Editing of the wheat *cox*III transcript: evidence for twelve C to U and one U to C conversions and for sequence similarities around editing sites

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ABSTRACT

The complete cDNA sequence corresponding to the wheat *coxIII* gene transcript (coding for subunit 3 of cytochrome oxidase) has been determined by a method involving cDNA synthesis using specific oligonucleotides as primers followed by PCR amplification, cloning and sequencing of the amplification products. In 12 different clones, the same 13 nucleotide modifications have been found as compared to the genomic mitochondrial DNA sequence. Among these modifications, 12 are C→U conversions which change codons identities, thereby increasing the homology between the wheat COXIII protein and the corresponding protein of non-plant organisms. The 13th modification is a silent U→C conversion which seems to be an unfrequent editing eventin plant mitochondria. Homologies can be found between sequences surrounding editing sites in the *coxIII* transcript and in other wheat mitochondrial transcripts. The presence of such homology suggests that these sequences could base-pair with a common RNA molecule which might be involved in editing site recognition.

INTRODUCTION

Plant mitochondrial (mt) genomes are different from those of other organisms. Their large size, the presence of repeated sequences involved in recombination processes, the existence of promiscuous chloroplastic DNA sequences, the differences in genome organization even between closely related species and the presence of specific genes (such as those coding for several mitochondrial ribosomal proteins) which are not found in fungal or mammalian mitochondrial genomes, are some of the characteristic features of plant mt genomes (1). Although their structure complexity has been extensively studied, the regulation of plant mt genes expression is still poorly understood, and the sequences or factors involved have not yet been identified. The recent finding that an RNA editing mechanism is active in plant mitochondria (2–4) has revealed another degree of complexity in the control of plant mt genes expression. This activity results in the specific conversion of some cytidine residues to uridines, and is essential for the correct expression of plant mt protein genes. The reasons for the existence of RNA editing in plant mitochondria, and for the existence of other RNA editing mechanisms in other organisms (5–9) is not clear, but it is likely that RNA editing confers functional advantages as a modulator of gene expression. In agreement with this idea, it appears that RNA editing in plant mitochondria plays a role in the conservation of protein sequences during evolution, as most of the nucleotide conversions result in modifications of the codons which then specify amino acids that are conserved in the mt proteins of non-plant organisms (2). The basis for the specificity of the editing system is still not clear, but recent results in the study of the editing processes occurring in the mitochondria of *Leshmania tarentolae* (10) and in *Xenopus laevis* (9) suggest that anti-sense RNAs, transcribed either from the same genomic sequences or from other regions of the genome, might be involved in the RNA editing recognition processes. However, up to now, such guide RNAs (gRNA, 10), which could be involved in editing site recognition, have not been detected in plant mitochondria.

Because of editing in plant mitochondria, amino acid sequences of plant mt proteins cannot be correctly deduced from the corresponding gene sequences. Therefore, the determination of the mRNA sequence, either directely from the RNA or deduced from a cloned cDNA sequence, is a necessary step, if sequencing of the protein is not possible.

In this report we present the complete cDNA sequence corresponding to the wheat mitochondrial *coxIII* transcript. This sequence shows that 13 nucleotides are different from the genomic sequence. Among these modifications, 12 are C→U conversions, whereas one is a U→C silent modification. This demonstrates that a plant mt RNA editing process can carry out the reverse U→C conversion. In order to understand the basis for the RNA editing specificity, the sequences of the regions surrounding editing sites have been compared. It appears that some of these sequences can be grouped into a number of related families according to their sequence homology. Moreover, sequences belonging to these families can be found around editing site

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positions in other wheat mt RNAs. This is an indication that the recognition mechanism operating in plant mt RNA editing might involve antisense RNAs.

MATERIAL AND METHODS

cDNA synthesis and amplification

Mitochondrial DNA and RNA were prepared from 6 to 8 day old etiolated seedlings as previously described (11). Total mitochondrial RNA (0.5 mg/ml) was treated with 100 U/ml of RNase-free DNase (Pharmacia) during 15 min at 37°C. After phenol extraction and ethanol precipitation, 20 μg of mt RNA were mixed with 50 ng of primer 426 (a 27-mer oligonucleotide complementary to the 3′ end of the *coxIII* mRNA, which has the sequence GGAGAATTCTCTTTGTCTTCGAGCTTT) in 10 μl of 0.4 M NaCl, 10 mM Pipes pH 6.4, 1 mM EDTA. After denaturation at 80°C for 5 min, the reaction mixture was incubated 2 hours at 50°C for primer hybridization. First strand synthesis was initiated by addition of 90 μl of 50 mM Tris-HCl ph 8.3, 6 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP and 10 U of AMV reverse transcriptase (Life Sciences). The resulting cDNAs were amplified by PCR, using primers 426 and 422 (a 25-mer oligonucleotide whose sequence was derived from the 5′ end of the mRNA, and which has the sequence GAGAATTCATGTTCACGCCGGAGTG). The primers contained mismatches as compared to the DNA sequence, in order to create EcoRI sites at the ends of the DNA fragment amplified by PCR (Fig. 1). The amplification mix contained, in 50 μl, 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 67 mM Tris-HCl pH 8.8, 80 μg/ml of BSA, 10% dimethyl sulfoxide, 300 μM of each dNTP, 100 pmol of each oligonucleotide primer and 2.5 U of *Taq* DNA polymerase (Biolabs). Amplification was obtained by 30 cycles of the following steps : denaturation (92°C; 1 min), annealing (47°C; 2 min) and DNA polymerization (72°C; 3 min), followed by a final incubation at 72°C for 10 min. Amplification products were purified on NACS columns (BRL). After digestion with EcoRI, amplified cDNAs were cloned in M13mp19 and sequenced. Primer 613 (a 16-mer which has the sequence TCCACGTTGGAAGGGC, corresponding to positions 749 to 754 in the published gene sequence, ref. 12), was used for internal sequence priming.

Direct sequence of uncloned genomic mt DNA

The fragment to be sequenced was amplified by PCR, as described above, using 1 μg of total mt DNA as template. The amplified DNA fragment was purified from the excess of primers by chromatography on a NACS column and sequenced, using the end-labelled internal primer 613 and T7 DNA polymerase (Pharmacia).

Probe for anti-sense transcripts

The 2.1 kb XhoI fragment containing the wheat *coxIII* gene had been cloned in anti-sense orientation in M13mp19 (12). The labelled sense strand was synthesized, using the universal sequence primer and the Klenow enzyme and, after digestion with EcoRI, the single strand labelled fragment was isolated in a strand separation agarose gel. After elution, the fragment was used as a probe in Northern experiments in order to specifically detect anti-sense transcripts.

RESULTS

Cloning of *coxIII* cDNAs

To specifically synthesize and amplify *coxIII* cDNA sequences, two oligonucleotides (422 and 426) were prepared, with sequences in opposite orientation, corresponding to the extremities of the wheat *coxIII* mRNA (determined by primer extension and S1-nuclease mapping, ref. 12). Before cDNA synthesis, mt DNA contaminating mt RNA preparations was eliminated by DNase treatment, since it would also be amplified during the PCR step. Oligonucleotide 426, complementary to the 3′ end of the *coxIII* mRNA, was used to prime cDNA synthesis, as described in 'Methods'. The primer extension products were subsequently amplified by PCR. As a control, DNase-treated mt RNA was submitted to PCR without the reverse transcriptase step. Upon analysis in agarose gel, only the sample containing reverse transcriptase showed an amplification product, demonstrating that no amplification resulted from residual mt DNA. The 1.2 kb amplified fragment was cloned in the EcoRI site of vector M13mp19. The analysis of the cDNA sequence was performed in several clones, as the high error frequency of *Taq* DNA polymerase results in nucleotide misincorporations in the amplified products. Considering the estimated rate of error of *Taq* DNA polymerase (13), after 30 cycles of amplification an error in each 800−400 nucleotides can be expected. Therefore, we only considered the nucleotides different from those in the genomic DNA sequence which were found in more than one cDNA clone. In the 12 clones sequenced, several modifications were found which were attributed to errors introduced by the *Taq* DNA polymerase. None of those modifications was found at the same position in more than one of the clones. These modifications were mostly transitions, but some transversions could also be found.

Editing sites in the wheat *coxIII* cDNAs

Each of the 12 clones had in common 13 nucleotide modifications as compared to the genomic DNA sequence. These modifications were all located in the coding sequence; no modification was found in the untranslated flanking region, namely in the 323 nucleotide long leading sequence. Among these 13 modifications, 12 are C→T conversions, which are likely to result from the C→U editing activity. Two of them have previously been identified (2). All 12 C→U conversions result in a modification of the specified amino acid (Figures 1 and 2), in agreement with our previous observation that editing in plant mitochondria is involved in modulating the expression of the DNA-encoded message (2). A single nucleotide modification which doesn't result from a C→U conversion is a C present in all cDNA clones and which must derive from a T present in the genomic sequence (Figures 1 and 2). This apparent U→C conversion is a silent modification and does not occur at the same position in the *Oenothera coxIII* transcript (4). Given the similarities between wheat and *Oenothera coxIII* sequences, we first considered that this difference between wheat DNA and cDNA sequences could result from an error in the wheat mt DNA sequence, introduced during the cloning and sub-cloning steps. To check this hypothesis, we determined the genomic sequence of this region on uncloned mt DNA. The same oligonucleotides used to amplify cDNAs were used for amplification of mt DNA sequences, as described in 'Methods'. The direct mt DNA sequence revealed that no error had been introduced during the genomic sequence

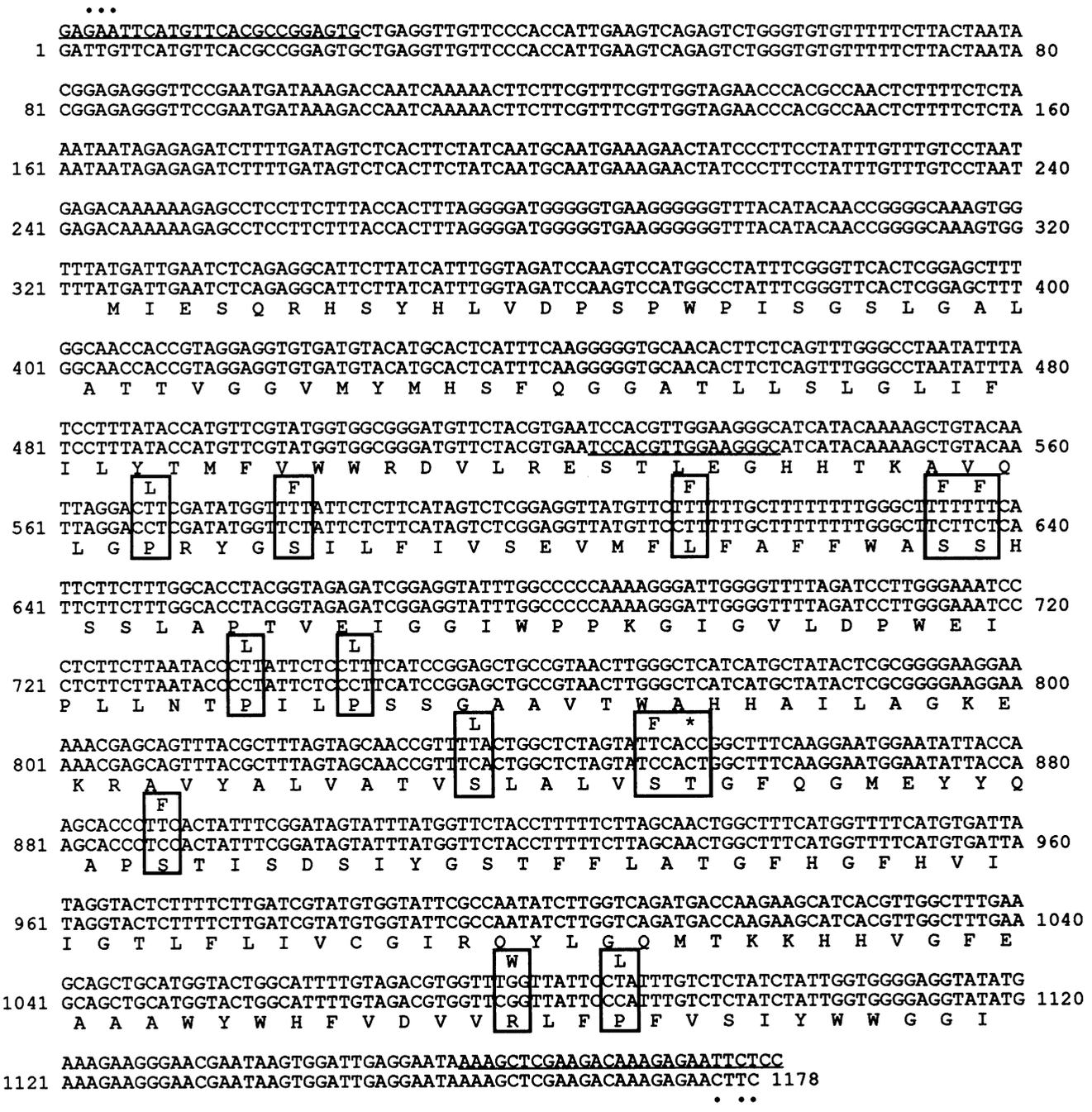

Figure 1. Comparison between wheat mitochondrial genomic and cDNA *coxIII* sequences. The upper sequence is the consensus sequence obtained from 12 independent *coxIII* cDNA clones, while the lower sequence is the corresponding mt DNA sequence. The sequences corresponding to oligonucleotides 422 and 426 used for cDNA amplification by PCR are underlined, and the sequence of oligonucleotide 613 used as internal sequence primer is underlined with an arrow. The amino acid sequence deduced from the genomic sequence using the universal genetic code is presented. Codons modified by editing are boxed, with the corresponding amino acid modifications indicated above the cDNA sequence. The silent T→C modification found is represented by an asterisk. Dots indicate nucleotide mismatches introduced in the oligonucleotide sequences in order to create EcoRI sites.

studies (Fig. 2 b), and therefore that the U→C conversion results from an editing activity in the mitochondria.

Conservation of COXIII protein in plants

Most amino acid modifications resulting from C·U editing increase the homology between the wheat COXIII protein and the corresponding protein of non-plant organisms (Fig. 3). Comparison of the wheat cDNA and genomic *coxIII* sequences with *coxIII* genomic sequences determined in other plant species (*Oenothera*, maize, soybean and broad bean, 14–17) reveals strong sequence homologies between the genomic sequences of wheat and other plants, suggesting that at positions where editing occurs in wheat *coxIII* RNA, the same editing process is operating in those plants. If the protein sequence deduced from the wheat cDNA sequence is assumed to be highly conserved in the other plant species, it is possible to predict other putative sites of editing

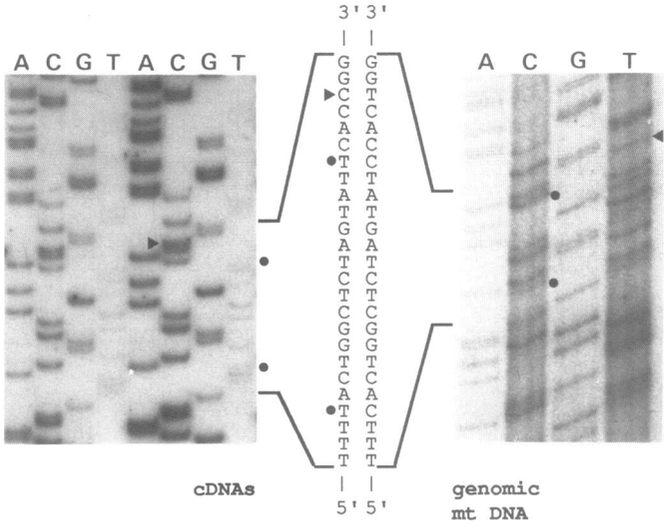

Figure 2. Comparison between *coxIII* cDNA sequences and the corresponding gene sequence determined on uncloned genomic DNA. Part of the sequence of two of the cDNA clones are presented, corresponding to nucleotides 1050 to 1105 of the published *coxIII* mt DNA sequence. The sequence from uncloned mt DNA is shown in parallel. Positions where C to T modifications are found are indicated by dots, whereas the position where a T to C conversion is found is indicated by an arrowhead.

in the *coxIII* transcripts of those plants. These modifications would result in specifying the same amino acid as the one deduced from the wheat *coxIII* cDNA sequence (Fig. 3). This reasoning seems to be valid, as it correctly predicts four editing sites in the coding sequence of the *Oenothera coxIII* transcript, which have indeed been identified by partial cDNA sequence (4). Considering these possible editing sites in *coxIII* RNA sequences, the homology between plant COXIII proteins is increased. As an example, homology between the wheat COXIII protein and the *Oenothera* and broad bean COXIII proteins would increase respectively from 93.6 to 96.6% and from 96.2 to 98.5%.

In some plants the amino acid which is specified only after editing in wheat mitochondria is already encoded by the mitochondrial DNA sequence (for instance, aminoacid positions 82, 97, 141, 176 and 189 in Figure 3). This observation implies that, during evolution, DNA mutations can be recognized and corrected by editing or, alternatively, that edited sequences have been integrated in the mt genome of some plants which no longer exhibit editing at these positions.

Consensus sequences

The sequences surrounding editing sites found in the wheat *coxIII* sequence have been aligned in order to identify possible conserved sequences or secondary structures that could be implicated in the editing specificity. No such sequence could be found, and the

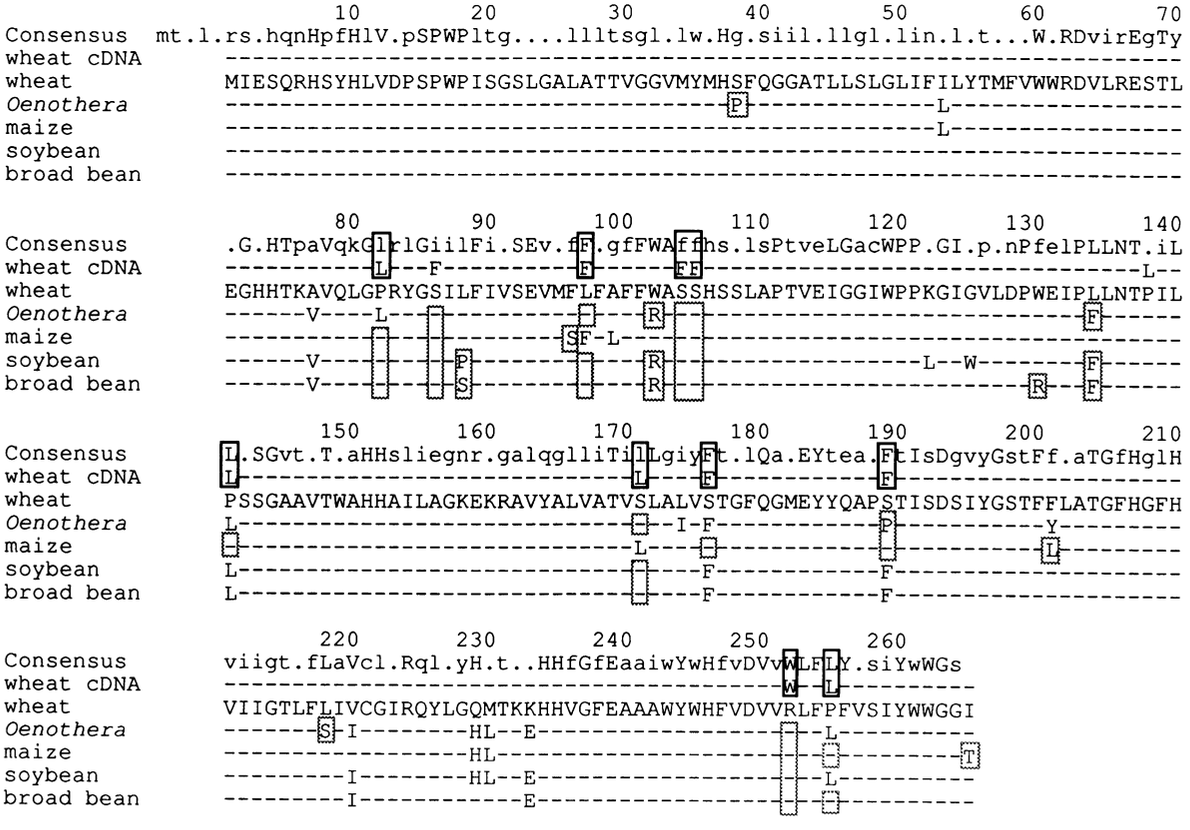

Figure 3. Amino acid homologies between wheat COXIII protein deduced from the nucleotide sequence and other mitochondrial COXIII proteins. The aminoacid sequence deduced from the wheat *coxIII* genomic sequence, using the universal genetic code, is compared with: 1) the consensus COXIII amino acid sequence based on mt *coxIII* sequences from non-plant species (found in GenEMBL Data Bank). Dots in the consensus sequence indicate non-conserved positions, lower case letters indicate partial conservation and capitals indicate complete conservation. 2) the amino acid sequence deduced from the wheat *coxIII* cDNA sequence. 3) the amino acid sequences deduced from other plant mitochondrial *coxIII* sequences. A dash indicates identity to the amino acid deduced from the wheat mt DNA sequence. Solid line boxes indicate increase of homology between the wheat COXIII protein and the consensus sequence, as a result of RNA editing. Dotted line boxes indicate putative editing positions in the other plant sequences, which would result in specifying the same amino acid as the one specified by the wheat *coxIII* cDNA sequence.

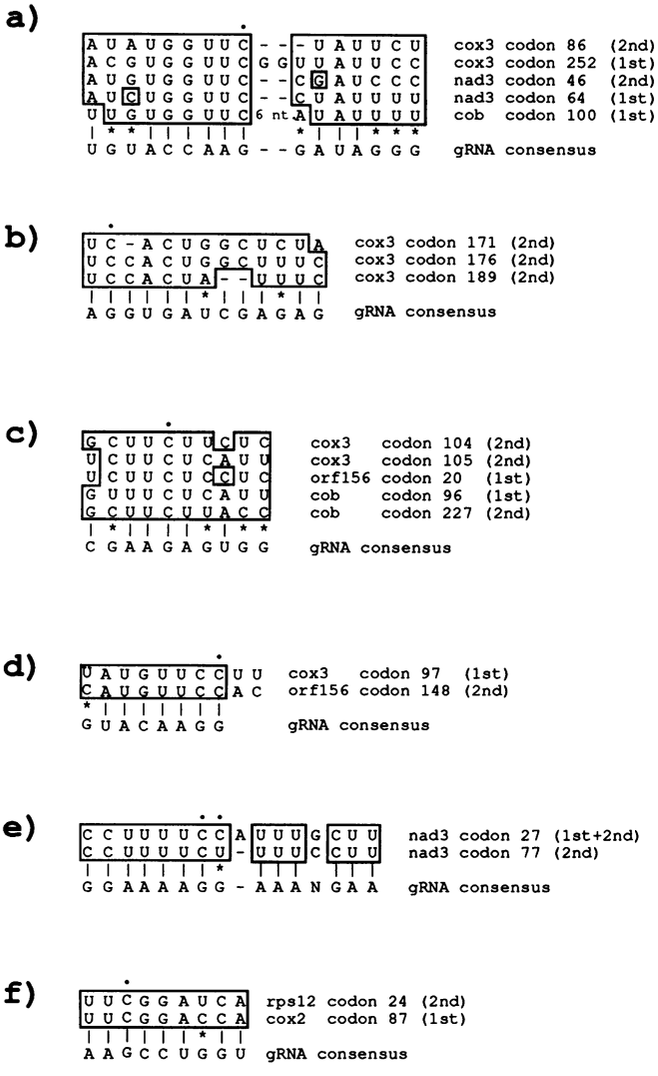

Figure 4. Homologies between sequences surrounding wheat mt editing sites. Sequences flanking different editing positions in wheat mitochondrial transcripts (designated by the position of the modified codon) are grouped by sequence homologies (a–f). In each group of sequences, the position of the edited C is indicated by a dot above the upper sequence and its position (1st or 2nd) in the modified codon is indicated. The editing sites compared have been found in the wheat mt transcripts of *coxIII* (this work), *coxII*(2), *cob* (2), *nad3*, *rps12* and *orf156* (in preparation). Nucleotides which could be involved in base-pairing with a same putative guide RNA (indicated below each family) are boxed. Positions where G:U base-pairing would be required for complementarity are indicated by asterisks.

only conserved feature revealed is that edited cytidines in *coxIII* are always preceded by a pyrimidine. It is therefore not likely that the editing machinery recognize a single nucleotide sequence motif. However, comparisons between individual editing sites allowed us to classify several of these motifs, based upon sequence homology, into families of editing related sites (Fig. 4). Between these related sequences, homologies extend over few nucleotides if one considers U equivalent to C, as if such sequences would be involved in RNA-RNA interactions accepting G : U base-pairing in addition to the standard G : C base-pairing. Homologies between editing sites does not necessarily involve editing sites located on the same transcript and does not depend on the amino acid being specified after editing. As an example, editing sites involving codons 86 and 252 of the wheat *coxIII* sequence have similar flanking sequences, but respectively modify a serine codon into a phenylalanine codon and an arginine codon into a tryptophan codon. Comparison of editing sites present in other wheat mt transcripts also revealed homologies between the sequences surrounding some of these editing sites (Fig. 4 e–f).

Searching for anti-sense transcripts

There are several examples of specific RNA modification activities involving RNA-RNA interactions (e.g. for pre-mRNA splicing and for RNA processing by RNase MRP; see ref. 18 and 19), and it is possible that the specificity of RNA editing is dependant upon an antisense template. Considering this possibility, a probe able to hybridize specifically with a putative *coxIII* anti-sense sequence was prepared (see 'Methods'). Unfractionated mt RNA, containing high and low molecular weight RNAs, was used in Northern hybridizations. No anti-sense transcript could be detected, and if such RNA molecules exist, either they are present in low concentration (at the level of background hybridization signals) or they are transcribed from different genomic loci which have little overall complementarity to the gene transcript.

DISCUSSION

The discovery of RNA editing in plant mitochondria raises several important questions, especially concerning the basis for editing specificity and the nature of the enzymatic activities involved.

In wheat *coxIII*, 13 edited positions were found in the 12 cDNAs which were sequenced. As cDNAs have been amplified by PCR before cloning, it could be argued that most of the clones originated from a same cDNA molecule. Although we cannot test this hypothesis, it is unlikely because the coxIII mRNA is abundant in wheat mitochondria (12), so that a great number of full size cDNAs must have been synthesized. Furthermore, using the same method to clone *nad3* cDNAs (manuscript in preparation), we obtained many different clones, most of them partially edited, and could not find two partially edited cDNAs of identical sequence. Partially edited *nad3* transcripts also exist in *Oenothera* mitochondria, as a number of independent cDNA clones were found to be differentially edited (20). The meaning for the accumulation of partially edited *nad3* RNAs is not clear, since translation of those transcripts would result in proteins with different amino acid sequences. For wheat *coxIII*, no unedited or partially edited cDNA was found.

The existence of a U→C conversion in wheat *coxIII* transcripts suggests a possible reverse activity of the C→U editing system. In trypanosome, although U additions are much more frequent, deletion of U residues can also be carried out by the editing system (6). The plant mt editing system may also be able to carry out the reverse editing reaction. However, in plant mitochondria, the reverse reaction seems to be a very rare event as no other example has been found so far in other wheat mt transcripts. The U→C modification found in wheat *coxIII* does not change the codon identity, as ACU and ACC are both specifying threonine. Other editing events occurring in non-coding regions or resulting in silent codon modifications have also been found in other plant mitochondrial transcripts (3, 4, 20, 21). These events seem to be exceptions and may introduce translational advantages to the edited RNAs. For example, in *Oenothera*, an editing site in the leader sequence of *rps14* RNA results in a 'stronger' ribosome binding site (21). It is also possible that editing having no apparent translation advantage is the result of RNA over-editing, a

phenomenom that seems to occur in trypanosome mitochondria, where editing intermediates can contain stretches of Us presumably inserted in excess (22).

All 12 C→U substitutions in wheat *coxIII* transcripts result in changes of codon identities, increasing the homology between the wheat COXIII protein sequence and the consensus COXIII sequence of non-plant organismes (Fig. 3). Putative editing in other plant *coxIII* sequences, as deduced from gene sequences comparisons, would also result in the synthesis of proteins more similar to the wheat one. Editing may have had in evolution an important function in correcting mutations occurring in coding sequences. This would explain that in some plant mt DNA sequences, a T is found at positions where a DNA-encoded C is edited into a U in the wheat RNA sequence.

The most intriguing problem concerning RNA editing is how specificity is achieved. Possible explanations for editing specificity have been proposed for the trypanosome mitochondrial and *Xenopus* editing systems, implying that specificity is due to complementarity with antisense RNAs (9,10). In *Xenopus*, an editing mechanism responsible for conversion of adenine residues into inosines has been shown to occur only in the double-stranded region of two complementarity transcripts (9). However, a *coxIII* sense probe failed to detect any *coxIII* antisense transcript. Therefore, if antisense RNAs are required for wheat *coxIII* RNA editing, either they exist in very low amounts or they have limited complementarity to the gene sequence. In trypanosome mitochondria, a model has been proposed which associates the editing enzymes with RNA templates partially complementary to the edited RNAs (10). Complementarity with those guide RNAs (gRNAs) would determine the region of editing and the number of U residues to be added or deleted. Sequence homologies can be found around different wheat mt editing sites. These homologous sequences could be recognized by factors required for editing specificity. It is therefore possible that putative gRNAs transcribed from a different region of the mt genome or from the nuclear DNA recognize more than one editing site in wheat mt transcripts. The mitochondria would just need a restricted set of gRNAs sequences for the recognition of a large number of editing positions. If this hypothesis is correct, complementarity between gRNAs and mRNAs in plant mitochondria would be restricted to a few nucleotides, smaller than in trypanosome where complementarity between gRNAs and mRNAs involves 14 to 51 nucleotides. However, little complementarity between RNAs does not exclude strong specificity. It is possible that if such gRNAs exist in plant mitochondria, they share the same sequence motifs required for interaction with the editing enzymes, while only a limited sequence is necessary for the editing specificity. As examples of high specificity achieved through limited RNA-RNA complementarity, procaryotic ribosome binding sites are restricted to a few nucleotides in mRNAs leader sequences, and the RNA component of mouse mt RNase MRP has only a decamer sequence complementary to a conserved region of the RNA substrate (19). Therefore, in plant mitochondria, recognition of editing sites may involve gRNAs showing limited homology with the sequences to be edited, and a same guide RNA could be used to recognize different editing sites, in the same or in different transcripts.

Although differences exist between the editing systems found in trypanosome and plant mitochondria, it is possible that both systems derived from an ancestral mitochondrial mechanism, when information contained in RNA sequences had a more fundamental role, perhaps before the appearance of DNA (RNA world). That editing activities are present in plant mitochondria, but not in mammalian or yeast mitochondria, may reflect the peculiar phylogenetic origin of the plant mitochondrial genome (23).

After this report was completed, a U→C editing conversion was reported to occur in the cytochrome b cDNA of *Oenothera bertiriana* (24). Unlike the conversion reported in this paper, this modification results in changing the identity of the encoded aminoacid. However, in wheat mt *cob* cDNA sequence (2), the same nucleotide position of the same codon is not involved in such a conversion. The reverse situation is found for the U→C editing position reported in this paper which is not found at the same position of the coxIII cDNAs from *Oenothera bertiriana* (4). This leads to the idea that these unfrequent conversions are not important for the expression of the corresponding genes in plant mitochondria.

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