally detected in the kinetoplast genetic system of trypanosomes (1) and was subsequently observed in nucleus-encoded mRNA (2) and mRNA encoded by mitochondrial genes from higher plants (3–5). More recently, several chloroplast transcripts of higher plants have been shown to be subjected to RNA editing (6,7, for a review see 8). Editing has been found in virtually all mRNAs of plant mitochondria (9). However, observations of editing have been restricted to the chloroplasts of seed plants, with the exception of rbcL transcripts of Anthoceros (10). All of these editing events in chloroplasts involve C→U conversions, except in Anthoceros. We report broad RNA editing of atpB transcripts of Anthoceros formosae; this is the first evidence for the existence of this gene product and the most extensive editing of chloroplast transcripts observed to date.

MATERIALS AND METHODS

The following oligonucleotide primers designed from the genomic DNA sequence of A. formosae were obtained from Biologica (Nagoya, Japan): P1, 5′-ATGCGAAA TTTAGCTAAGCAA T (–55 to –34); P2, 5′-CAAATCTACAATGAGTACCGTTTTT (549 to 525); P3, 5′-TTAGTAGATGGGAACAATCTCTTCAA (1464 to 1440). Numbering is from the translation start site of atpB.

Five clones containing atpB of A. formosae chloroplasts were prepared and identified as described previously (10). All contained an identical 7556 bp KpnI fragment, the structure of which is shown in Figure 1.

Total RNA was isolated and cDNA was synthesized as described previously (10). The cDNA amplified using the primer pairs P1/P2 and P1/P3 was ligated to pUC18 as described (10) and introduced into Escherichia coli DH5α by means of E. coli Pulsar (BioRad).

The nucleotide sequences of genomic DNA and cDNA were determined by dideoxy chain termination methods (11) using 7-deaza Sequenase v.2.0 (US Biochemical) as described (10). The resulting sequences were analyzed with Genetyx-MAC 8.5 software (SDC, Tokyo).

RESULTS

Unusual nucleotide sequence of chloroplast DNA from A. formosae

Five clones assumed to contain rbcL were selected from a DNA library of A. formosae by colony and Southern hybridization as described previously (10). Restriction enzyme mapping and partial DNA sequencing revealed that all clones contained a common 7556 bp KpnI fragment, with sequences homologous to accD, trnR, rbcL, atpB, atpE, trnM, trnV, ndhC and ndhK, in that order (Fig. 1). The gene arrangement is the same as that of

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Evidence of RNA editing

To examine the possibility of RNA editing we determined the mRNA sequence by analyzing the cDNA. The nucleotide sequences of the resulting five clones containing the entire coding region of *atpB* were determined and compared with that of genomic DNA. Three nonsense codons in the genomic sequence were changed into sense codons by a U→C conversion at positions 193, 415 and 1408. The unusual ACG sequence at the initiation codon was changed to AUG by a C→T conversion, even though TAA is used as the stop codon in *Angiopteris* (12) and *Marchantia* (13), suggesting that the sequence is that of chloroplast DNA. The complete nucleotide sequence of plasmid pK79 containing the 7556 bp KpnI fragment was determined (accession no. D43695). The sequence contains three nonsense codons of TAA in the putative coding region of *atpB*, though TAA is used as the stop codon in *rbcL* (10). In addition to these, ACG instead of ATG was identified at the putative initiation codon. These findings indicate that the RNA transcripts are edited.

**DISCUSSION**

Many processes requiring interaction with specific RNAs recognize not only primary sequence but also the secondary and higher order structures of the target RNAs. Guide RNA complementary to the segments of the edited mRNA has been detected in the trypanosome mitochondrial system (17) and mispairing in RNA was corrected by RNA editing in the stem of mitochondrial tRNA (18) and in the introns of mitochondrial transcripts (19). Therefore, we tried to find *cis*-acting determinants for the transcripts of *atpB*. Partial two-dimensional structures of the mRNA were estimated using Genetyx software, which showed stem structures containing editing sites at 2, 799, 820, 1223 and 1415 (Fig. 3). They were longer than 7 bp, although three of them contained G-U base pairs. The other editing sites were located in loop structures containing editing sites at 2, 799, 820, 1223 and 1415 (Fig. 3). They were longer than 7 bp, although three of them contained G-U base pairs. The other editing sites were located in loop structures.
or relatively short stem structures. Sequences complementary to them were found in a similar structure of mRNA using homology search software. They could form Watson–Crick and G-U base pairs which were longer than 5 bp. The G-U pairs usually seen in tRNA species and in the editing system of trypanosome (20) were located outside the edited nucleotides in the possible double-stranded structures.

One sequence of 9 bp, two of 8 bp, six of 7 bp, eight of 6 bp and six of 5 bp formed in the transcript of \( \text{atpB} \) without G-U base pairs. A sequence complementary to the pentamer which contains editing sites could be found within an ~1 kb sequence: the possibility of finding a specific sequence of 5 nt within a random sequence is \( \frac{1}{4^5} \). However, it would require 16 and 65 kb to find a specific sequence of 7 and 8 nt respectively. It would be difficult to find such a long specific sequence within the 1572 bases of \( \text{atpB} \) mRNA examined if the mRNA did not possess a functional sequence to interact with the editing site. This mRNA is highly biased towards A and U content (63%), therefore the probability of finding a specific AU biased sequence in the mRNA is increased. For example, to find a heptamer sequence the probability within a random non-biased sequence is \( \frac{2^7}{4^7} = 6.10 \times 10^{-5} \). For example, to find a heptamer sequence the probability within a random non-biased sequence is \( \frac{2^7}{4^7} = 6.10 \times 10^{-5} \). This value is not high and thus it is not easy to find a heptamer sequence within the mRNA. In addition, the sequences observed in the possible double-stranded structures do not overlap, suggesting that they interact with only one partner, though they contain 350 nt in total, which corresponds to 22% of the analyzed sequence. Similar double-stranded structures containing all the editing sites could form by Watson–Crick base pairing in the rbcL mRNA of \( \text{Anthoceros} \) (10), in which three sequences of 8 bp, four of 7 bp, five of 6 bp and five of 5 bp were detected.

Therefore, we believe the complementary sequences within the same mRNA to be factors in selective recognition of the nucleotide to be edited in \( \text{Anthoceros} \), though such factors have been reported to be of extraplastic origin in higher plants (21). Mispairing can be corrected by an editing enzyme such as cytidine deaminase (22) or transaminase. This hypothesis can explain several phenomena of RNA editing which have been observed in chloroplasts and mitochondria. No general consensus sequence has been deduced from aligning heterologous sites, thus implying the existence of \( \text{trans} \)-acting specificity factors for individual editing sites (23). Our hypothesis can provide sequences complementary to individual editing sites, although some such functional sequences have not been found in higher plant chloroplast genomes (24). That complementary sequences exist in the chloroplasts of the hornwort but not in those of higher plants could be explained as follows: copies of the chloroplast genes containing the complementary sequence were transferred to the nucleus and the remaining genes lost the sequence during evolution. Several cases of gene transfer from chloroplast to nucleus have been documented; the \( \text{tufA} \) gene was transferred within the green algal lineage giving rise to land plants (25) and spinach nucleus contains integrated sequences that are homologous to chloroplast DNA sequences (26).

The amino acid sequences deduced from the genomic DNA and cDNA of \( \text{Anthoceros} \) were compared with those of other plants. As shown in Figure 4, the homology of the amino acid sequence deduced from cDNA was always higher than that deduced from genomic DNA, suggesting that the former sequence reflects the functional protein sequence. Amino acids at 286, 287 and 344 were changed from Ser to Leu by RNA editing in \( \text{Anthoceros} \). In these sites TCA codons deduced from genomic DNA were changed to TTA. The same codons were found at 286, 287 and 344 in \( \text{Angiopteris} \) and \( \text{Pinus} \) indicating that at least three sites in \( \text{Angiopteris} \) and one in \( \text{Pinus} \) are edited. RNA editing occurs at 29 sites in the hornwort, three in the fern, one in the gymnosperm and at no sites in the angiosperm, indicating a tendency towards a reduction in the number of editing sites of \( \text{atpB} \) transcripts during the evolution of plants, with the exception of the liverwort. This suggests that either RNA editing arose in chloroplasts of the first land plants and disappeared from the liverwort or, alternatively, that it arose in the common ancestor of \( \text{Anthoceros} \) and vascular plants (10). The study of RNA editing in chloroplasts has been rather restricted in angiosperms. However, that more editing sites were observed in the gymnosperm than in the angiosperm (27), with the most extensive editing
observed in the hornwort, suggests that RNA editing is distributed widely in the transcripts of many genes of primitive land plants.

Hiesel et al. (28) showed RNA editing in mitochondria of all major groups of land plants and Malek et al. (29) showed the highest frequency in a lycopsid Isoetes and the highest frequency of reverse events in the hornwort Anthoceros, which is among the first land plants in their phylogenetic tree. They showed the existence of RNA editing in some bryophytes but not in the green alga Chara. We also analyzed editing events in rbcL transcripts of the green alga Nitella spp., but could not detect any. These results support a scenario in which the mechanism of RNA editing arose in chloroplasts and mitochondria of the first land plants. RNA editing would affect the evolution of land plants, though further studies of this event in primitive green plants are required.

As already described in the rbcL transcripts of hornwort chloroplasts (10), atpB transcripts show extensive U→C conversion. This was likely to have been prevalent among primitive land plants and to have disappeared more quickly than C→U conversion, because several events of reverse conversion have been observed in the mitochondria of bryophytes and pteridophytes (29).

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