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Analysis of *cis*-elements for RNA editing at two sites in the *atp*9 mRNA

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1. Abstract

To analyse the *cis*-requirements for RNA editing sites a newly developed *in-vitro* RNA editing system was employed. The system is based on a sensitive mismatch detection by the thymine glycosylase (TDG) and allows quantification of RNA editing products generated in mitochondrial extracts.

The *cis*-requirements for editing of *atp*9 site 1 in pea were investigated by templates deleted, sequence exchanged templates, and competition reactions. In the pea system, deleted templates in steps of 10 nucleotides between –40 to -20 edit correctly but with decreased efficiency, while deletions with less than 20 upstream nucleotides do not support editing. 3' deletions have little effect in pea, suggesting little influence on recognition. These results suggest that 20 nucleotides upstream are necessary and sufficient for recognition of the editing site. Stepwise mutated RNAs as templates or competitors reveal distinct substructures of the *cis*-elements. In pea a sequence element situated -40 to –35 enhances editing. The essential core region for recognition is restricted to the 10 nucleotides between -15 to –5. Experiments show that the enhancing effect of the region –40 to –35 can be titrated. This suggests, that either an abundant *trans*-factor is participating in recognition or an *in-vitro* artificial sequence effect is observed. The *trans*-factors interacting with the core region are present in restricted amounts, since they are sensitive to competition.

To investigate the evolutionary adaptation of sequence variations 5' of an editing site in another plant, *atp*9 (1) was investigated in cauliflower. Like in pea, 20 upstream nucleotides are essential and sufficient for editing. In cauliflower 3' deletions affect editing up to 50 %, suggesting a (sterical) influence of the +1 nucleotide. This inference is experimentally supported by the effect of +1 exchange mutants. Relative to pea, the core recognition region extends further upstream, suggesting adaptation of the *trans*-element(s). All recognition elements are located within the conserved area. To investigate whether neighbouring sites can influence each other, two RNA editing sites *atp*9 (1) and *atp*9 (2) located 30 nucleotides apart were analysed in the cauliflower mitochondrial lysate. Deletion and competition experiments reveal specific determinants for the second site. The *cis*-recognition elements are confined to 20 nucleotides upstream of the second C, which is edited independently from the first site. The deletion of an enhancer located 50 to 70 nucleotides upstream decreases editing efficiency. This suggests a *cis*-/*trans*- interaction effect over a larger distance.

To provide *cis*-recognition targets for *trans*-elements screening for further editing sites processed *in-vitro* was done theoretically and experimentally. Several editing sites in the two genes *ccb*206 and *nad*5 were identified as new *in-vitro* accessible targets.

1. Abstract (German)

Die *cis*-Elemente einer RNA-Editingstelle wurden in einem neuen *in-vitro* RNA-Editing-System analysiert. Dieses System basiert auf einer sensitiven Analyse von Fehlpaarungen, die durch die Thymine-DNA-Glykosylase detektiert werden und erlaubt die sensitive Quantifizierung der *in-vitro* RNA-Editing-Produkte.

In der Erbse wurden die *cis*-Elemente für die Editierung von *atp*9 (Editing-Stelle 1) durch deletierte Substrate, durch Sequenzaustausch mit Hilfe von partiell mutierten Substraten und durch Experimente mit konkurrierenden mutierten Substraten untersucht.

Im Erbsensystem editieren Substrate, die in Schritten von 10 Nukleotiden von –40 bis –0 deletiert wurden, korrekt, jedoch mit verminderter Effizienz, wenn bis zu 20 Nukleotiden 5' erhalten bleiben. Deletionen mit weniger als 20 verbleibenden Nukleotiden unterstützen das Editing nicht mehr. 3' Deletionen dieser Editierungsstelle haben einen geringen Effekt in der Erbse, was ebenso auf einen geringen Einfluss dieser Region hinweist. Die Ergebnisse deuten darauf hin, dass 20 Nukleotide 5' notwendig und ausreichend für die Erkennung der Editierungsstelle sind. Schrittweise mutierte Substrate, als Mutanten oder Kompetitoren eingesetzt, zeigen die Anordnung der *cis*-Elemente innerhalb abgegrenzter Bereiche. In der Erbse befindet sich ein Sequenz-Element im Bereich –40 bis -35, welches die Editing-Aktivität steigert. Die essentielle Kernregion für die Erkennung ist begrenzt auf ein Gebiet von ca. 10 Nukleotiden zwischen –15 bis –5. Experimente zeigen, dass der verstärkende Effekt der Region –40 bis –35 titriert werden kann. Dies legt nahe, dass entweder ein im Überfluss vorhandener *trans*-Faktor in die Erkennung eingebunden ist, oder ein künstlicher *in-vitro* Sequenzeffekt beobachtet wird. Die *trans*-Faktoren, die mit der Kernregion interagieren sind nur begrenzt vorhanden, da sie konkurrenzempfindlich sind.

Um die evolutionäre Anpassung an Sequenzvariationen vor der zu editierenden Stelle in verschiedenen Pflanzen zu untersuchen, wurde *atp*9 (1) im Blumenkohl untersucht. Wie in der Erbse, sind 20 Nukleotide 5' nötig und auch ausreichend, um erfolgreich zu editieren. In Blumenkohl reduziert die vollständige 3'-Deletion das Editing um 50%, was auf einen (sterischen) Einfluss des +1-Nukleotids hindeutet. Dieser Einfluss wurde experimentell durch den negativen Effekt der +1-Mutantionen bestätigt. In Erbse, erweitert sich die Kernregion weiter 5', was auf eine Anpassung der beteiligten *trans*-Elemente hinweist. Alle Erkennungsregionen befinden sich innerhalb der konservierten Gebiete. Um zu untersuchen, ob benachbarte Editierungsstellen einander beeinflussen, wurden zwei RNA-Editingstellen, *atp*9 (1) und *atp*9 (2), die 30 Nukleotide voneinander entfernt liegen, im mitochondrialen Blumenkohllysat untersucht. Deletions- und Konkurrenzexperimente zeigen von *atp*9 (1) unabhängige, spezifische Faktoren für die Erkennung der zweiten Editierungsstelle. Die *cis*-

Erkennungselemente sind begrenzt auf eine Sequenzumgebung von 20 Nukleotiden 5’ des zweiten editierten Cs. Die Deletion eines potentiellen Verstärkerelements, das sich 50 bis 70 Nukleotide 5’ befindet, vermindert die Editierungseffizienz. Dies legt einen *cis*-/*trans*- Effekt über einen längeren Sequenzabstand nahe.

Um *cis*-Erkennungs-Ziele für *trans*-Elemente bereitzustellen, wurde ein umfangreiches theoretisches und experimentelles Screening nach zusätzlichen *in-vitro* Editierungsstellen durchgeführt. Mehrere Editingstellen in zwei Genen, *ccb*206 und *nad*5 wurden während des Prozesses als *in-vitro* prozessierte Ziele ausgemacht.

2. Introduction

The central dogma of molecular genetics states that the information given in DNA is used to produce mRNA, which in turn will be translated into protein. DNA, mRNA and protein contain in essence the same information in their respective forms. A similar statement was made by the "one-gene-one-protein-hypothesis": Only one protein is translated from one gene.
In plant mitochondria the analysis of DNA sequences of the subunit II of the cytocrome c oxidase (*cox*II) showed that essential tryptophans conserved in non-plant species were exchanged to arginines, which would severely damage the protein structure (Fox and Leaver, 1981). To explain this problem a deviation from the genetic code was discussed. It was intriguing though, that only a small fraction of the CGG triplets behave abnormally. At the mRNA level, direct sequence analysis showed that the UGG codon for the indispensable tryptophan was restored: The codon CGG (DNA) has been changed to UGG in the mRNA. This formally expected triplet coding for tryptophan showed that plant mitochondria use the universal genetic code.
How has the codon been changed? Could it be that there is a system to correct "sequence mistakes" seen on the DNA-level in the RNA during or after transcription?
Plant organelles contain a machinery to modify the sequence of transcripts. The as yet unknown process changing C to U in the RNA has been called RNA editing.

2.1 General aspects of RNA editing in plants

The common type of editing in plants is the deamination of cytidine to uridine (Schuster and Brennicke 1994). There are also examples for a high "reverse" editing activity (Malek et al. 1996), e.g. in Bryophytes seen for example in the hornwort *Anthoceros formosae* (Yoshinaga et al. 1996 and 1997) or in ferns and related plants (Malek and Knoop 1997). In flowering plants the U to C editing is extremely rare (Fig. 1.1).
No editing has been observed in *Marchantia polymorpha,* a liverwort and one of the first living land plants (Oda et al. 1992) or in green algae (Wolff et al, 1994). Because *Marchantia* branches early during the evolution of land plants, it has been suggested that editing developed in the early stage of land plant development (Knoop 2004).
Every plant showing reverse editing also edits "forward". Interestingly, C to U conversions exist independent. This seems to point to the conclusion that even though the biochemical machinery for an amination process should be quite different from the one of a deamination process, reverse editing is based on the biochemical tools of C to U editing. It was outlined, that the

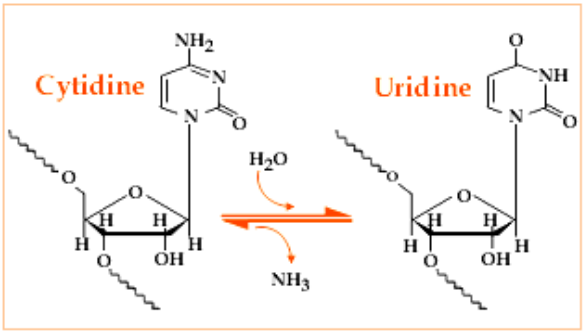


Figure 1.1: Deamination process during RNA editing in plants. The actual biochemical course remains unsolved.
Source: http://nobelprize.org/educational_games/medicine/dna/a/splicing/rna_editing.html

scenario of one enzyme involved in both, the forward and reverse reactions, is biochemically improbable (Takenaka et al. 2006, review). But it is not impossible. At least if not the performing enzyme, the recognition system could be shared.
Which of the events, C to U editing or reverse editing, was established first during evolution is still a point of discussion. It can be assumed that during evolution either U to C editing got lost while the C to U editing still persists in higher land pants, or U to C was never established in higher land plants.

As described above, RNA editing was first reported in mitochondrial transcripts (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al. 1989) and later on in mRNAs of chloroplast transcripts. A comparison of editing in mitochondria and in chloroplasts has shown differences in editing frequencies. During mitochondrial genome sequencing projects of *Arabidopsis thaliana* (Giegé and Brennicke, 1999), *Brassica napus* (Handa 2003) and *Oryza sativa* (Notsu et al. 2002) the occurrence of RNA editing was investigated. The total number of edited mitochondrial sites in *Arabidopsis*, rapeseed and rice is reported to be 441, 427 and 491 respectively in protein coding transcripts (excluding introns, leader and trailer regions). In chloroplasts of *Arabidopsis*, tobacco and rice, editing sites sum up to 28, 34 and 21 (Tillich et al. 2005; Schmitz-Linneweber et al. 2002; Corneille et al. 2000). This shows that editing in chloroplasts is a comparatively rare event, which alters on average about 1 out of 1000 nucleotides. In mitochondria the situation is different. Since in coding regions as many as ten times more editing events are observed, the distance between editing sites is relatively small, sometimes editing sites can even be adjacent. Each of the nucleotides altered must be recognized specifically since only some of the total complement of C-nucleotides are editied. Interestingly, RNA editing in plant mitochondria seems to exclude rRNA molecules for yet unclear reasons, except for potentially rare events.
Partially edited transcripts are detected in plant mitochondria. After the discovery of RNA editing it was believed that only completely edited mRNA were translated into proteins assumed from an analysis of the polysomal mRNA population (Gualberto et al., 1991) and from protein sequencing of the protein ATP9 (Bégu et al., 1991) and NAD9 (Grohmann et al., 1994). In other investigations, partially edited transcripts were found in the polysomal fractions of plant mitochondria. These are potentially translated into proteins (Mulligan 2004) and corresponding

polymorphic proteins produced from partially edited mRNAs have been reported for maize mitochondrial RPS12 (Lu et al. 1996). Up to now only fully edited proteins have been seen to be incorporated into protein complexes (Grohmann et al., 1994, Mulligan 2004).

2.2 Functional effects of RNA editing

The process of RNA editing is presumably expensive for the plant cell considering that a given genetic system can live well without it. Editing needs a biochemical machinery to exclusively recognize and modify the C of choice. This machine must consist of recognition elements for the respective C, e. g. specific *trans*-elements, and an enzyme (or possibly an enzyme complex), which attaches to the editing site to modify the repective nucleotide identity, which presumably requires energy, at least for the U to C direction. This energy is needed, firstly, to produce and maintain the components of the presumed enzyme complex, secondly, to supply the energy for the actual procedure of deamination or transamination and thirdly, to produce and recycle partially edited transcripts and/or even their translated and dysfunctional proteins, if the partially edited transcripts are degraded rather then matured, and if their proteins are indeed produced.
Though costly, RNA editing is still necessary for the survival of the plant. For as yet unclear reasons, the primary genetic information of mitochondria and chloroplasts makes these multiple alterations in the RNA necessary to produce functional proteins and to maintain the functionality of their systems.

Depending on the position of the C to be altered, different functional consequences can result (Fig. 1.2):
(1) Through RNA editing, start codons (AUG) can be generated from a threonine codon (ACG) as reported in the of *psb*L gene in chloroplasts (Kudla et al., 1992).
(2) Stop codons can be created by modification of CAG, CAA and CGA triplets to their UAG, UAA and UGA “counterparts”, respectively. The most impressive example of this effect of RNA editing, though not in plant mitochondria but in mammal nuclear encoded mRNAs, is the creation of a truncated form of the apolipoprotein B100. The occurrence of the edited and non-edited versions of the apolipoprotein is dependent on the surrounding milieu in the specific tissue in which it is expressed. The mRNA is edited in the small intestine in humans and results in a 48 kD protein through creation of a stop codon. The transcript is not edited in the human liver and the full-length version of the protein (100kD) is synthesised.

(3) RNA editing can change the identity of the encoded amino acid. In plant mitochondria the most frequent amino acid substitutions generated by editing are changes of encoded prolines or serines to leucins or serines to phenylalanines. In functional consequence, most of the amino acid changes by editing restore a primary sequence of a given protein, which after editing shows higher similarity compared to homologs of the same protein in other organisms.

Furthermore, an important consequence of RNA editing can be the restoration of secondary structures of their RNA-substrates. It has been shown that editing plays a substantial role in forming secondary structures in tRNAs (Marchéal-Drouard et al., 1993). Many examples show that only through editing essential stem structures can be formed correctly, while in loop structures conserved bases are restored. In Group II Introns, editing events eliminate mismatches, restore correct folding and allow correct excision of the intron (Binder et al., 1992).

Not all editing events in the nucleotide sequence have an obvious effect. Some changes are silent with respect to the encoded amino acid and are not situated in an area forming detectable secondary structures. The reason for the presence of silent editings is still obscure. They should be liable to selective pressure against their presence and should disappear during evolution. Whether the silent editing site is edited or not does not influence the protein sequence.

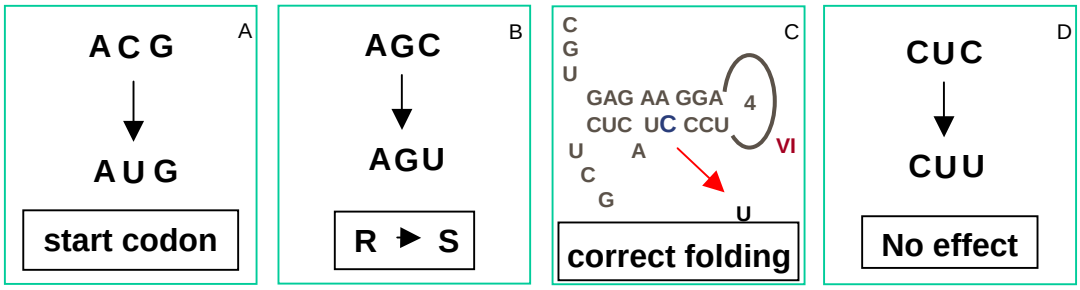


Figure 1.2: Effects of RNA editing. Several effects on the specified amino acid sequence are result after different individual C to U alterations. Start codons can be created (A). Amino acid exchanges are observed (B) and in some cases editing is indispensable for correct folding, e.g of Introns (C) or tRNAs. If a third codon position affected by editing, it has in most cases no effect on the amino acid sequence as shown here with an example of a leucine codon (D).

2.3 Reasons for RNA editing and its potential advantages

The consequences of RNA editing have been described in the previous chapter. The reason for the presence and the selective advantage of editing is, after 17 years of research, still speculative. Why would a plant “create” or “allow” many “mistakes” in their essential organellar DNAs only to correct these mistakes by employing specific (protein) molecules for recognition and repair and spent considerable costs on maintenance.

It has been suggested that the system of RNA editing became established by a chance mutation of a household de- or transaminase which then became able to accept polynucleotides as substrates and modify certain RNA positions. This capability had to develop before, or at least parallel to the mutation of information on the DNA level (Covollo and Gray, 1993). After RNA editing was established by correcting at least one otherwise negative mutation, editing became essential and had to be kept.

This chain of events might explain how editing developed, but it is still unclear if there are additional functions for RNA editing.

A regulatory function of editing has been considered, e.g. in transcripts where a start codon is generated by RNA editing. Synthesis of a protein suddenly required in larger quantity can be started rapidly by editing an ACG codon to a functional AUG start codon in an already present RNA-molecule.

In other systems RNA editing is an important mechanism for the creation of genetic plasticity through the generation of alternative protein products from a single structural gene. Stop codons, created through editing lead to proteins different in length and possibly also in function (Blanc and Davidson; 2002). However, postulating an effect of saving space as an advantage of editing might be deceiving. The biochemical tools needed for RNA editing might use more coding space than would be required for the additional gene information saved in the primary gene, if any at all. Especially in plant mitochondria, editing clearly does not save space in the genome. In fact genome sizes in plant mitochondria appear to be rather unimportant, since chondriom sizes have been and are being expanded through duplications and integration of coding and non-coding DNA from nucleus and chloroplasts.

RNA editing has been discussed to possibly play a role inhibiting sequence transfers to the nucleus. The export of coding regions to the nucleus and the secondary loss of the functionality of the respective gene in the organelle is a common event. Since RNA editing has not been found in the nucleus of plants, numerous alteration processes would be necessary to successfully establish a functional protein from a gene exported from the mitochondrial genome to the nuclear genome. Most easily, cDNA transcripts of an exported fully edited and spliced mRNA molecule could be correctly expressed in the nucleus. In this respect mitochondria keep editing in order to protect their chondriom.

As a further function of RNA editing it has been suggested (Ian Small) to compensate for mutations in the mitochondrial DNA which lead to a CMS phenotype (cytoplasmic male sterility). According to this theory the nucleus invented editing as a corrective to be able to produce functional pollen in a kind of internal arms race between the genomes in organelles and the

nucleus. This idea may be supported by the observation that editing sites evolve and change rapidly in the plant kingdom.

. SInce each of the discussed possible advantages can be achieved otherwise there is presently no obvious functional need for RNA editing. In fact, RNA editing appears to be a selfish process, established by chance events and now impossible to loose (This is also drawn as conclusion in Takenaka et al. 2006).

2.4 The biochemical process of RNA editing

While the biochemical processes of some types of editing have already been worked out, the biochemistry of plant RNA editing remains unsolved.

C-to-U changes may take place via four different biochemical mechanisms: RNA cleavage followed by cytidine release, uridine incorporation and RNA ligation (1); transglycosylation (2); transamination (3) or cytidine deamination (4).

RNA editing in plants is clearly unrelated to the RNA editing in trypanosome kinetoplasts. Here, defined numbers of uridines are inserted into the pre-mRNA. This changes the resulting encoded proteins so much that the originating DNA sequence can not predict the protein. For editing, a “guide” RNA pairs 3´ of the position to be edited and defines the place and number of U´s to be inserted. An enzyme complex, the editosome, cuts the sugar-phosphate backbone of the pre-mRNA and inserts the additional nucleotides according to the number of A’s present in the respective gRNA (1) (Benne et al; 1986).

RNA editing in plant mitochondria and also chloroplasts does not insert nucleotides but modifies present bases. During this process the sugar backbone remains intact (2) and the base is not replaced (Rajasekhar and Mulligan 1993; Yu and Schuster 1995). Consequently the cytidine must be de- or transaminated.

Although very different processes, the involvement of analogous guide RNAs in plant editing has been discussed. Unpublished results (van der Merwe, pers. comunnication) show an increase of the *in-vitro* RNA editing activity when total mitochondrial RNA is added to an *in-vitro* editing assay. This observation could hint at an involvement of guide-RNAs. However, the employed mitochondrial lysate is extensively dialysed and small RNA molecules should be eliminated, which should inhibit the *in-vitro* reaction if no mitochondrial RNA is added.

Dialysis of the *in-vitro* active mitochondrial lysate furthermore argues against an involvement of a classic transaminase (3), since this enzyme needs an acceptor for the transferred amino group.

Such acceptors are usually small molecules such as oxaloacetate or alpha-ketogluterate, which have been eliminated by the dialysis. Furthermore, addition of the acceptors to the *in-vitro* reaction (Takenaka, pers. communication) does not influence the reaction.

Since breakage of the sugar backbone (1), transglycosylation (2) and transamination (3) are unlikely, the remaining candidate process that has to be investigated is a deamination reaction (4).

Precedence for such a system which involves the activity of an RNA-specific C-deaminase is the apobec1 enzyme complex. This enzyme deaminates specifically the apoB site, targeting a single C in a transcript of more than 1400 nucleotides. A so called “mooring sequence” of about 30 nucleotides provides the information to bind the enzyme complex. In addition, targeting of the correct editing site also may require a stem-loop-structure, which exposes the C to be edited to the editosome (Araya et al., 1998, review).

In extensive computer analysis of the surrounding sequences of the editing sites in *Arabidopsis* no common secondary structure forming sequences could be outlined, suggesting that RNA editing in plant mitochondria does not require such a sterically exposed C.

So what are potential *trans*-acting components of the plant mitochondrial editing complex and what are the *cis*-elements, which play a role in targeting the C to be edited?

A deaminase: This enzyme is most likely to execute the process of altering C to U. Experiments in chloroplasts have shown that *in-vitro* editing can be inhibited by a zinc chelator (Hegemann et al. 2005). Zinc is the central atom of deaminases. In mitochondria this effect has not been seen (Takenaka and Brennicke 2003). The discrepancy can be explained by either of two possibilities. Either chloroplasts and mitochondria use different deaminases of which the mitochondrial enzyme does not expose the central ion. Alternatively a different type of enzyme may be involved, for which the CTP-synthases have been discussed (Takenaka et al 2006).

A helicase: Evidence of an RNA-helicase involved in RNA editing has been obtained through protein sequencing projects (Takenaka, unpublished results). RNA-helicases are accessory enzymes, essential in almost every biochemical reaction in RNA metabolism. They resolve secondary structures and remove protein molecules bound to the RNA.

Transfactors: The lately most discussed factors recognizing given *cis*-elements, are the PPR-proteins. The pentatricopeptide repeat (PPR) motif is a degenerated unit of 35 amino acids and is present in tandem repeats in proteins involved in RNA maturation steps in mitochondria and plastids. The PPR-protein family encoded in the *Arabidopsis* genome includes about 500 members, which are considered to bind nucleotide sequences. Most of these PPR-proteins are predicted to be targeted to mitochondria and/or to chloroplasts (Small and Peeters 2000). These

proteins possibly act as *trans*-acting factors binding the *cis*-elements of the RNA editing sites. Experimental evidence for PPR proteins taking part in editing is a phenotype expressed by an *Arabidopsis* mutant. RNA editing is abolished by mutation of one specific PPR-Protein (Kotera et al, 2005). This PPR-protein is thus a necessary *trans*-factor to generate the translation initiation codon of the chloroplast *ndhD* mRNA.

2.5 An in-vitro *RNA editing system to analyse cis-requirements of RNA editing*

To investigate the *cis*- and *trans*-elements as well as the requirements for successful editing, it is necessary to employ an *in-vitro* system, which imitates the processes taking place *in-vivo* as closely as possible.
The first assays used *in-organello* systems first established for wheat as model organism (Farré and Araya 2001) and subsequently also developed for maize and sorghum (Staudinger and Kempken 2003). In these experiments RNA editing was studied using electrotransformation to introduce foreign DNA into purified mitochondria. Cognate and non-cognate genes were inserted employing a plasmid under the control of a plant mitochondrial promoter. The construct was electroporated into isolated mitochondria, where it was correctly transcribed and altered by the editing machinery.
The method established permits the uptake of DNA of up to 11 kb into the mitochondrial matrix. *In-vitro* incubation of maize mitochondria permits editing of *de novo* synthesized mRNA. Transcripts of cognate mitochondrial genes (*atp*6, *cox*2) and transcripts from a non-cognate *Arabidopsis thaliana cox*2 gene were correctly edited at many sites.

Parallel to the promising results of *in-organello* systems, *in-vitro* systems were developed. While *in-organello* experiments require freshly prepared organelles, *in-vitro* systems can use deep frozen aliquots of a previously prepared lysate and allow an analysis of the biochemical processes.

One of the first efficient *in-vitro* RNA editing systems was established in chloroplasts by the group of Sugiura (Hirose and Sugiura, 2001). Using chloroplast extracts of tabacco *cis*-acting-elements of *psb*L and *ndh*B were investigated. Site-specific labeling of the mRNA at the editing site was used to detect the edited nucleotide products. After *in-vitro* incubation, RNA was

isolated, digested to mononucleotides and RNA editing was monitored by analysis of labelled U by thin layer chromatography.

Based on this method attempts were made to establish a mitochondrial *in-vitro* system. These experiments with a mitochondrial extract of pea seedlings with an accordingly labelled mitochondrial template (*atp*9) showed only very low levels of RNA editing.

Another method had to be found to visualize editing *in-vitro*. A novel and more sensitive procedure was developed by Mizuki Takenaka in a pea system with specific methods to label the template and to monitor a given editing event (Takenaka and Brennicke, 2003). This *in-vitro* system provides the foundation for the here presented work

2.6 Objective of the presented work

In this study, the *cis*-elements guiding RNA editing in plant mitochondria are investigated with this *in-vitro* RNA editing system.

One of the major open questions about the biochemical mechanism of RNA editing is how the machinery actually recognizes the targeted C to be edited. Which *cis*-elements play a role? How are they distributed around the editing site? Previous studies in chloroplast systems have shown that a region of more than 200 nucleotides 5' of the editing site can play a role in recognition, while for other sites 30 nucleotides can be enough for faithful editing. The 3' sequence of the targeted C generally seems to be less important.

To gather information about the recognition elements used by the RNA editing machinery, *cis*-elements important for RNA editing of two sites of a mitochondrial gene (*atp*9) were studied and compared. Different mutated templates have been used in this *in-vitro* system. The location of *cis*-elements of the first editing site in *atp*9 was studied by consecutive mutated templates and competition analysis.

Another open question are the *trans*-elements involved in RNA editing. To identify these, one approach focuses on the proteins, which bind specifically to a given editing site. To compare different protein patterns, it is necessary to be able to monitor different sites *in-vitro*. Therefore a second task of this work was to identify more editing sites, recognized by *trans*-factors and edited *in-vitro*. A screening for different editing sites recognized in an *in-vitro* lysate, should give a starting point for analysis of *trans*-elements involved in the editing process.

3. Results

3.1. Spadework

This work is based on the development of an *in-vitro* RNA editing system by Mizuki Takenaka (Takenaka and Brennicke, 2003). At first, a pea mitochondrial extract was employed to investigate part of the pea *atp*9 gene, including the first and second editing sites present in pea and *Arabidopsis*. *Atp*9 was chosen as a standard substrate based on the work of "Kuhn et al., 2001" and "Hoffmann and Binder, 2002", which described and investigated the gene in detail with respect to its promoter and transcript stability. *In-vivo*, it is efficiently edited at most of its editing sites.

3.2 Determination of cis-*elements in the pea* in-vitro *RNA editing system*

Exploration of the 5' *cis*-recognition elements using deletion templates

The first pea *atp*9 template tested *in-vitro* (Mizuki Takenaka, pers. communication) contains 173 native nucleotides upstream and 49 native nucleotides downstream of the editing site of interest, the first site in the *atp*9 mRNA (*atp*9 (1)). To investigate the minimal requirements for editing shorter templates in the form of different 5' deletion clones were constructed and tested *in-vitro*. The deletion clone containing 40 native nucleotides upstream and 49 nucleotides downstream (-40/+49) was edited as efficiently as the previously tested template –173/+49. This suggests that 40 nucleotides upstream of the editing site are sufficient for the editing of this site. The next deletions were done in steps of 10, which results in the clones –30, -20, -10, -0, which always contain 49 nucleotides downstream (+49) (Figure 3.1). Clone –30/+49 shows an editing efficiency of only about 50 % (Figure 3.2A), implying that 30 nucleotides upstream are still sufficient for recognition of the editing site, but not optimal.

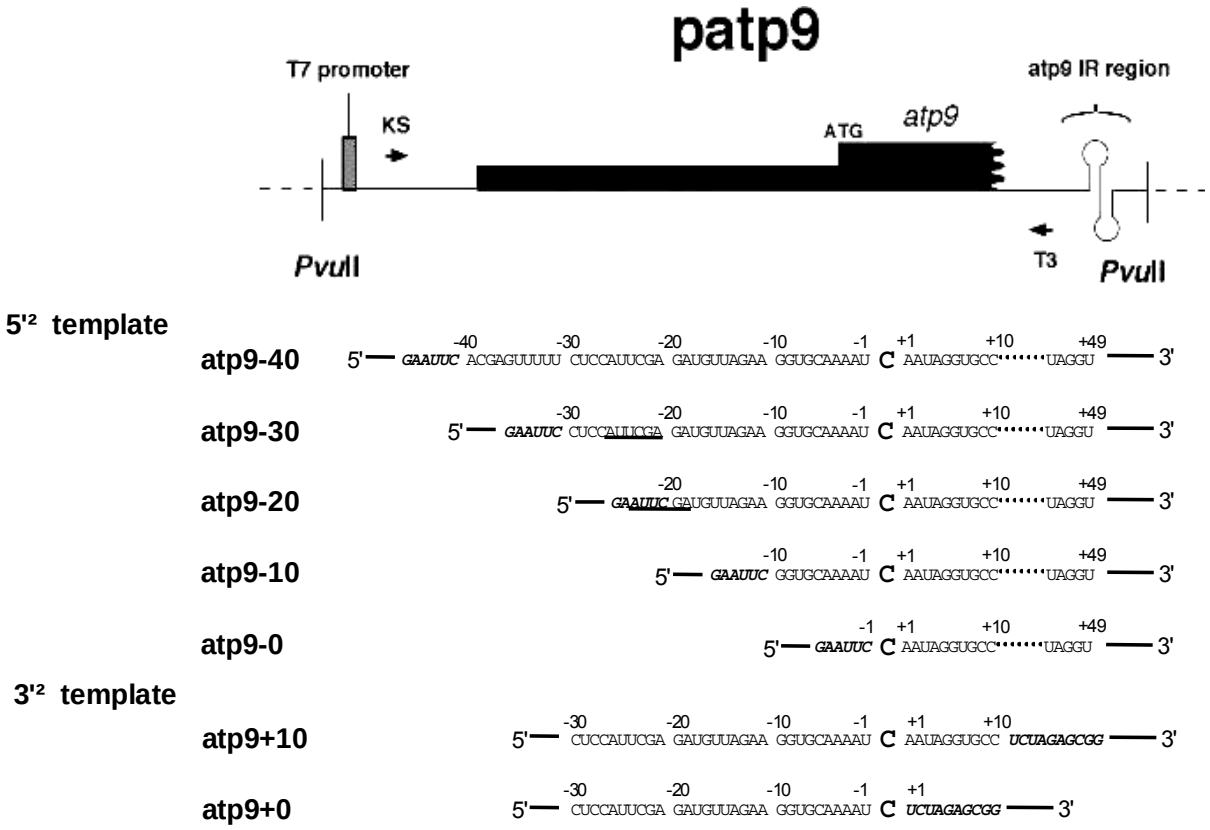


Figure 3.1: Structure of the pea *atp*9 (1) RNA editing template and the constructed deletion clones. The top line shows schematically the structure of the construct clones in the plasmid pVec. The *atp*9 fragment (bold black bar) is transcribed in a run-off transcription from the T7 promotor. It is flanked by bacterial sequences and includes a stabilizing atp9 IR region.
The shortened templates containing –40, -30, -20, -10, -0 nucleotides 5' and +49 nucleotides 3', or -30 nucleotides 5' with +10 and +0 nucleotides 3' are pictured below. The bacterial sequences replacing native nucleotides are given in italics. A hexanucleotide sequence in clone –20/+49 , which is by chance identical to the wild type sequence is underlined. The 5' and 3' black lines indicate vector sequence.

The removal of native nucleotides in effect moves the plasmid sequence closer to the editing site. This might lead to unwanted chance sequence similarities (Figure 3.1). The result of the -20/+49 deletion clone seems to be influenced by this effect. Even though the editing efficiency decreased further with the –20/+49 clone, it is still comparatively high. A chance similarity of 6 nucleotides of the plasmid sequence upstream of the –20 deletion with the –30 deletion clone, might be a possible explanation for this effect. Nevertheless, about 20 nucleotides upstream of the editing site seem to be sufficient for editing. The removal of 10 more nucleotides abolished editing completely. These results suggest that for this site in cauliflower, 20 nucleotides upstream are sufficient and necessary to identify the nucleotide to be altered. The sequence between –40 and –20 seems to modulate or increase editing efficiency.

3’ deletions to investigate downstream requirements for editing

The 3’ region of this editing site was similarly investigated in the pea lysate by generating deletion mutants –30/+10 and –30/+0 (Figure 3.1). As control the –30/+49 deletion mutant was used, which had been tested in the 5’ deletion clone analysis. All 3’ deletion mutants were edited accurately (Figure 3.2B). Surprisingly, the editing activity seems to increase, the more native nucleotides are removed. It seems that the sequence given around this editing site is only sub-optimal at least for this *in-vitro* system and that the editing efficiency can be accordingly improved by exchanging some nucleotides to, as done here a bacterial sequence, which may have yielded chance-optimization.

These experiments allow the conclusion that the minimum number of nucleotides necessary for successful editing are 20 nucleotides upstream and none downstream. However, the sequence region of –40 to –20 (5’ of the edited C) and +0 to +49 (3’) seems to influence and modulate the editing efficiency.

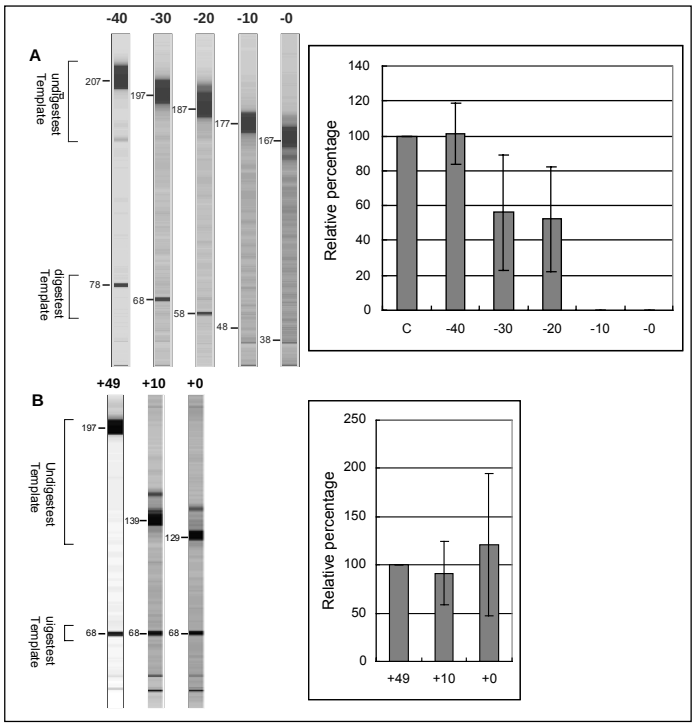


Figure 3.2: *In-vitro* editing of deletion mutants in the pea lysate. (A) The TDG mismatch detection products are analysed with a sequencing gel, shown on the left site. The sizes of the uncut fragments and the predicted cut fragments after amplification with RT-PCR using KS (Cy5 labelled) and T3 Primers are given in nucleotides beside the fragment signals in the gel lanes. The graph shows that deletion mutant -20/+49 still edits, while in mutant –10/+49 no editing event is detectable. Three independent experiments were quantified relative to the control, averaged and standard deviations were calculated. (B) The results of the 3’ deletion clone analysis suggests that the downstream nucleotides are not involved in the recognition of the pea *atp*9 (1). The length of the deleted mutants is indicated by the running time of the undigested templates in the gel lanes. The editing activity is similar in each of the 3’ mutants.

Exchange mutations as a tool to determine the sequence requirements

To take a closer look at the individual sequence requirements for editing and those which modulate the editing activity, mutants with consecutive sequence exchanges were constructed (kind gift of Mizuki Takenaka). In steps of 5 nucleotides, sequences were mutated to their respective anti-sense pendants (Figure 3.3A). The exchange of nucleotides –40 to –35 reduces editing severely to less than 10 % of the wild type control (Figure 3.3B). This suggests that this region of 5 nucleotides contains an important sequence information. The comparable deletion clone, -40 to –30, for comparison reduces editing only to 50 % which raises the question whether different factors such as an effect of the mutated sequence could play a role. In contrast, the following 10 nucleotides (M2, M3) do not affect the editing to such an extent, lowering editing only down to 60 and 70 % percent, respectively. Mutants M4 and M5 show a much stronger effect, their alteration reduces the editing rate to about 5 to 15 %. Out of 4 independent *in-vitro* assays using different mitochondrial lysate preparations, editing of M6 was abolished in 3 experiments entirely, while in one assay very little residual editing was observed. This shows that these nucleotides –15 to -10 are crucially important, yet editing can occur occasionally without them. The sequence covered by the M7 mutant (-10 to –5) is essential for editing. This mutant never showed any editing activity. If the 5 nucleotides immediately upstream of the editing site are exchanged (M8), editing is still observed although with a severely reduced activity of about 10 %.

Mutations downstream of the editing site (M9, M10) hardly affect editing. The little reduction (30 to 40 %) is comparable to M2 and M3 suggesting only a moderate effect of the sequence.

According to these results following conclusions can be drawn: The sequence elements –40 to -35 and –25 to –10 enhance or modulate the RNA editing of the respective site. The region, essential for recognition of the editing site is located between nucleotides -5 and –15, most likely extending several nucleotides beyond. The 3’ sequence seems to be able to slightly modulate editing efficiency. In summary the regions around the first editing site in pea *atp*9 have been investigated in detail by scanning mutants, which confirm and extend the results of the deletion clones.

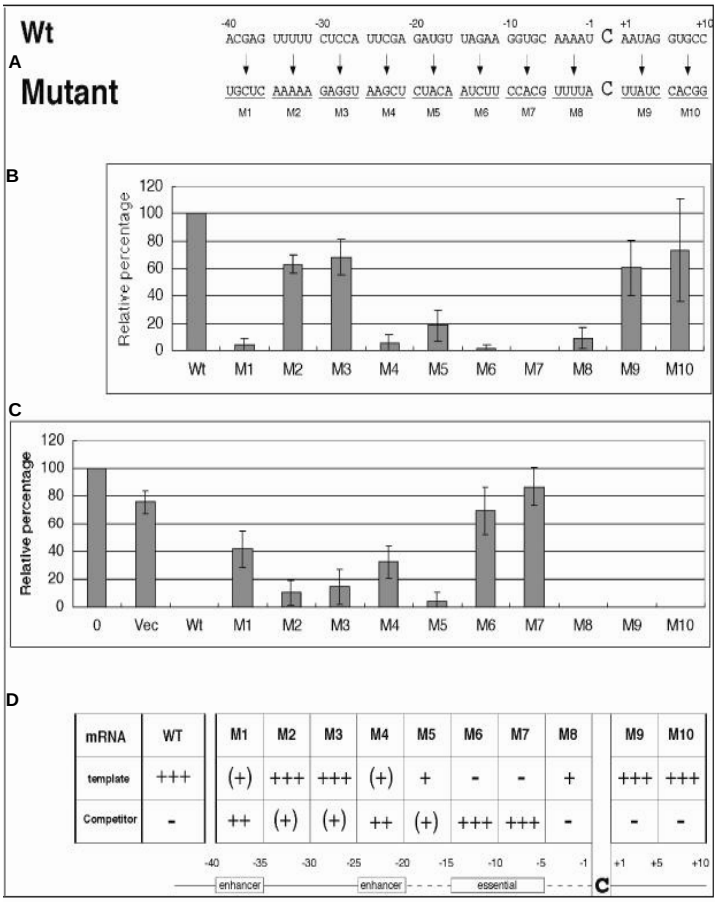


Figure 3.3: Comparison between experiments with mutant templates and competition experiments in the pea lysate. The comparison reveals distinct regions for recognition and efficiency of the editing reaction. (A) Mutant templates were constructed by scanning mutagenesis. Nucleotides were altered in steps of 5 to their consecutive complement. (B) 4 independent experiments were averaged after comparing the respective editing efficiency to the wild type. Standard errors were calculated. M1, M4 and M6 only show very little editing, in some experiments no editing is detectable. (C) Mutant templates were used as competitors in 1000-fold excess to the wild type sequence template. Results of 3 out of 4 experiments were calculated. Competition with M8, M9 and M10 indicates that essential *trans*-elements upstream can be completely titrated. (D) The comparison of the results of the two experiments reveals one core recognition element (-15 to –5) and two potential enhancer regions. Editing efficiencies are classified as full (+++), reduced (++), little (+), occasionally very little ((+)) and not detectable (-). Point D was kindly provided by M. Takenaka. Details are discussed in the text.

Competition with mutated templates

To determine which sequence is actually interacting with a potentially limited amount of *trans*-factors a set of experiments was performed using the mutated templates M1 to M10 as competitors for the wild-type RNA-template. The following influence on editing was expected: competed with a mutated template containing an exchange of a non-essential region like M3, editing should be blocked entirely, since the essential sequence elements are present in both templates competitor and compete with each other. *Vice versa* competitors mutated in essential sequence elements should not influence the editing activity severely.

This pattern delineates essential and non-essential regions and the abundance of respective *trans*-factors (Figure 3.3C). The competitor templates mutated in non-essential regions, like M5, M8, M9 and M10 abolish editing entirely. In contrast, mutant M7, which lacks the essential region between –15 to –10 shows little competition effect. Mutant M6, in which the region immediately upstream to the C to be edited has been exchanged, likewise only slightly reduces editing. Surprisingly, mutants covering the sequence –40 to –20 (M1 to M4) inhibit the editing efficiency with varying intensity although the essential sequence –15 to –5 is present.

As a control a wild-type competitor was used, which blocks the reaction entirely. Using the vector sequence as a negative control little reduction of editing was observed, which might reflect the presence of by chance sequence similarities between the vector sequence and the template detailed above.

3.3 Rice as a candidate plant for an in-vitro *RNA editing system*

Beyond investigation of *cis*-elements of the first editing site of *atp*9 in pea mitochondria there are two complementing ways of proceeding towards new insights into plant mitochondrial editing. The first approach is, to gather further information about *cis*-requirements of different editing sites. This requires to determine further editing sites employing the pea or cauliflower editing system. This attempt is described under point 3.6.

The second possibility to extend our knowledge about the recognition system used to determine the C to be edited and its differences and evoluntions in plant mitochondria is, to try to establish an *in-vitro* editing system with a different plant species.

Rice was chosen as possible candidate because the mitochondrial genome has been sequenced (Notsu et al. 2002), seeds are easily available and growing conditions of etiolated shoots are easy to establish. Furthermore, as a monocot, rice could give a closer view on the differences between *cis*-element distribution as well as variations of RNA editing in dicots and monocots.

At first, the protocol of Neuburger et al. (1982) was used to extract mitochondria of rice shoots grown 10 days in the dark (30°C). A mitochondrial lysate was prepared according to the protocol for pea mitochondria and tested in the *in-vitro* system. The control (*atp*9, –40/+49) showed no detectable editing. One possible explanation for the failure of this procedure is the fact that rice contains various secondary metabolites, which might disturb the sensitive editing system. To diminish their amount and concentration, the growth time of the seedlings was reduced to 7 days and the volume of the buffer used was increased from 1 l per 400 g shoots to 4 l per 400 g shoots, and additional centrifugation steps were performed. Furthermore, the time of dialysis was extended from 5 hours to 6 hours with a change of buffer every hour. These attempts were without success, the resulting lysate didn't show editing activity.

Another explanation could be that the activity of RNases or their concentration could be higher in the rice mitochondrial lysate, which would damage and destroy the template, explaining occasional PCR drop outs, where no amplification products could be detected. For that reason

the amount of RNase inhibitor was doubled in the *in-vitro* assay, which however did not influence the result.
Next, a different gene was examined. *Rpl*2 was cloned and tested in the rice *in-vitro* system, but did not reveal an editing event.
In summary it can be said that in spite of efforts to establish an *in-vitro* system in *Oryza sativa*, it was not possible to overcome the difficulties. Potentially since rice is a monocot, the mechanism of editing has evolved in a different way, and it is not possible to establish an *in-vitro* system under these conditions. For that reason another plant, *Brassica oleracea* was chosen to establish a new system (J. A. van der Merwe).

3.4 The RNA editing system from cauliflower to determine editing site recognition parameters in a different plant species

For further analysis it would be helpful to have a second *in-vitro* system of another plant spiecies. The first attempt using etiolated rice shoots was not successful. Also the mitochondrial lysate of an *Arabidopsis* cell culture did not show *in-vitro* editing activity (J. A. van der Merwe, pers. communication). Finally, by using a cauliflower mitochondrial lysate, a second *in-vitro* editing system, was successfully developed (van der Merwe, pers. communication; Neuwirt et al., 2005).
Cauliflower inflorescences are a convenient source for mitochondria. They offer several advantages to obtain large amounts of comparatively clean mitochondria from plants: 1. They contain few secondary compounds, which notoriously make biochemical and molecular analyses difficult in plants. 2. Chloroplasts are not found in these white tissues. 3. Another advantage is the economic source of material, which does not have to be cultivated in greenhouses but can be easily and cheaply purchased at local markets (Neuwirt et al., 2005). In addition to these technical advantages, the chondriom sequence of a closely related plant, *Brassica napus*, has been entirely sequenced (Handa, 2003), which is helpful in the analysis of data. Cauliflower (*Brassica oleracea*) is closely related to *Arabidopsis thaliana* which is intensively studied as a model plant species and which provides numerous data including the fully sequenced genomes (Unseld et al, 1997, e.g.).
An editing *in-vitro* system has been developed according to the protocol of Takenaka 2003 (J. A. van der Merwe, pers. communication; Neuwirt et al. 2005). The respective lysates were used in the RNA editing *in-vitro* reaction which is illustrated in figure 5.1.

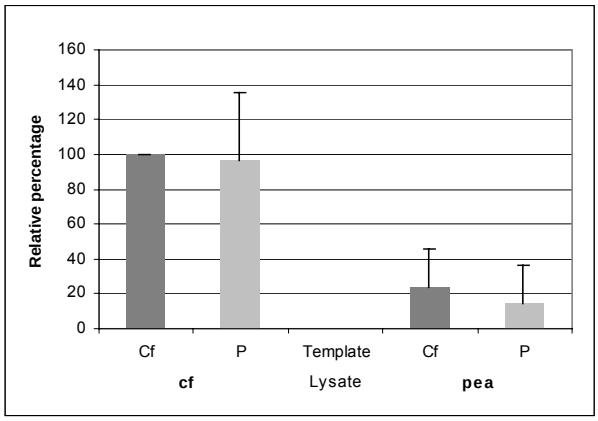


Figure 3.4: Comparison of *in-vitro* RNA editing activities in cauliflower (Cf) and pea (P) lysates with pea and cauliflower mitochondrial RNA derived *atp*9 site 1 templates. The cauliflower lysate shows an approximately four times higher efficiency than the pea lysate. In the cauliflower lysate the pea and the cauliflower template are edited with similar efficiencies. *Vice versa* similar editing efficiencies for pea and cauliflower templates are observed in the pea system.

Because the condriome of *Brassica oleracea* has not yet been sequenced, the DNA and cDNA sequences had to be compared to determine editing sites. The sequences of the construct -40/+49 of *atp*9 are conserved between pea and cauliflower from position –23 to +49 where only nucleotide +46 differs. The region 5' of -23 diverges, possibly due to the location of the AUG codon, in pea and cauliflower at position –19 relative to the C to be edited. The 5' leader regions vary between the species, potentially because of relaxed evolutionary pressure.

Prior to detailed *cis*-element analysis the activites of the respective lysates were tested (Figure 3.4).

The cauliflower mitochondrial extract proved to be more active than the pea extract, which supported the results of J. A. van der Merwe (pers. communication). Both templates, pea as well as cauliflower, are about three times more efficiently edited in the cauliflower system. This might be due to the higher protein concentration in cauliflower mitochondrial preparations (8,3 µg/µl) than in pea preparations (1,9 µg/µl) (J. A. van der Merwe, pers. communication).

In both plant species 23 nucleotides upstream of the editing sites are sufficient for recognition of the editing site. The cauliflower template seems to be preferred in both lysates but the differences are within the experimental error.

Analysis of deletion mutants in the cauliflower *in-vitro* editing system

For the following experiments the pea *atp*9 template was employed, since it had been successfully examined in the pea editing system. The conservation of the *cis*-elements between the species and by extrapolation of the *trans*-elements recognizing an editing site can be compared within the conserved sequence area.

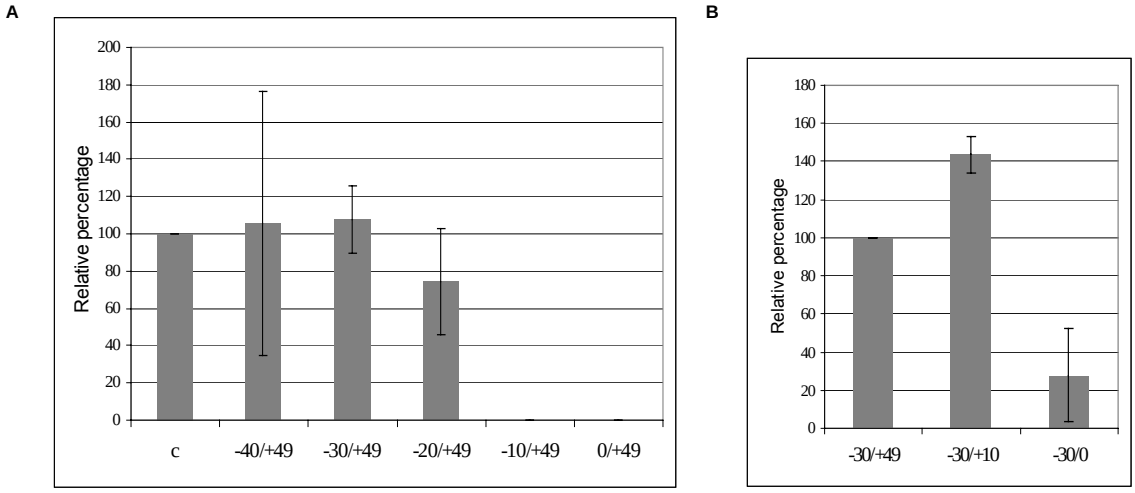


Figure 3.5: Distinguishing the *cis*-recognition elements by deletion clones in the cauliflower lysate. (A) The 5' deletion clones were done in steps of 10 nucleotides as indicated in the graph. In effect the bacterial sequences replace the deleted nucleotides. Deletions up to –20 nucleotides to the C to be edited still allow editing, while further deletion abolishes editing intirely. As control served the template –40 / +10. Pea mutants were employed to allow direct comparison of the *cis*-elements. (B) 3' deletions show little effect up to position –10. Deletion up to nucleotide +0 shows severe inhibition of the editing activity. The template –30 / +49 was used as standard.

The templates successively deleted in steps of 10 nucleotides (see also 3.2) were examined in the cauliflower system. 20 nucleotides upstream of the C to be edited are sufficient for editing (Figure 3.5A) of the cauliflower template. Further deletion clones –10/+49 and –0/+49 do not show editing, which suggests that as in pea 20 nucleotides upstream are necessary and sufficient for recognition of the site.

Complete substitution 3' of the C to be edited by bacterial sequence still allows editing, but, different to the pea lysate, much of the activity is lost if the nucleotides +0 to +10, like in pea, are exchanged to bacterial sequences.

Mutation analysis in the cauliflower system in comparison to pea reveals a similar pattern of *cis*-elements for RNA editing

To compare the *cis*-recognition elements, which are necessary and sufficient in the two systems, the mutated templates described under 3.2 were investigated in the cauliflower *in-vitro* RNA editing system. An overall similar pattern of editing requirements emerges in the comparison of

the relative activities of these templates in pea and cauliflower lysates, which underlines and extends the previous results (Fig. 3.6B).

The mutagenized region –40 to –35 (M1) shows a similar decreasing effect on editing in pea and cauliflower. This fact is especially interesting, because the pea template employed does not correspond to the native sequence of cauliflower in this area. Not showing any effect in the deletion clone analysis, the –40/+49 template was expected to be recognized equally well, even in its mutated form. However, this region, which serves as an enhancer element in pea, seems to fulfil a similar function in cauliflower, because the sequence alteration in construct M1 effects a similar decrease in the editing efficiency in both species. This is particularly stunning, since the native sequence of cauliflower is different from the pea template which was used for these experiments.

Another difference was observed in the mutant template M5 (-20 to –15). In pea, editing activity is still detected. The cauliflower template M5 does not show any editing at all, suggesting that in cauliflower the major *cis*-recognition element extends beyond –16 nucleotides upstream.

Another difference is seen in mutant 9 (M9, +0 to +5). Mutation of this region severely inhibits editing in cauliflower, while in pea this template looses little activity.

Competition experiments in the cauliflower lysate

Competition of the wild type template of pea with wild type competitor in the pea lysate had left residual editing activity with a 1000-fold excess of the competitor. The higher capacity of the cauliflower extract is reflected in the analog experiments: Even a 1500-fold excess of the competitor template still shows residual editing activity. The results shown in Figure 3.6C have to be interpreted accordingly and have to take the remaining editing of the wild type into consideration.

In general, the differences in the extent of the *cis*-elements, which are observed with the mutated templates in the two plant species, are also reflected in the competition experiments. Generally, higher levels of editing in cauliflower of M8, M9 and M10 thus can be interpreted as similar, since they reflect the level of inhibition of wild type competitor.

A major difference with respect to the extension of the *cis*-elements in cauliflower and pea is reflected by competitor M5, which covers the essential region from –16 to –20. While the pea system reveals a reduction only to 90% residual activity if these nucleotides are mutated, in cauliflower a remaining activity of about 50% is observed.

The requirement of the nucleotides between –15 to –5 is supported by the competition experiments in pea as well as in cauliflower, which show little effect on the editing reaction with the respective competitors are in the reaction. The level of reduction is comparable to the effect of vector sequence used as competitor in a control reaction.

M1 (-40 to –35) shows as competitor a similar effect on the wild type editing efficiency in the pea and cauliflower *in-vitro* systems. In both plants, the M1 competitors inhibit considerably less than the next downstream mutants. This shows that the M1 region serves as an enhancer in both species, as previously discussed.

Competitors mutated 3' of the editing site fully inhibit the editing reaction, showing that the upstream sequences are essential for the recognition of the editing site.

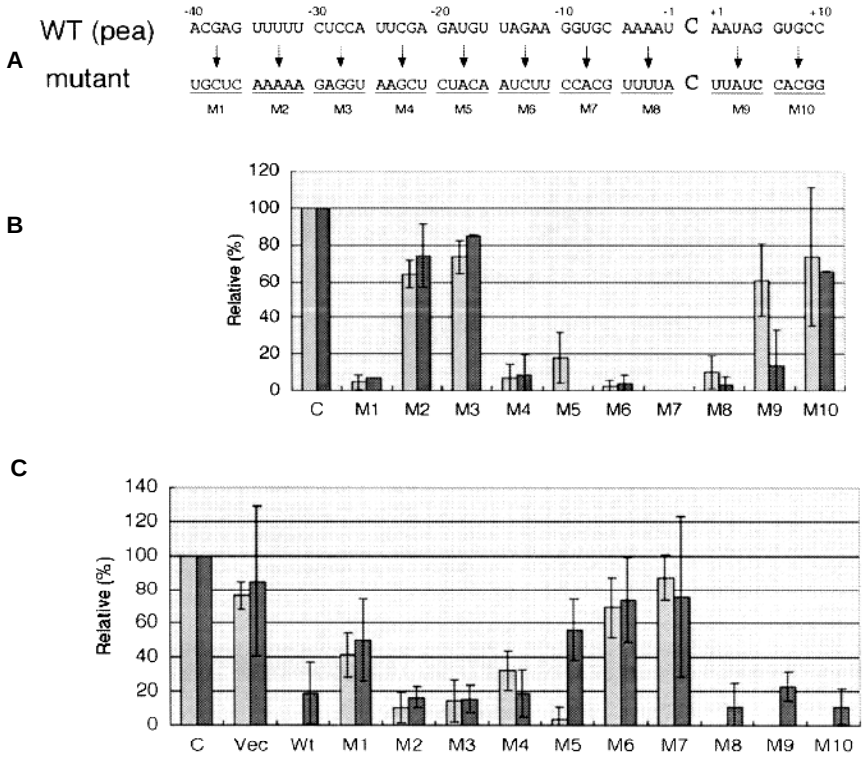


Figure 3.6: Comparison of the effects of mutations in pea and cauliflower to distinguish differences between the *cis*-elements. (A) Design of the mutated sequences which are altered in steps of 5 nucleotides to the respective complementary sequence. (B) The editing efficiencies of the respective mutated templates in cauliflower lysate (dark grey bars on the right) are compared to the efficiencies of the pea lysate (light grey bars on the left; see also in Figure 3.3). Obvious differences are seen between mutants M5 and M9 in the cauliflower and pea lysate, respectively. M1 does not show differences in editing activity in the respective lysates. (C) Competition reactions using the wild type pea template and 1500-fold excess competitor of the mutated templates M1 to M10 in the cauliflower lysate (dark bars) and 1000-fold excess competitor in the pea lysate. The vector sequences as a control compete very little, while the wild type competitor (pea, -40/+49) competes. Please note, that suppression of the wild type sequence of cauliflower is not complete. The most striking difference between the lysates of these two plant species is noticed with the competitor M5. Further details are discussed in the text.

Point mutations at the +1 position in cauliflower

The mutation experiments in the cauliflower extract showed differences to pea, which were further investigated. Firstly, the 3' deletion mutant –30/+0 showed about 70 % reduction of editing. This was supported by the mutant analysis of M9, which also showed only about 15 % remaining editing activity compared to about 60 % in the respective pea lysate.
The result allows the conclusion that one or more nucleotides in the area between +1 and +5 are important for the editing complex.

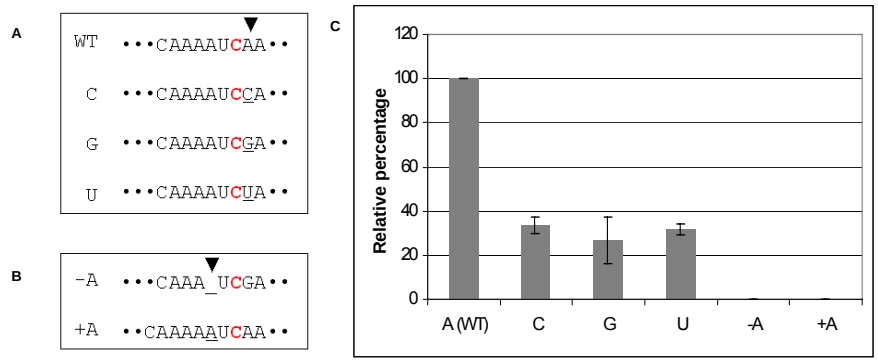


Figure 3.7: Importance of the identity of the +1 nucleotide and the distance to the respective editing site by deleting or adding a nucleotide in cauliflower for the efficiency of editing. Site specific mutation was employed to modify the +1 nucleotide to each of the alternative nucleotides and to delete or add one nucleotide (A,C) at position –2. (A) Any of the nucleotide changes result in a loss of editing activity up to 70%. This indicates the restricted tolerance at the +1 position. Only one nucleotide identity is optimal for editing of this site. (B,C) Neither the addition nor the deletion of a nucleotide was tolerated by the editing enzyme in case of *atp*9 (1) in cauliflower showing that the distance between the *cis*-elements and the C to be edited is crucial. Please note the concomitant point mutation in the –A template.

To further investigate the importance of the adjacent nucleotide, exchange mutants of the +1 nucleotide were made. All four possible nucleotides were tested in the *in-vitro* system (in cooperation with M. Takenaka). If the native A was exchanged to G (like in the deletion mutant), which is also a purine, editing got severely reduced. A similar effect was seen when the A was changed to either pyrimidine U (like in M9) or C. This result implicates that the wild type adenosine is crucial at this position (Figure 3.7A/C).

Analysis of the spacing tolerance between the *cis*-elements and the C to be edited

To investigate the importance of the distance between the edited C and the major *cis*-element for recognition, two *atp*9 (1) mutants were made, one with a nucleotide inserted the other with one nucleotide removed (Fig 3.7B). These mutations result in + and - one nucleotide distance alterations between the editing site and the *cis*-element.

These experiments show that the editing complex does not accept any alteration of the distance to the C to be edited, since no editing is observed after deletion or insertion of one nucleotide (Figure 3.7C). This shows, that the wild type distance of the *cis*-element to the edited C is the only functional configuration and crucial for editing.

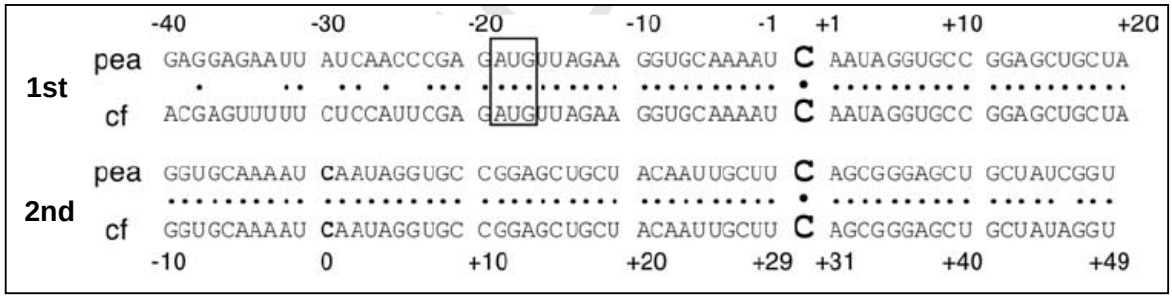


Figure 3.8: Two neighbouring sites *atp*9 (1) and (2) are processed in the cauliflower *in-vitro* lysate. Sequence alignment of the vicinities of *atp*9 (1st, 2nd) which are edited in the cauliflower *in-vitro* system. The editing sites are indicated in big bold letters. The first site is indicated as position 0 in both of the alignments. In all experiments nucleotide positions in the templates are numbered relative to the first site (0). (Picture: J. A. van der Merwe)

3.5 Investigation of cis*-elements of two neighbouring sites*

Thirty nucleotides determine the second editing site

In the process of establishing the reaction conditions for the new *in-vitro* RNA editing system from cauliflower the template -40/+49 was closely investigated (J. A. van der Merwe). This template contains two native editing sites spaced by 30 nucleotides (Figure 3.8 by J. A. van der Merwe).

In the pea mitochondrial lysate the second editing site has never been observed. Editing of the second site was first detected using the pea template in the cauliflower system. With this heterologous non-native template, editing of the second site appeared only occasionally. Editing of the second editing site in the pea template was observed in only one out of three *in-vitro* reactions and then only with much less efficiency than in the native cauliflower template. Employing the cauliflower template the second site was usually observed with confidence. This suggests that the editing of the second site depends on a species-specific template in the cauliflower lysate (results by van der Merwe).

During investigation of the *cis*-elements responsible for editing of the first site, 5' deletion clones were made, which still contain this second editing site 30 nucleotides downstream of the first site.

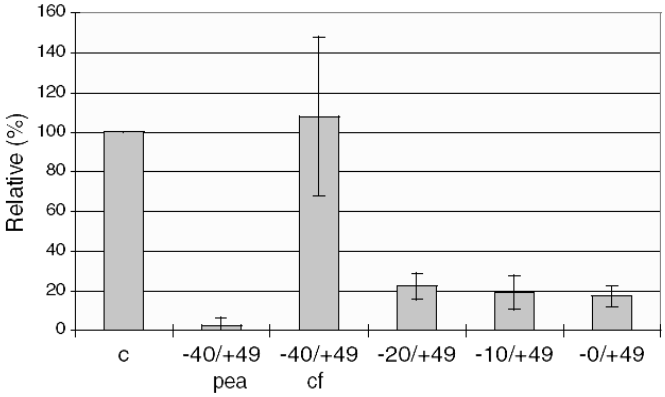


Figure 3.9: Analysis of the *cis*-requirements for *in-vitro* RNA editing at the *atp*9 second site. The pea derived RNA templates employed for each reaction are indicated underneath each bar. The first editing site serves as a control for the respective second sites in each experiment. The deletion clones are derived from the pea sequence which, except for position +46, is identical to cauliflower.

Figure 3.9 shows the experiments with the deletion clones in the cauliflower lysate as the average of three independent assays (five with the native cauliflower -40/+49). As a control the first editing site in pea is used.

The level of editing of the second site in cauliflower is comparable to the level of editing of the first site in pea, but drops to only 25 % upon deletion up to -20 nucleotides relative to the first site. Further shortening of the clone in steps of 10 nucleotides up to -0 relative to the first site does not show any additional effect on the editing of the second site. *Atp*9 (2) only requires 30 or less nucleotides to be still recognized although at a low level.

Comparing the levels of editing in the pea and cauliflower –40/+49 template, it seems that pea template contains a sequence in the area –40 to –20 which inhibits editing of the second site, since after deletion of this region higher levels of editing are observed in the now homologous cauliflower template.

Competitions to investigate *cis*-requirements for the second site

Figure 3.10 shows the effect of the in A described competitors on the cauliflower template containing both, the first (B) and second (C) editing sites. As expected the editing of the first site is inhibited by the competitors –40/+10 of cauliflower and pea. The -0 /+49 competitor has no effect on editing of the first site, which supports the conclusion that the important *cis*-elements required for recognition of the second site are situated upstream of the first editing site.

Meanwhile, the second site is inhibited by the competitor (-0 /+49), which further confirms the results of Figure 3.9 showing that this element (+0 to +29) contains the *cis*-elements important for recognition of the second site.

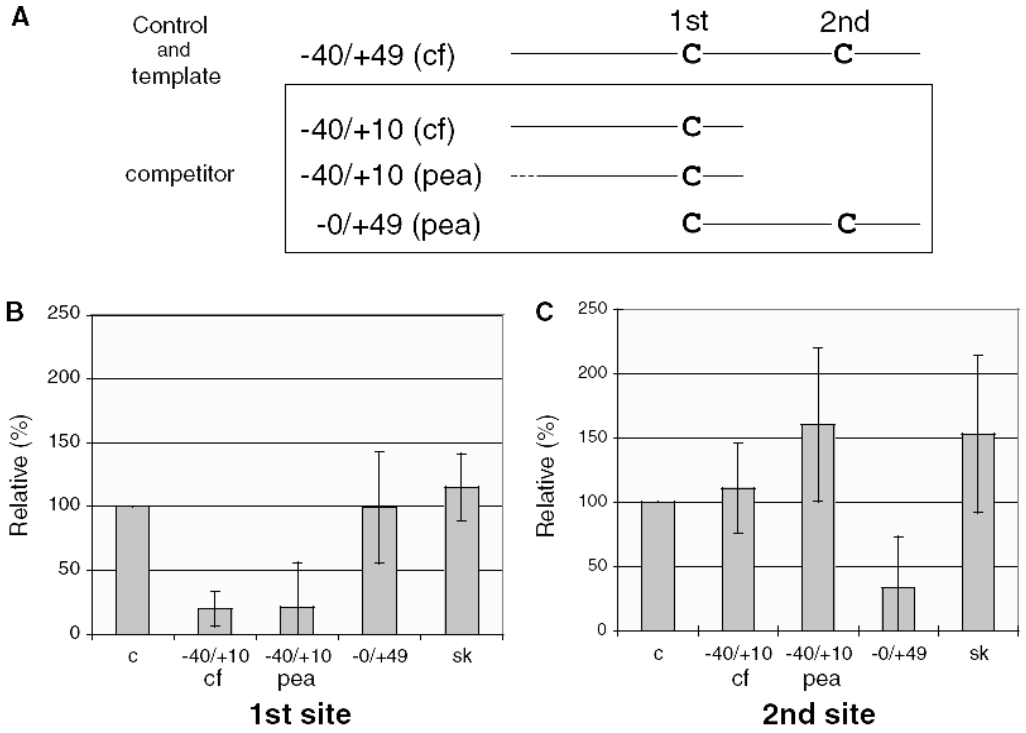


Figure 3.10: Determination of competition for *trans*-factors between the respective core recognition sequences of the two neighbouring sites. (A) The cartoon shows the structure of the template *atp*9 RNA from cauliflower in the top line and the respective competitors for the upstream site from cauliflower (second line) and from pea (third line), the dashed line indicates the different sequence in pea. Editing sites are indicated by a bold C. Figure (B) shows the competition of *atp*9 (1). A 1500-fold excess of the upstream sequence (-40/+10) from cauliflower and pea was used as competitor. The competitor of the downstream region (0/+49), which contains the recognition sequence of the second site was put into the reaction. Effects on RNA editing were monitored. The control reaction c is run without competitor. The effect of a 1500-fold excess of a bacterial plasmid sequence is shown as sk. (C) The effect on *in-vitro* editing of the downstream site by 1500-fold competitor excess of its own recognition sequence (0/+49) and of the pea or cauliflower upstream site recognition sequences (-40/+ 10) is investigated. The data for cf (–40/+10) is kindly provided by D. Verbitskiy.

As seen in the deletion experiments, a sequence element 70 nucleotides upstream of the second editing site increases editing significantly compared to the adjacent deletions, which do not contain this region. This effect is species specific: The –40/+49 native cauliflower sequence shows an around 20 times higher editing rate than the heterologous pea template of the same length.

Surprisingly, if used as a competitor, neither the pea nor the cauliflower sequence show an effect on editing of the second editing site of *atp*9 in cauliflower.

This effect –no inhibition- allows the following conclusions:

The pea competitor does not inhibit editing of the cauliflower template, so the pea distal element does not compete with the cauliflower sequence.

The *cis*-element of cauliflower is indeed, as previously suggested, shorter than 30 nucleotides, since the competitor –40/+10 includes 10 more nucleotides than the shortest deletion clone (-0/+49). This shows that 20 nucleotides are sufficient for recognition of the second editing site.
The recognition elements responsible for the first site have no detrimental effect on editing of the second site in competition experiments.
In some competition experiments the editing activity is higher with added competitor, than in the control. This could be explained by the control experiments, in which RNA derived from only vector sequence is added. This increase in editing could be due to unspecific RNA, which inhibits or "catches" unspecific RNA-binding-proteins and bars them from binding. In this way the specific *trans*-factors can bind easier, which is reflected in a higher editing efficiency.

The cauliflower –40/+10 competitor does not compete with the second editing site of the cauliflower template (-40/+49), which allows conclusions about the *trans*-factors addressing the long distance supporting motive situated in the region –40 to –20. Adding competitor excessively, the first site was competed, while the second editing site remained edited. One but not the other was affected suggesting that the *trans*-factors promoting editing on the first but not the second site from this same distal region are or act distinct. The *trans*-factor enhancing editing on the second site seems to be abundant, since an excess of –40/+10 competitor does not influence the editing of second site.

3.6 Screening for different in-vitro *editing sites*

The editing site, which was used to establish the *in-vitro* RNA editing system in pea (M. Takenaka) and cauliflower (J. A. van der Merwe), *atp*9 (1) has been characterized with a focus on the *cis*-elements involved in RNA editing of this site. A*tp*9 (2), the second editing site in *atp*9, has been detected, described (J. A. van der Merwe) and investigated for its analogous *cis*-elements.
Current and future research on RNA editing will need to identify the *trans*-factors involved in the process and to assemble the RNA editing enzyme complex.
One approach to get closer to this aim is to find and characterize different editing sites, which are edited in our *in-vitro* editing system. The *cis*-elements required for correct editing of these sites can then be compared and conclusions about the *trans*-factors can be drawn. Additionally, by comparing the results of cross-linking experiments, proteins (including potential *trans*-factors) binding to the *cis*-elements of these different editing sites can be identified.

The next part of the thesis work was accordingly aimed to identify further editing sites for *in-vitro* analysis.

Investigation of randomly selected editing sites in the pea *in-vitro* system

This work was started before the cauliflower *in-vitro* system was developed and is therefore based on the pea RNA editing system.

To find other editing sites edited by the system, different genes were cloned into the pVec vector. The primers used originated from the work of Phillipe Giegé (Giegé and Brennicke, 1999) which had included all actively transcribed genes. *Atp*9 (1) was used as control for the *in-vitro* system. A blind control of the respective probed gene, were no TDG-enzyme was add, was used to exclude unspecific RT-PCR products. The reaction was performed according to the protocol described in Takenaka and Brennicke 2003. Out of 9 genes including approximately 150 different editing sites chosen to test in the pea *in-vitro* system, none was edited under standard conditions.

After several tries, *nad*3 was chosen because of its comparably small size for testing different reaction conditions. *Nad*3 contains 12 editing sites within 359 nucleotides (Giegé and Brennicke, 1999).

Firstly, the incubation time of the *in-vitro* reaction was varied. The reaction was stopped after 2,5 hours, 3 hours, the standard 4 hours, 4,5 hours and 5 hours, respectively. No editing of any *nad*3 editing site was visible, while the control, *atp*9 (1), was edited to various degrees as described (Takenaka and Brennicke, 2003).

Secondly, *in-vitro* editing was tested at different incubation temperatures: 26°C, 28°C (the standard temperature), 30°C and 32 °C for 4 hours. No editing was observed with *nad*9.

Thirdly, the reaction was performed at different NTP concentrations: 0; 2,5; 5; 7,5; 10 and 20 mM of ATP were added. No editing was observed with *nad*3.

With these experiments no further editing site could be detected in the pea system.

Computer screenings

Since the random *in-vitro* screening for editing sites accessible *in-vitro* turned out not to be successful, a computer-based comparison of the editing sites was employed. It is possible that editing sites share factors for their recognition. Thus, these sites could potentially have the same

or similar *cis*-elements. If randomly chosen editing sites are not addressed in this *in-vitro* system, it might be that an editing site with sequence similarities to the *cis*-elements of the sites, which have been investigated, and which are potentially recognized by the same *trans*-factors, are edited *in-vitro*.

To check for common patterns of *cis*-elements, the sequence contexts around all 441 mitochondrial editing sites of *Arabidopsis* between –100 and +20 respective to the C to be edited were compared with a computer program (DNA star) (in cooperation with J. A. van der Merwe). Mr. van der Merwe and Mr. Reimer developed a program to compare the editing sites and find common features.

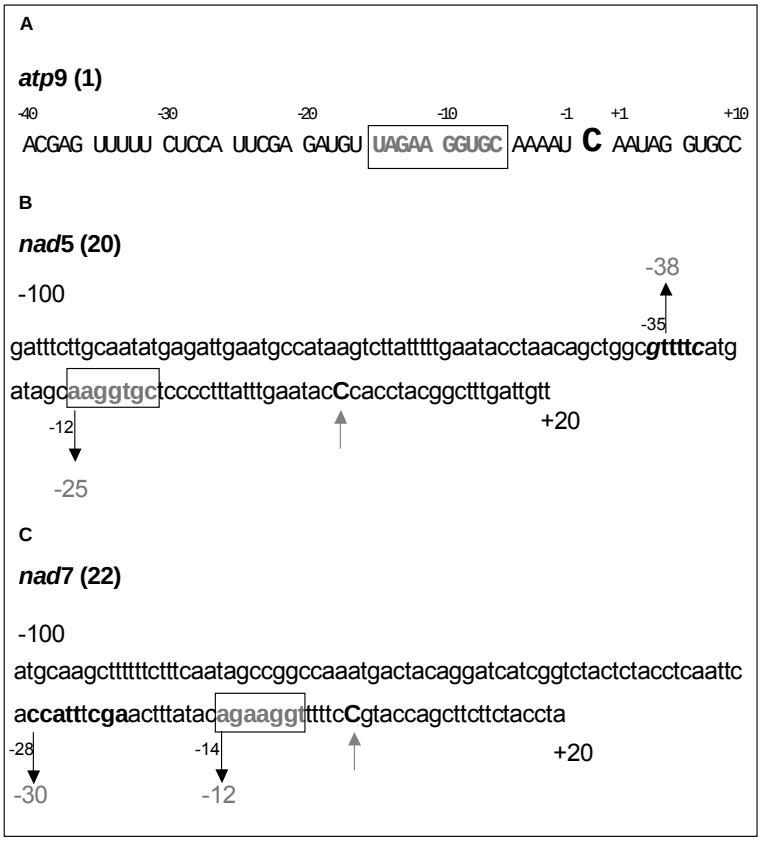


Figure 3.11: Computer screening reveals two editing sites with sequence similarities to the *atp*9 core recognition region in comparable distance. (A) Cartoon of the sequence of the core recognition region, which was taken for screening against all editing sites in *Arabidopsis thaliana*. (B) The 20th editing site in *nad*5 shows similarity to the in the core recognition region nucleotides in comparable distance (-12 in *atp*9, -25 in *nad*5 respective to the edited C). The sequence –39 to –35 (and –38 to –34 respectively) also shows similarity to –36 to –32 (and –35 to –31 respectively) (bold) depending on the starting point of the sequence. (C) The 22nd editing site in *nad*7 shows a similarity to the core recognition region of *atp*9 beginning at position –12 in *nad*7 (-14 in *atp*9). The nucleotide sequence –28 to –21 of *atp*9 is present in *nad*7 starting at position -30 (bold letters). This sequence includes one additional nucleotide at position -25 (not in bold).

Screening for *cis*-elements similar to the important recognition elements of *atp*9 upstream of the C to be edited was done. *In-vitro* analysis of the consecutively mutated templates had shown that the crucial recognition elements are situated in the sequence region covered by mutation clones M6 and M7 (see under point 3.2 and Figure 3.11A). These 10 nucleotides were run in a sequence comparison to all 441 editing sites in *Arabidopsis thaliana*.

Of these 10 nucleotides, a maximum of 7 were found to be situated upstream of 2 different editing sites in the genes *nad*5 (editing site 20) and *nad*7 (editing site 22) (Fig 3.11).

*Nad*5 (20) and *nad*7 (22) were cloned and tested in the pea *in-vitro* system. These editing sites contain a sequence similarity of 7 nucleotides to the core recognition area of *atp*9 in a distance comparable to the respective edited C. Further sequence similarities to different nucleotide sequences of *atp*9, e.g. to region M1 (Fig. 3.11), were found at *nad*5 (20) and *nad*7 (22).
RNA-templates (-100/+20) containing these two editing sites (*nad*5 (20); *nad*7 (22)) was tested under various conditions (see "Investigation of randomly selected editing sites in the pea *in-vitro* system".) in the pea *in-vitro* system.

Part of *ccb*206 as template for the *in-vitro* systems

A third strategy to find a new editing site recognized in the pea *in-vitro* editing system, was to choose an editing site which was previously characterized in a different RNA editing *in-vitro*-system. For this reason *ccb*206 was chosen, which was examined by the group of Schuster (Yu and Schuster, 1995). The group used mitochondria of pea seedlings and potato tubers and developed an RNA editing system to follow the fate of the α-Phosphate of the cytidine to be edited by radioactive labeling.
According to this publication, a 105 bp long fragment containing 8 editing sites (pea) was cloned and tested in the pea RNA editing system but no editing was observed.

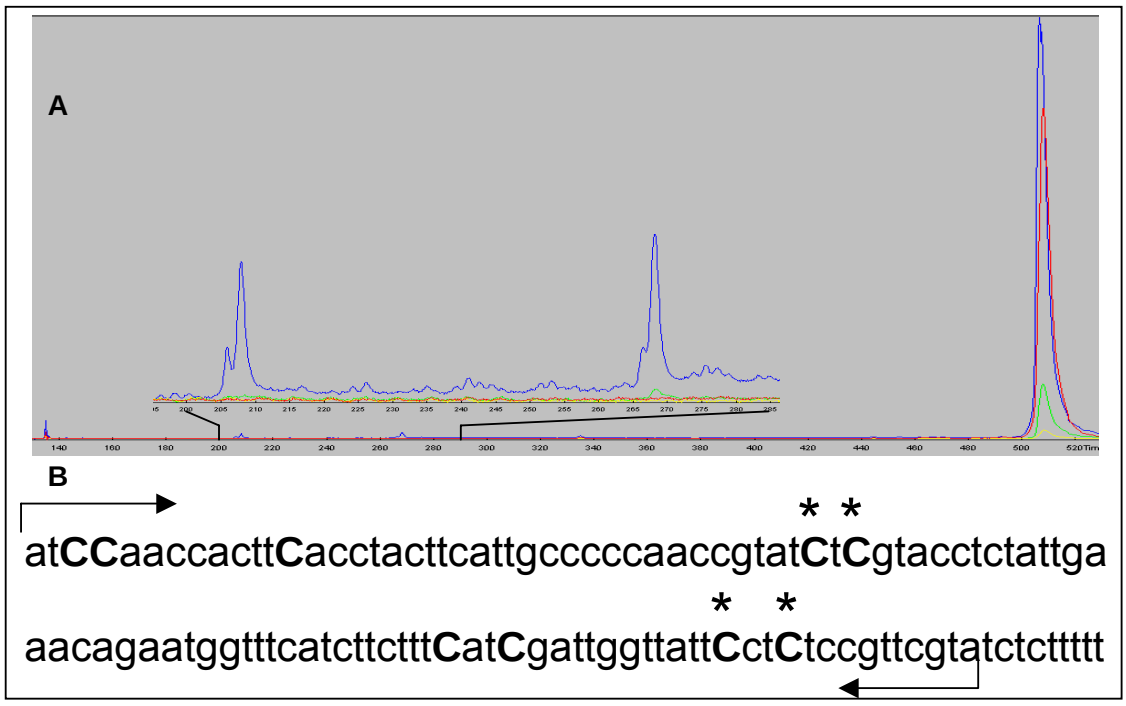


Figure 3.12: In a *ccb*206 derived template RNA 4 out of 9 different editing sites are edited in the cauliflower *in-vitro* system. (A) Computer analysis of the sequencing gel shows 4 editing peaks at 205, 208 and 266 and 269 minutes. (B) These edited sites correspond to the editing sites labelled with an asterix *.

Testing the different approaches with the newly developed cauliflower RNA editing system

The efforts to find more editing sites which are correctly addressed *in-vitro* show, that the pea system is not the system of choice for detecting additional editing sites. Successfully addressing of RNA editing sites *in-vitro* probably depends on the concentration of respective proteins and therefore will not be achieved in lysates with only low activity. The previously discussed advantages of the later developed RNA editing system of cauliflower indeed solved this problem (D. Verbitskiy, JA van der Merwe, pers. communication).
After the development of the cauliflower system the templates made before and newly constructed templates (D. Verbitsky, pers. communication) were tested with the cauliflower system. Editing sites, which were addressed in the cauliflower system could be detected (D. Verbitsky, pers. communication).

In *ccb*206 (pea template) 4 sites, could be detected to be edited in the cauliflower system. In the cauliflower *ccb*206 9 editing sites are present (Fig. 3.12A), of which 4 were found to be edited *in-vitro* (see Figure 3.12B). In Yu and Schuster, 1995, 7 out of 8 editing sites present in pea were edited with different percentages in the pea template. Site 4 was found to be edited with the highest percentage, second highest editing showed site 6. In our system, sites 3 and 5 seem to be edited efficiently, while sites 2 and 4 are processed less efficiently, further investigation of the editing sites was done by Anja Zehrmann in her diploma thesis (Zehrmann, 2006).
Another site, which was investigated, is *nad*5, which was found to be edited in the pea system, though at very low activity (Takenaka and Brennicke, 2003). The site was better edited in the cauliflower *in-vitro* system, which was further investigated by J. A. van der Merwe.

4. Discussion

In this thesis a newly developed plant mitochondrial *in-vitro* system was employed to investigate *cis*-elements involved in the recognition of editing sites. Editing has previously been studied in plastids using *in-vivo* (Chaudhuri et al., 1995; Bock et al., 1996) and in mitochondria using *in-organello* approaches (Staudinger and Kempken, 2003; To et al. 1996). The advantages of an *in-vivo* system are the possibility to observe a phenotype and to do reverse genetics, while a drawback is the time consuming strategy of the selection of a plant with the introduced gene to be investigated. The *in- organello* system provides the possibility to investigate a reaction inside a native system and to look at several post-transcriptional processes in one system and one reaction. Unfortunately, in this kind of system it is not possible to change or modulate the reaction conditions, since it is a closed system which does not allow the manipulation of biochemical contents.

After the development of an *in-vitro* system for chloroplasts (Hirose and Sugiura, 2001) our system was the first *in-vitro* system to be developed to detect RNA editing activity in mitochondria (Takenaka and Brennicke, 2003). It is a tool to investigate *cis*-elements, which determine the editing site. The advantages of *in-vitro* systems compared to *in-organello* or *in-vivo* systems for the investigation of RNA editing are numerous: At first the possibility to execute competition experiments has to be pointed out. Secondly the concentrations of the components of the system can be varied and the proteins potentially participating can be inhibited.

In this thesis, the extensions of *cis*-elements around an editing site in two plant species are investigated. Through the comparison of these two species, pea and cauliflower, species-specific effects, like differences in the extension of *cis*-elements, are detected. The comparison of two consecutive sites shows that these editing sites are addressed independently.

4.1 Determination of the basic recognition elements in pea

The major *cis*-elements involved in recognition are situated 5’ of the editing site (*atp*9 (1))

To investigate the extension of *cis*-elements 5’ and 3’ of the respective editing site the original template of pea (-173/+ 49) was deleted progressively first down to a –40/+49 template. These deletion clones (-173- and –40/+49) show similar editing activity. Therefore a set of consecutive deletion clones in steps of 10 nucleotides 5’ from –40 to –0 were constructed.

These deletions expose in pea an enhancer region situated between nucleotides –40 to –30 and a crucial recognition element between –20 and –10. Deletions 3' of the C to be edited have no negative influence on the editing efficiency. The slightly increased editing activity, when deleting the nucleotides 3' up to the +0 position, might be triggered by the substitution of the complete 3' region by bacterial sequences. This suggests that the original sequence might only be sub-optimal, at least *in-vitro*. This might be due to evolutionary reasons. The sequences of a gene can only be adapted until the changes influence the functionality of the resulting protein, which restrains the possibility of sequence modulation in favour of the editing rate. *Vice versa* the *trans*-factors could be involved in recognition of more than one editing site. This might result in a sub-optimal fit to each of the editing sites addressed by one *trans*-factor. This effect of increasing the editing rate by an exchange of nucleotides, was also observed by Sugiuras group (Miyamoto et al., 2003) with a template edited in the chloroplast, which was also not optimal for (*in-vitro*) editing.

These results are comparable to the observations made with an *in-organello* RNA-editing system at least concerning the 5' region, which reveals -16 nucleotides upstream of a site in the *cox*II mRNA to be sufficient for successful editing. In contrast +6 nucleotides downstream of this editing site are reported to be necessary for editing (Farré et al., 2001), while in *atp*9 (1) in pea no nucleotide is necessary.

Enhancer region and a crucial recognition element are separated by several nucleotides

How are the *cis*-elements crucial for recognition structured in front of the editing site? To address this question consecutive nucleotide exchange mutants were analysed.

Mutation of nucleotides –40 to –35 abolishes editing almost completely in pea, which suggests that this sequence includes an important sequence motive. The adjacent mutated nucleotides -35 to –25 (M2 and M3) seem not to be involved in recognition but might serve as spacing elements. The following 10 nucleotides (-25 to –15; M4, M5) reveal a higher importance since the editing rate is strongly decreased, if these are mutated. The competition experiments further define this region to be located mostly between –25 to –20 (M4), since M5 (-20 to –15) competes considerably stronger than M4. However, the *cis*-element covered by M4 (-25 to -20) probably extents further downstream, since this neighboring sequence appears to be important for recognition, but not sufficient to compete for binding with the recognizing *trans*-factor (Figure 3.3 B and C).

The less important nucleotides –20 to –15 are followed by the crucial recognition region around –10, covered by mutant M7. However the recognition element seems to extend further upstream (-5 to -1), since the mutated template (M8) dramatically reduces editing. If

the mutated template of –5 to –1 (M8) is used as a competitor, editing is abolished entirely, which means that the few nucleotides of M8 involved in recognition are not sufficient to rescue recognition for the adjacent upstream element.

The 3´region does not seem to be important for editing in pea *atp*9 site 1, since mutations in this region show full editing activity.

Comparison between the influence of the deletion of –40 to –30 and the exchange mutant -40 to –35 on the editing activity shows following results: While the deletion clone only suppresses editing up to 50%, the editing rate of the mutant (M1) with exchanged sequences drops to less then 10%. These results seem to contradict each other. There are two possible explanations for this effect: Either, the bacterial sequences replacing the deleted area contain a by chance similarity, which replaces the necessary sequence in the deletion clones and thereby recovers part of the editing activity or, the sequence exchange in connection with the adjacent bacterial sequence forms a secondary structure or an inhibiting sequence element, which lowers the *in-vitro* editing activity.

The editing complex does not tolerate either increase or decrease of the distance between recognition element and editing site

Single nucleotide insertions or deletions were examined to investigate the importance of the distance of the *cis*-elements to the respective editing site, particularly the major recognition region covered by –15 to –1 (M6 to M8). Shifting the editing site by one nucleotide in either direction abolishes the editing activity entirely showing that the editing enzyme complex in case the of *atp*9 (1) does not tolerate any shift in the distance between recognition element and editing site.

This is supported by the results of 3.6 where the seven nucleotides which are contained in the core region and responsible for recognition (M7 extended to M6) have been found upstream of *nad*5 (20) and *nad*7 (22), however in altered distance to the C to be edited (Fig. 3.12). In case of *nad*5 (20) the sequence element showing identical nucleotide identities is situated at position –19 to –26 whereas in *atp*9 (1) it is at position –6 to –13. In *nad*7 (22) the sequence found at position –6 to –13 starts in *atp*9 (1) at position –8 to position -15. Under the same conditions no editing activity was detectable, neither in *nad*5 (20) nor in *nad*7 (22), suggesting that different *trans*-factor(s) might be responsible for editing of these sites. If the editing complex in *nad*7 (22) is the same as in *atp*9 (1) (and *nad*5 (20)) and if this complex tolerates a nucleotide shift of two nucleotides respective to *atp*9 (1) this *trans*-factor could also be employed for editing. If so, one specific *trans*-factor is needed for these sites which is missing in our lysate, since not editing activity is detectable.

4.2 Variants between pea and cauliflower point to species-specific recognition

Cis-elements 5' of the homologous editing site can vary between species

The evolutionary development of the RNA-editing machinery and of the *cis*-elements involved in recognition is an interesting question. How do the recognition patterns diverge between plant species and how dynamic is the *cis*-recognition system? To address the question how specificities alter, *atp*9 (1) was chosen as homologous editing site present in pea and cauliflower and the *cis*-recognition elements were compared. The reasons to study *atp*9 (1) are: Firstly, this site has been described already in the pea system and secondly, the sequence upstream of the editing site rapidly diverges between the two plants: 23 nucleotides 5' of the edited C the sequence is entirely different (Fig. 3.8).

The variations between the *cis*-elements in pea and cauliflower are the following:

- Deletion mutants, exchange mutants and competition analysis reveal that the extent of the core region of recognition between the two plants slightly differs. While in pea the main recognition element seems to be covered by nucleotides –15 to –5, the cauliflower *cis*-element extends further into the 5' region expanding to the –20 to –15 area. The area –5 to +0 seems to play a similar role in both species.
- Deletion mutants of the 3' region of *atp*9 (1) of cauliflower display in contrast to pea a severe drop of editing activity if the original sequence is replaced by bacterial sequence up to position +0. Also the exchange mutant M9 shows less activity compared to pea supporting this result. A set of mutations of nucleotide +1 adjacent to the editing site showed that in cauliflower the editing activity depends on the identity of this nucleotide.

These results suggest that the *trans*-elements responsible for recognition have evolved between the two species to the adapted *cis*-elements. The editing event itself has necessarily been conserved to maintain the functionality of the *ATP*9 protein and in consequence the ATPase complex.

Is the enhancing region –40/-35 of pea present in cauliflower?

Comparison of the sequences of pea and cauliflower *atp*9 (1) regions shows a divergent sequence from position –40 to –23. This difference suggests that if this area is part of the recognition system for editing, the respective *trans*-factors must have evolved differently.
In pea the region between –40 to –35 was suggested to enhance editing, since the deletion clone –30/+49 shows a severe reduction in editing. Pea and cauliflower templates are edited equally well in the cauliflower lysate.

Most interesting within these divergent sequences are the nucleotides –40 to –35, which are absent from deletion mutant (1) and affected by mutant M1. The experiments show the following results: The deletion mutant –30/+49 tested in the pea lysate reduces editing up to about 50 %, while the editing rate in the cauliflower lysate is not affected. This result shows that in cauliflower this sequence does not have an effect, which is supported by the extent of editing of pea and cauliflower templates, respectively, which are equal in the cauliflower lysate.
But both the mutation as well as the competition experiments with M1 (-40 to –35) show similar results in both lysates, notably the drop of editing to 10 % with the mutated template and the reduction of editing by competition. There are various possibilities to explain these effects:
Firstly, the effect is an artifact triggered by the combination of bacterial sequence upstream of the cloned mutations and the mutated sequence M1, which might form a sequence motif that attracts RNA-binding proteins, which through binding block the *cis*-elements necessary for recognition. This might explain that the mutated sequence in the pea template has a similar effect in pea and cauliflower even though in cauliflower this sequence is not necessary for efficient editing.
The cauliflower sequence is equally well edited in the pea lysate even without the enhancing sequence. There might be a *trans*-factor in pea mitochondria which also recognizes the cauliflower sequence element. In the cauliflower *atp*9 (1) template RNA no enhancing effect is observed. All recognition elements are confined 5' of the editing site. However, the *cis*-elements of most of the editing sites so far investigated usually range between approximately 20 nucleotides upstream to 6 nucleotides downstream which is reported, e.g. for *cox*II (77) where the area –16 to +6 is crucial for editing (Choury et al., 2004). It could be that we look at a unitised assembly of the hypothetical editing complex. The enhancer binding to the M1 area might not be in contact with the *trans*-factors which bind to the core recognition region. It could just contact the editing enzyme and bring it into closeness to the *trans*-factors responsible for recognition. Once there, the RNA-editing complex consisting of the enzyme

and the *trans*-factors responsible for recognition of the core *cis*-elements, if they are distinct, can form easier. This might boost the editing activity.
However, in pea the M1 area has an enhancing effect on editing of the cauliflower and the pea templates, suggesting that through an unknown interaction editing of either template is boosted, possibly through preferred binding of *trans*-factors to the area –40 to -35. M1 in cauliflower, which means the mutated pea sequence, attracts RNA-binding proteins which would explain the mutation and competition experiments.

4.3 Investigation of two consecutive editing sites

Editing sites are addressed individually

With the template construct of *atp*9 (1) –40/+49 it was possible to monitor a second editing site, which is situated at nucleotide position +30 respective to the first site. A possible connection between the two sites was investigated.
In a template RNA, in which the first site (-0/+49) has been deleted, the second site can still be recognized and edited *in-vitro*. If the second site is deleted (-40/+10) the first site is still edited showing that both sites can be edited independently in the absence of the respective other site.
These experiments suggest that there is no stringent order in which the respective editing sites have to be addressed. If such an effect is observed it possibly depends on the affinities and concentration of the respective *trans*-factors – the higher the affinity of a *trans*-factor to a *cis*-element in front of an editing site or the higher the respective *trans*-factor is concentrated, the greater is the possibility for it to bind.

Specific *trans*-factors recognize neighbouring sites independently

The *trans*-factors for these consecutive editing sites are different. Comparison of the sequences 5' of the two editing sites shows no sequence similarities (Fig. 3.8), which suggests that the *trans*-factors addressing the two sites are different. This is supported by cross competition experiments. Competing the first site with an excess of 1500-fold competitor, the second site edits as well as the control, while the first site is competed as expected and *vice versa* (Fig. 3.10). This shows that only the cognate sequences interfere with editing at either site. The different extents of competition of the first site and the second site with their respective competitors, using the same concentrations of competitor and template in an experiment also show that distinct *trans*-factors must be present. The

observation that in competition experiments with a non-influencing competitor (SK, Fig. 3.10 B,C) the editing efficiency increases is a result of additional RNA that snatches away unspecific binding proteins which might inhibit the editing reaction (J. A. van der Merwe, pers. communication).

Additionally, since the competitors used contain the necessary *cis*-elements to be edited, they are expected to not only compete, but also to be edited in the *in-vitro* system. For that reason, the enzyme and all *trans*-factors, shared by the consecutive editing sites have to be present in excess, since editing of one site is not affected by competition of the respective other site.

A species-specific *trans*-factor acts over a distance of 70 nucleotides

Why is the second editing site of *atp*9 only edited in the cauliflower *in-vitro* system?

The second editing site in cauliflower is only edited efficiently in the native –40/+49 cauliflower template. Using either of the templates (cauliflower and pea) in the pea lysate, no editing of the second site is observed. With the pea template in the cauliflower lysate this site is only observed to be edited occasionally and only with an efficiency lower than in the respective cauliflower template (about 5 % in the pea template *versus* about 110 % in the cauliflower template, Fig. 3.10). Deleting 20 nucleotides of this pea template (to -20/+49), the sequences 5' of the second editing sites are identical between pea and cauliflower. With this template (-20/+49) the editing efficiency of the second site increases compared to the native pea template, while it is still much lower than the native cauliflower template. The editing activity remains constant from template –20/+49 up to the final deletion step -0/+49 (Figure 3.10).

The differences in editing activity between the cauliflower and pea –40/+49 templates, suggest that the region deleted in template –20/+49 contains an important sequence motif triggering or enhancing editing. This deleted region includes the nucleotides –40 to –35, respective to site 1 (-70 to –65 respective to site 2), which have previously been found to affect editing activity of *atp*9 (1) in the pea template. When these nucleotides are changed or deleted, editing activity drops significantly, suggesting that this area is a candidate for having a comparable effect on the second site of the cauliflower template. It has not been reported so far that a *cis*-element situated at such a distance can influence editing activity of a site in mitochondria. The native cauliflower sequence seems to attract a *trans*-factor which can act over a distance of 50 to 70 nucleotides.

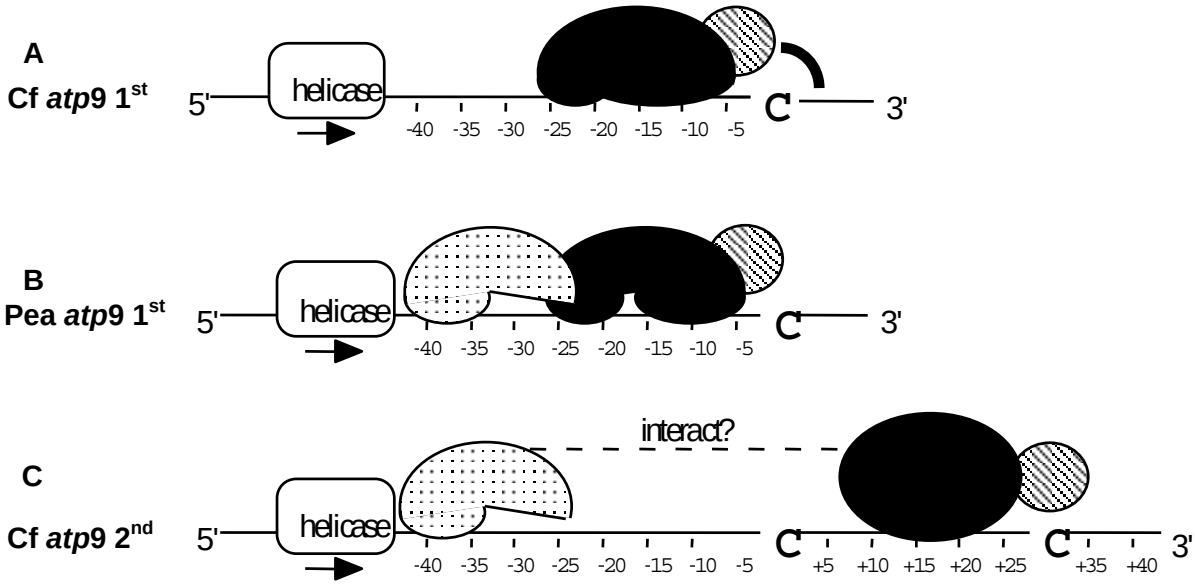
The effect is species-specific, since in the cauliflower *in-vitro* assay the non-native pea template sequence can not substitute the positive effect of the cauliflower sequence. It

seems that two different *trans*-factors are attracted by the same sequence area, since the positive effect of this region can be titrated for the first site but not for the second.

4.4 A model of the editing complex

The three editing sites investigated *in-vitro* show variations of *cis*-elements, which suggests different patterns of recognition regarding the *trans*-factors involved. The active *trans*-elements, which address the described *cis*-elements can be separated into (1) non-limited enhancer elements, (2) essential and limiting recognition elements and (3) the actual editing enzyme (Fig. 4.1).
The location of *cis*-elements of the 3 editing sites investigated leads to the model of *trans*-factors transcribed in Fig. 4.1, which varies slightly between the respective sites.

(1) The involvement of enhancer elements was investigated in pea *atp*9 (1) and cauliflower *atp*9 (2), which are separated by 30 nucleotides. Interestingly, in both cases the same sequence between -40 to –35 respective to the first editing site is the potential *cis*-element participating as enhancer. It is not clear how the potential enhancing factor attracted by this sequence and the *trans*-factor(s) recognizing the core region interact, especially for the second editing site which is addressed over a distance of 50 to 70 nucleotides. Apparently these elements are not limited in amount. One conclusion could be that the enhancing effect is due to by chance effects of the sequence present.
(2) The *trans*-elements, which are responsible for recognition of the core region are limited in amount, since they can be competed. They show a high specificity, since mutation of the *cis*-elements responsible for recognition abolishes editing. From the results of the experiments in different plants for *atp*9 (1) it can be concluded that the binding or recognition sequences in the core region slightly vary in extent between these plant species (Fig. 4.1 A,B). While in pea apparently the *trans*-factor(s) responsible for recognition bind(s) in the core region of –15 to –5, supported by a stabilizing binding in the area –25 to -20, in *atp*9 (1) in cauliflower the shape of the *trans*-factor (most likely a protein) is different since the core region extends to –25 covering also the approximately 5 nucleotides which are not necessary for recognition in pea. This suggests that either species-specific recognition proteins are involved or the *trans*-factors evolved between the two plants. These proteins might have been the same in an ancestor but by adjusting to the rapidly evolving 5’ sequence in the non-coding area of cauliflower and pea they assumed a different shape. Similarly, for *atp*9 (2) of cauliflower the core recognition area also covers an area up to 20 nucleotides upstream to the edited C (Fig. 4.1, C), which is comparable to pea *atp*9 (1).



***Trans*-factor**. Responsible for recognition of the core *cis*-element. Essential (limited amount and high specificity).

Potential enhancer element. Responsible for boosting the editing reaction. Not limited in quantity.

Editing enzyme. Responsible for the deamination reaction. Not limited in amount. Little specificity.

Figure 4.1: Models of possible interactions between hypothetical *cis*- and *trans*- factors of the three investigated editing sites. (A) The 1st editing site in cauliflower *atp*9. One (or more) *trans*-factor(s) bind to the core recognition region –25 to –10. The enzyme binds the *trans*-factor and edits. This enzyme seems to depend on the identity of the +1 nucleotide for sterical or inhibiting reasons. (B) The 1st editing site of *atp*9 in pea. The *trans*-factor(s) show two major binding sites: The core area between –15 and –10 and a supporting binding site (-25 to –20). An enhancing element boosts the editing reaction possibly by interacting with the *trans*-elements. The +1 area does not influence the editing reaction. (C) The 2nd site of *atp*9 in cauliflower. The extension of the core recognition area is comparable to A,B. The sequence –40 to –35, a potential enhancing sequence for the first editing site of pea, seems to play a similar role in the 2nd site of cauliflower. Details of the different models are given in the text.
Picture kindly provided by M. Takenaka.

As candidates for recognition proteins members of the family of PPR-proteins have been discussed as promising candidates (if indeed *trans*-factors participating in editing are proteins) because of several features: The family of PPR-proteins consists of several hundred members, e.g. about 450 respective genes have been identified in *Arabidopsis* and 650 in *Oryza,* of which the majority is predicted to be targeted to mitochondria and/or plastids. The PPR-motif is a highly degenerate unit of 35 amino acids that usually appears as a tandem repeat. Although PPR-proteins are widely distributed in eukaryotes but are not found in prokaryotes the number of genes is limited in non-plants (Small and Peeters, 2000). Most are predicted to bind RNA sequences and some members have been found to be involved in various RNA-processing reactions such as RNA cleavage, splicing, transcription and translation processes. These inferences, and a mutant of a PPR-protein which was

found to be deficient in RNA-editing of *ndh*D (1) in chloroplasts, suggest that PPR-proteins may be the *trans*-acting factors in editing (Shikanai, 2006). Indeed, PPR-proteins have been found to bind to *atp*9 RNA-editing templates in cross-linking experiments (Mizuki Takenaka, unpublished data).

(3) Since the PPR-proteins themselves do not contain a domain with clear similarity to a deaminase which could be responsible for the actual deamination reaction, it is likely that a third component is responsible for the enzymatic reaction. *In-vitro* experiments in pea have shown that the enzyme involved is insensitive to a zinc-chelator (Takenaka and Brennicke, 2003), which makes the participation of a zinc-dependent deaminase unlikely. On the other hand, zinc sensitivity has been observed in chloroplast *in-vitro* assays (Hegeman et al. 2005), suggesting that the editing enzymes in the two organelles differ in some aspects.

In the present model (Fig. 4.1), the actual editing enzyme is thought to form a complex with the *trans*-factor(s) binding to the core region. Possibly, depending on the sterical conformation of the *trans*-acting factor(s), the enzyme can interact with different sequence parts of the substrate, respectively. In pea *atp*9 (1) the region 3' to the edited C does not seem to be important for recognition, while in cauliflower *atp*9 (1) the identity of the +1 nucleotide plays a crucial role. Either the enzyme itself attaches to this nucleotide or, which is more likely, the identity of the first 3' nucleotide influences binding of the enzyme sterically. In cauliflower *atp*9 (1) only one of the four nucleotide identities is optimal.

Whether or not the postulated *trans*-factors actually stably assemble can not be answered yet. It is possible that the *trans*-factor(s) and enhancing proteins only contact the enzyme.

The hypothetical editing complex of *atp*9 (1) of cauliflower template in cauliflower lysate does not tolerate an alteration of the distance to the C to be edited, while in *atp*4 (2-4) such a tolerance is suspected (Verbitsky et al., 2006). Here, two editing sites spaced by 2 nucleotides are postulated to be edited by the same editing enzyme - *trans*-factor complex, which suggests that this stretching might depend rather on the *trans*-factors involved than on the editing enzyme.

A further question is whether editing sites are addressed independently or whether they are edited in a certain order. The investigation of the two consecutive editing sites in *atp*9 showed that these sites are edited independently. The editing complex seems to work in a hit-and-run manner rather than driven by progressive screening along the RNA molecule.

The possible involvement of a helicase cleaning up the template for *trans*-factor attachment has been discussed, based on the observation that NTP's are necessary for the reaction and that cross-linking reveals a helicase being bound to the RNA template.

In summary: Firstly, the RNA-editing machinery in mitochondria possibly consists of one or more *trans*-element(s). These *trans*-elements bind to the *cis*-recognition area, which includes up to 25 nucleotides upstream of a given RNA editing site. Secondly, an enzymatic activity is attached, which might prefer a specific nucleotide identity downstream. Thirdly, the RNA editing activity can be enhanced by an specific sequence. It is still unclear, whether this effect is restricted to an *in-vitro* reaction. Lastly, RNA editing site recognition can apparently be modified from plant to plant and also from editing site to editing site.

5. Materials and Methods

5.1 Material

Plant material

Pea seedlings (*Pisum sativum* L., var) were grown at 24°C in the dark for 6 days. Etiolated shoots were harvested.
Rice seedlings (*Oryza sativa*) were grown at 30°C in the dark for 10 days. Etiolated shoots were harvested.
Heads of cauliflowers were purchased at local markets.

Consumables

Commercially obtainable chemicals of the companies Duchefa Biochemie B.V. (Haarlem, Netherland), VWR International GmbH (Darmstadt, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany), PEQLAB Biotechnologie GmbH (Erlangen, Germany), Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), Merck KGaA (Darmstadt, Germany), SERVA Feinbiochemica GmbH & Co. (Heidelberg, Germany) und MP Biomedicals GmbH (Eschwege, Germany). All buffers were prepared in bidestilled water.

Enzymes

The enzymes used in this work were supplied by the companies Genecraft GmbH (Lüdinghausen, Germany), Fermentas GmbH (St. Leon-Rot, Germany), Roche Diagnostics GmbH (Mannheim, Germany), Stratagene GmbH (Heidelberg, Germany), and Trevigen, Inc. (Gaithersburg, USA).

Oligonucleotides

The oligonucleotides used in this work were commercially optained from the companies biomers.net (Ulm, Germany) und Invitrogen GmbH (Karlsruhe, Germany). Sequences can be provided upon request.

Devices

The equipment used is standart of a Molecular Biological laboratory. For PCR the GeneAmp® PCR System 9700 of Applied Biosystems was used. For sequencing an ALF-Sequencer of Amersham Bioscience was used.

5.2 General Methods

Standard methods of Molecular genetics

All molecular genetic methods, e.g. enzymatic treatment of nucleic acids, electrophoresis, are performed according to the standard protocols commonly available.
Standard PCR reactions are performed using 10 μM of each primer in a 50 μl reaction. Buffer and Taq Polymerase BioTherm™ are provided by Genecraft GmbH (Lüdinghausen, Germany). For exact description of the *in-vitro* system PCR see "Detection of RNA editing activity by mismatch analysis".
Escherichia coli was transformed by electroporation using the Gene Pulser from BioRad according to the recommendation of the provider.
Standard cloning was done using the *E. coli* laboratory strain DH5α and the vector Bluescript SKII$^+$ or the modified Bluescript SKII+ for the *in-vitro* substrates according to the description in Takenaka and Brennicke (2003).
For di-desoxy sequencing the T7 polymerase kit form Amersham Pharmacia (Freiburg) was used.
cDNA-synthesis was performed using the kit of Stratascript reverse transcriptase of Stratagene (Heidelberg) according to the protocol.

Preparation of mitochondrial extracts

Mitochondria of pea shoots and cauliflower inflorescences were isolated by differential centrifugation and purification on Percoll gradients as described previously (Binder et al., 1995). Mitochondria of rice shoots were isolated based on the protocol of Neuburger et al, 1982. Four hundred mg of purified mitochondria were lysed in 1,200 μl of extraction buffer (0.3 M HEPES-KOH pH 7.7, 3 mM magnesium-acetate, 2 M KCl, and 2 mM dithiothreitol) containing 0.2% Triton X-100. After 30 min of incubation on ice, the lysate was centrifuged at 22,000 x g for 20 min. The supernatant was recovered and dialyzed against 5 x 100 ml dialysis buffer (30 mM

HEPES-KOH pH 7.7, 3 mM magnesium-acetate, 45 mM potassium-acetate, 30 mM ammonium-acetate, and 10% glycerol) for a total of 5 h. All steps were carried out at 4 °C. The resulting extract (10-20 μg protein/μl) was rapidly frozen in liquid nitrogen. When stored at -80 °C the lysate was stable for at least 3 months.

RNA substrates

DNA clones (patp9) were constructed in an adapted pBluescript SK^+ to allow run-off transcription of the template RNA. The synthesized RNA molecule contains the first two pea *atp*9 editing sites flanked by bacterial sequences to allow specific amplification from these bacterial sequences against the background of internal mRNAs. 154 bp of the 5' untranslated region and the first 69 bp of the coding sequences of the pea mitochondrial *atp*9 gene were cloned into the *Pst*I site of the vector multiple cloning site between the T7 promoter upstream and the T3 promoter sequence in the downstream region. To probe other editing sites in the *in-vitro* system, in addition to the *atp9* template also an RNA substrate covering exon e of the *nad5* gene in pea was tested. The selected region was cloned into the arrangement described for *atp*9*.* The generated run-off RNA contains four RNA editing sites, the most 5'. 105 nucleotides of *ccb*206 containing 9 editing sites in cauliflower were cloned into the described arrangement for further investigation. Every other gene investigated was cloned full length, according to the description.

In-vitro RNA editing reactions

The *in-vitro* RNA editing reactions (Abb. 5.1) were performed in a total volume of 20 μl. The reaction mixture consisted of 30 mM HEPES-KOH pH 7.7, 3 mM magnesium-acetate, 45 mM potassium-acetate, 30 mM ammonium-acetate, 15 mM ATP, 2 mM dithiothreitol, 1% polyethyleneglycol 6000, 5% glycerol, 40 units RNase inhibitor (MBI), 1 x proteinase inhibitor mixture (Complete TM, Roche Applied Science, Mannheim), 100 amol (100×10^{-18} mol) mRNA substrate, and 6.0 μl mitochondrial extract. After incubation at 28 °C for 4 h, the substrate mRNA was extracted with the RNeasy kit (Qiagen, Hilden). Variations of individual concentrations and additions of various other compounds are indicated in the respective figure legends.

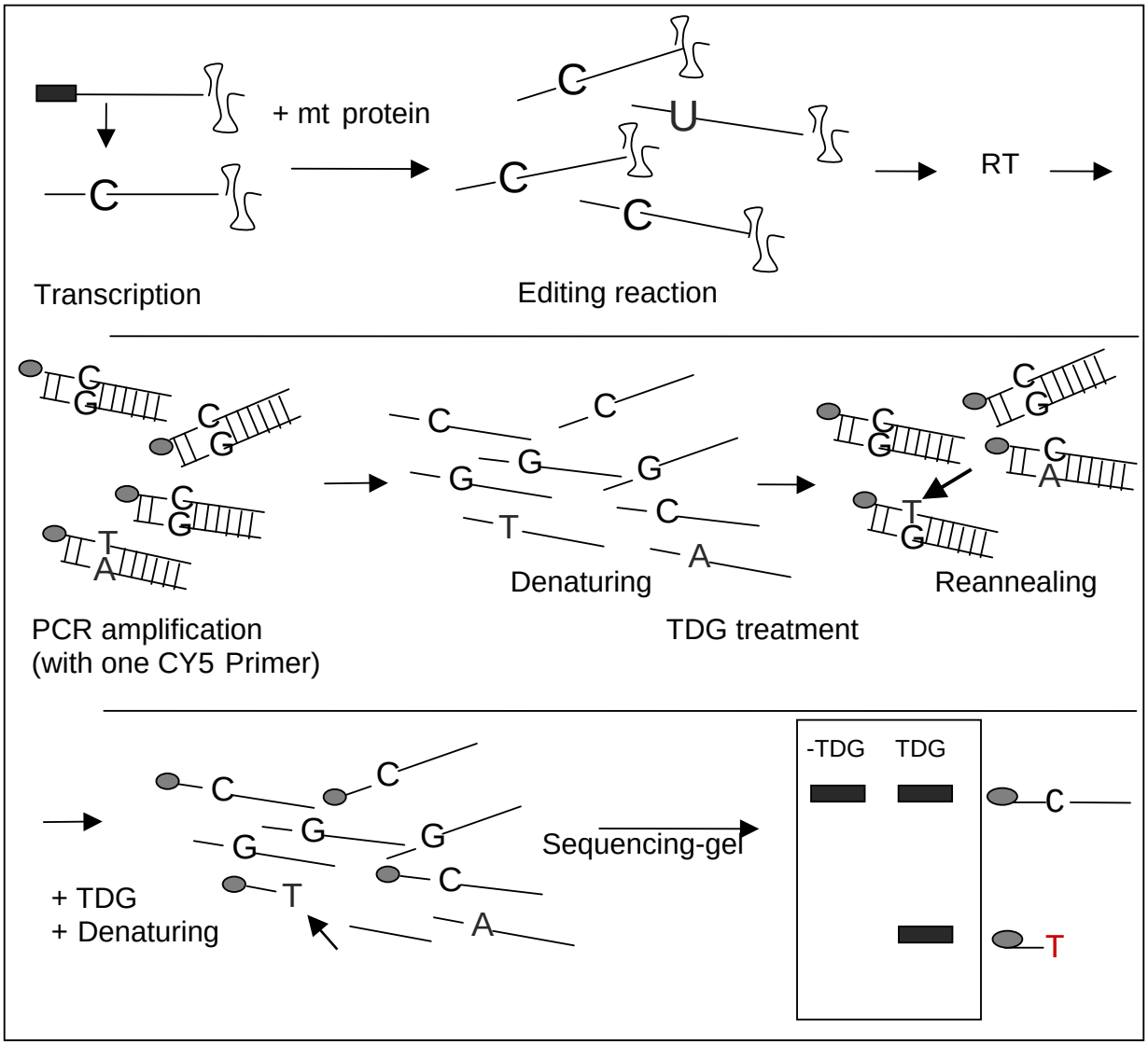


Figure 5.1: The RNA editing reaction.

Detection of the RNA editing activity by mismatch analysis

The cDNA was synthesized from the substrate mRNA with reverse transcriptase StrataScript (Stratagene, Heidelberg) from the T3 primer. The subsequent PCR reactions were performed with 0.1 units of Pwo polymerase (peqLab, Erlangen) and 0.5 units of Taq polymerase using a Cy5 labeled KS primer (Cy5-KS) and an unlabelled T3 primer. Cycling was performed as follows: 95 °C for 2 min; 5 cycles of touchdown PCR (95 °C for 30 s, 65 °C to 60°C decreasing by 1 °C per cycle for 30 s, and 72 °C for 1 min); 45 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min; finally 72 °C for 5 min. The PCR products were purified by 1% agarose gel

electrophoresis. Denaturation and reannealing were done as follows: 95 °C for 10 min; 90 °C to 70°C decreasing by 5 °C per cycle for 5 min; 65 °C for 1h. After reannealing, the resulting heteroduplexes were treated with 0.2 units of the enzyme TDG (thymine DNA glycosylase, Trevigen, Gaithersburg). The TDG-treated fragments were denatured for 5 min at 95 °C in alkali buffer (300 mM NaOH, 90% formamide and 0.2% bromphenol blue), and the strands of the DNA were separated by 6 M urea 6% PAGE. The Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (Amersham Biosciences, Freiburg). To quantify the efficiency of the *in-vitro* RNA editing reaction, the area under the peaks of the cleaved and uncut DNA fragments was determined. The ratio of cleaved, i.e. edited, fragment to uncut DNA was used to determine relative efficiencies of the investigated conditions in each experiment. To obtain comparable values to combine several independently repeated assays and to allow determination of variation bars, the ratios of cleaved to uncleaved fragments were displayed as percentages of the standard reaction conditions. Between individual experiments major sources of variation are the differences in RNA editing activity and RNase content of individual lysate preparations. In the gel analysis of the *in-vitro* editing products, smearing of the uncut fragment signal complicates the determination of the respective signal area for comparable quantification between individual gel runs. Therefore co-treatment and parallel resolution of the template under standard conditions was adopted for reference in each experiment.
The efficiency of detection has its optimum inside a window of a minimum editing percentage of 0,5 to 50%, and a substrate concentration of 100 amol using 60 to 120 µg of protein extract. Detecting two or more editing sites in one template, experimental detection inaccuracies have to be considered. The DNA template is labelled 5'. Every template, which contains more than one editing site can be edited at one or more sites in one molecule. The TDG-enzyme theoretically cuts every T/G mismatch, but only the labelled product will be detected. That way only the edited site which is situated closest to the labelled 5' end will be displayed.

The methods used were previously described in Takenaka and Brennicke, 2003.

Generation of mutant substrates

The deletion mutants were constructed using inverted PCR from patp9 with primers –40, –30, -20, –10 and –0, respectively on the one side and primer invertion1 on the other. The resulting fragments were digested with *Eco*RI to generate sticky ends in the primer contained *Eco*RI recognition site and were self-ligated. The deletion mutants were constructed using inverted PCR from clone atp9-30 with primer invertion2 and primers +10 and +0, respectively. The PCR

fragments were digested with *Xba*I and self-ligated. The mutant templates with defined sequence regions exchanged to their opposite sequence were constructed by introducing the respective complement pentanucleotide in primers M1–M10. PCR was performed on deletion clone atp9-40 with primer invertion1 and primers M1, M2, M3, M4 and M5, respectively, and in the second series with primer invertion2 and primers M6, M7, M8, M9 and M10, respectively. The resulting fragments were digested with *Eco*RI or *Xba*I, respectively, and self-ligated (Takenaka et al, 2004).

Competition assays

Wild-type competitor RNA was synthesized from the PCR product amplified with primers T7 and +10 from clone atp9-40. A complete plasmid-derived control RNA was synthesized from the PCR product amplified from pBluescriptIISK$^+$ with T7 and SK primers. The mutant competitors were synthesized from the PCR products amplified from clones M1 to M5 with the T7 primer and primer +10, and from clones M6, M7, M8, M9 and M10 with T7 and the respective mutant primers. One hundred attomoles of substrate and for pea competition experiments 1000 times (100 fmol) for cauliflower competition experiments 1500 times competitor RNA were first mixed and then incubated with the mitochondrial *in-vitro* assay as described above (Takenaka et al, 2004).

For further details on specific experiments see also the under point 7.1 listed publications.

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7. Appendix

7.1 Publications

Complex *cis*-elements determine an RNA editing site in pea mitochondria.

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Complex *cis*-elements determine an RNA editing site in pea mitochondria

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ABSTRACT

The *cis*-requirements for the first editing site in the *atp9* mRNA from pea mitochondria were investigated in an *in vitro* RNA editing system. Template RNAs deleted 5′ of −20 are edited correctly, but with decreased efficiency. Deletions between −20 and the edited nucleotide abolish editing activity. Substitution of the sequences 3′ of the editing site has little effect, which suggests that the major determinants reside upstream. Stepwise mutated RNA sequences were used as templates or competitors that divide the *cis*-elements into several distinct regions. In the template RNAs, mutation of the sequence between −40 and −35 reduces the editing activity, while the region from −15 to −5 is essential for the editing reaction. In competition experiments the upstream region can be titrated, while the essential sequence near the editing site is largely resistant to excess competitor. This observation suggests that either one *trans*-factor attaches to these separate *cis*-regions with different affinities or two distinct *trans*-factors bind to these sequences, and one of which is present in limited amounts, wheras the other one is more abundant in the lysate.

INTRODUCTION

RNA editing in plant mitochondria alters >400 nt identities (1). In mosses and ferns both C to U and U to C changes occur, while flowering plants nearly exclusively alter C to U. In chloroplasts of vascular plants ~30–40 analogous editing events are observed, raising the possibility that similar if not the same activities act in both organelles (2–5). Comparisons between the RNA editing parameters in the two different compartments are needed to clarify this question.

In both organelles, the recently renewed efforts to develop *in vitro* assays for RNA editing (6,7) have yielded considerable progress by extending the information gained from the first investigation in plant mitochondria almost a decade ago (8,9). These prior experiments provided evidence that the biochemical reaction underlying the C to U change is most probably a deamination step, which does not cut the sugar-phosphate backbone of the RNA (8,9). It is presently unclear whether one of the classic deaminases, several of which have been identified in *Arabidopsis* (10), is involved, since zinc-ion chelators have no effect on the *in vitro* reaction (7).

Determinants of the specificity in mitochondria have recently been investigated for several editing sites in an electroporation assay (11–13). Sequence requirements for editing sites have been analyzed in chloroplasts in transgenic plastids (14–18) as well as *in vitro* (6,19,20). These assays confirmed and extended the conclusions previously drawn from rearranged sequences in mitochondrial genomes and the editing states of their transcripts (21), which had suggested that the sequences 5′ of the edited nucleotide are the main determinants of site recognition. The crucial *cis*-region usually extends ~20–30 nt upstream of the editing site. Exceptions have been documented for plastids, where 84 upstream nucleotides may not be enough to specify a given site (14). The sequence region downstream of an edited nucleotide seems to contribute little, in some instances <5 nt identities appear to have an influence on the identification of the editing site.

In chloroplasts, cross-linking experiments in an *in vitro* system identified different proteins to bind specifically to the upstream sequence regions (6,19,20). Most individual RNA editing sites show little or no discernible sequence similarity and consistent with this high RNA sequence variation distinct proteins are found to interact with different sites. However, similarities between upstream sequences of groups of several editing sites suggest that sequence-specific *trans*-factors may be involved that can recognize several sites (15,22). In addition, common protein factors may be involved in binding a larger number of such sites, since one or more of the cross-linking protein moieties show similarity to general chloroplast RNA-binding proteins by their apparent size and by their reaction with the respective antibodies (6,19,20). Furthermore several *trans*-acting factors appear to be limited in quantity, since *in vitro* competition experiments as well as analyses of transplastomic plants revealed diminished editing at the sites with sequence similarities in their immediate 5′ regions, suggesting the depletion of a necessary *trans*-factor (6,14–17).

To gain further information about editing site recognition in plant mitochondria, we have now analyzed the *cis*-elements at the first RNA editing site in the *atp9* mRNA in our *in vitro* system developed recently for RNA editing in pea mitochondria (7). Deletions and mutations distinguish distinct elements upstream of the editing site. Some of these are essential for correct recognition, while others enhance the efficiency of the reaction.

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MATERIALS AND METHODS

Preparation of pea mitochondrial extracts

Pea seedlings (*Pisum sativum* L., var) were grown at 24°C in the dark for 6 days. Mitochondria were prepared by differential centrifugation and purified on Percoll gradients as described previously (7). An aliquot of 400 mg of isolated mitochondria were lysed in 1200 µl extraction buffer (0.3 M HEPES-KOH, pH 7.7, 3 mM magnesium acetate, 2 M KCl and 2 mM DTT) containing 0.2% Triton X-100. After 30 min incubation on ice, the lysates were centrifuged at 22 000 *g* for 20 min. The supernatant was recovered and dialyzed against 5 × 100 ml dialysis buffer (30 mM HEPES-KOH, pH 7.7, 3 mM magnesium acetate, 45 mM potasium acetate, 30 mM ammonium acetate and 10% glycerol) for a total of 5 h. All steps were carried out at 4°C. The resulting extract (10–20 µg of protein/µl) was rapidly frozen in liquid nitrogen.

RNA substrates

DNA clones (patp9) were constructed in an adapted pBluescript SK^+ to allow run-off transcription of the editing template RNA as described previously (7). Deletion clones were shortened by removing original mitochondrial sequences as indicated in the respective experiments. The outside bacterial anchors for the PCR amplification accordingly moved closer to the editing sites. Coincidental nucleotide similarities between these and the substituted mitochondrial sequences were taken into consideration when evaluating the nucleotide requirements for RNA editing.

In vitro RNA editing reactions

The *in vitro* RNA editing reactions were performed as described previously (7). After incubation, the template sequences were amplified using RT–PCR with one of the primers labeled with the Cy5 fluorophor. RNA editing activity was detected using mismatch analysis employing the thymine DNA glycosylase (TDG) enzyme activity (Trevigen). The TDG-treated fragments were separated and the Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (Amersham).

The efficiency of the *in vitro* RNA editing reaction was quantified by comparing the areas under the peaks of the cleaved and uncut DNA fragments. The ratio of the cleaved, i.e. edited, fragment to uncut DNA was used to determine relative efficiencies of the investigated conditions in each experiment. To allow comparisons and to determine the variation between individual experiments, the ratios of cleaved to uncleaved fragments were displayed as percentages of the standard reaction conditions.

Generation of mutant substrates

The 5′ deletion mutants were constructed using inverted PCR from patp9 with primers −40, −30, −20, −10 and −0, respectively on the one side and primer invertion1 on the other. The resulting fragments were digested with EcoRI to generate sticky ends in the primer contained EcoRI recognition site and were self-ligated. The 3′ deletion mutants were constructed using inverted PCR from clone atp9-30 with primer invertion2 and primers +10 and +0, respectively. The PCR fragments were digested with XbaI and self-ligated.

The mutant templates with defined sequence regions exchanged to their opposite sequence were constructed by introducing the respective complement pentanucleotide in primers M1–M10. PCR was performed on deletion clone atp9-40 with primer invertion1 and primers M1, M2, M3, M4 and M5, respectively, and in the second series with primer invertion2 and primers M6, M7, M8, M9 and M10, respectively. The resulting fragments were digested with EcoRI or XbaI, respectively, and self-ligated.

Competition assays

Wild-type competitor RNA was synthesized from the PCR product amplified with primers T7 and +10 from clone atp9-40. A complete plasmid-derived control RNA was synthesized from the PCR product amplified from pBluescriptIISK^+ with T7 and SK primers. The mutant competitors were synthesized from the PCR products amplified from clones M1 to M5 with the T7 primer and primer +10, and from clones M6, M7, M8, M9 and M10 with T7 and the respective mutant primers. One hundred attomoles of substrate and 1000 times (100 fmol) competitor RNA were first mixed and then incubated with the mitochondrial *in vitro* assay as described above.

RESULTS

Exploration of the 5′ *cis*-recognition region borders with deletion templates

The initial template tested *in vitro* contains 173 native mitochondrial nucleotides upstream and 49 original nucleotides downstream of the monitored RNA editing site in the pea mitochondrial *atp9* mRNA (Figure 1). To explore the limits of the necessary *cis*-sequence elements, we first tested a template containing 40 nt upstream of the editing site (−40 in Figure 2). This template was edited as efficiently as the original construct with 173 'native' upstream nucleotides (data not shown). We next constructed a series of deletion clones, in which the native sequences were removed in the steps of 10 nt up to the editing site (Figure 1B). Excision of the mitochondrial sequences in effect moves the 5′ plasmid sequences closer to the editing site. Since these replace the mitochondrial nucleotide identities, we took care to monitoring accidental sequence similarities (Figure 1B and discussed below).

In templates with only 30 nt conserved upstream of the editing site, RNA editing efficiency decreased to ∼50% (Figure 2A). Removal of the next 10 nt did not reduce the amount of editing further, which shows that the remaining 20 nt are sufficient to correctly identify the native editing site (−20 in Figure 2A). The comparatively high activity of the −20 deletion in comparison to the −30 deletion may be influenced by the chance similarity of 6 nt in the bacterial sequence with the sequence between −30 and −20 (Figure 1B, underlined sequences). Editing is completely lost when the next 10 nt upto −10, or all of the mitochondrial sequences are removed upstream of the edited nucleotide (−10 and 0 in Figure 2A).

This result suggests that the sequence arrangement of 20 nt upstream of the edited nucleotide is necessary and sufficient to

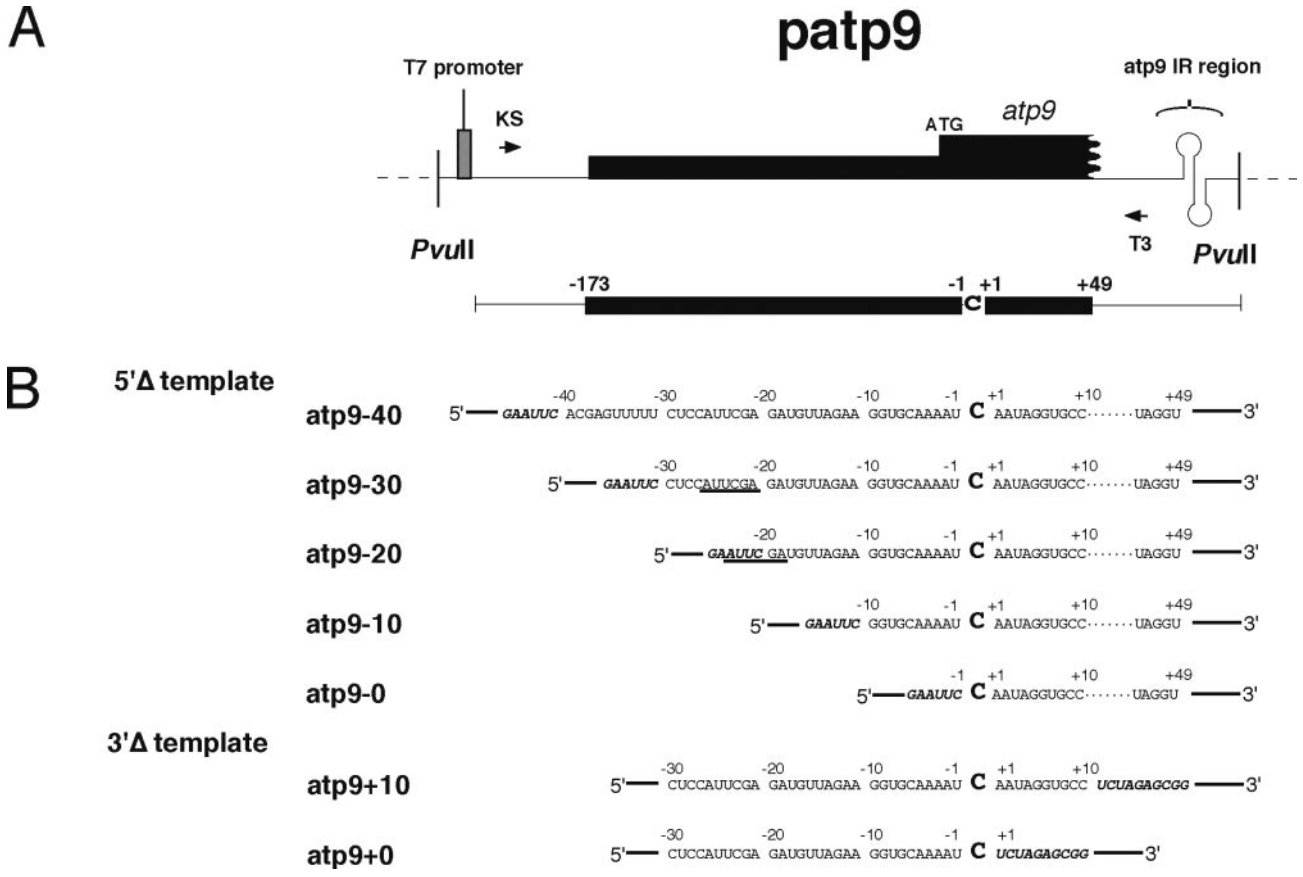

Figure 1. Structure of the RNA editing template and the construction of deletion clones. (**A**) The top line schematically depicts the plasmid embedded *atp9* gene fragment (bold black bar), off which the *in vitro* substrate is synthesized from the T7 promoter as a run-off RNA. In this RNA (shown in the line below), the *atp9* coding fragment is flanked by bacterial sequences and stabilized at the 3′ end by the *atp9* IR region (7). (**B**) Successively shortened templates were generated by deletions from the 5′ and 3′ ends, respectively. In these deletions, the excised *atp9* sequences move the primer binding bacterial regions closer to the targetted editing site. Each mutant respectively contains 40, 30, 20, 10 or 0 nt of the native *atp9* sequence upstream and 49 nt downstream of the editing site. A hexanucleotide sequence by chance identical between the deleted *atp9* sequence and the bacterial substitution (bold italics) in atp9-20 is underlined. The two 3′ deletion mutants tested include 30 nt upstream and 10 or 0 nt genuine *atp9* downstream sequence, respectively. The substituting bacterial sequence is given for 10 nt in bold italics. In the +0 clone the triplet UAG at positions +3 to +5 is incidentally present also in the bacterial sequence. The 5′ and 3′ black lines indicate the vector sequences containing KS and T3 promoters, respectively.

define the editing site and to identify the nucleotide to be altered. The upstream adjacent 20 nt between −40 and −20 contribute to an increase in the editing efficiency.

Charting the 3′ requirements with sequence deletions

The 3′ region downstream of the editing site was similarly investigated by templates processively deleted in the steps of 10 nt toward the edited nucleotide (Figure 3). In these constructs, the upstream region contained 30 nt identified as being sufficient to yield the accurate location of the editing reaction. To evaluate the sequence requirements in this region, three templates were tested containing +0, +10 and +49 nucleotides, respectively. All these deletions right up to the monitored C were edited correctly. Surprisingly, RNA editing became somewhat more efficient when more nucleotides were removed, i.e. substituted by bacterial sequences. This observation suggests that the *in vivo* sequence may be suboptimal at least in our *in vitro* assay and can be improved by changing some nucleotide identities.

From these experiments, we conclude that the minimal substrate region surrounding the editing site consists of 20 mitochondrial nucleotides upstream and no native nucleotides downstream of the edited C. Downstream sequences as well as the −40 to −20 upstream nucleotides, appear to modulate the *in vitro* editing reaction at this site.

Dissection of the requirements for editing site identification with mutated templates

To characterize the individual sequences necessary and/or supportive for editing of this site more in detail, we constructed mutants with consecutive sequence exchanges. In the steps of 5 nt, the native mitochondrial sequence was substituted by its respective antisense pendant (Figure 3A). The exchange of nucleotides −40 to −35 (M1) reduces the editing activity to <10% of the wild-type control, suggesting an important sequence element (Figure 3B). In contrast, the two exchanges between nucleotides −35 and −25 (M2 and M3) lower the editing efficiency only to 60 and 70%, respectively. The 10 nt between −25 and −15 upstream of the editing site are of comparable importance as the −40/−35 element, their alteration (M4 and M5) reduces the editing efficiency to ∼5–15%.

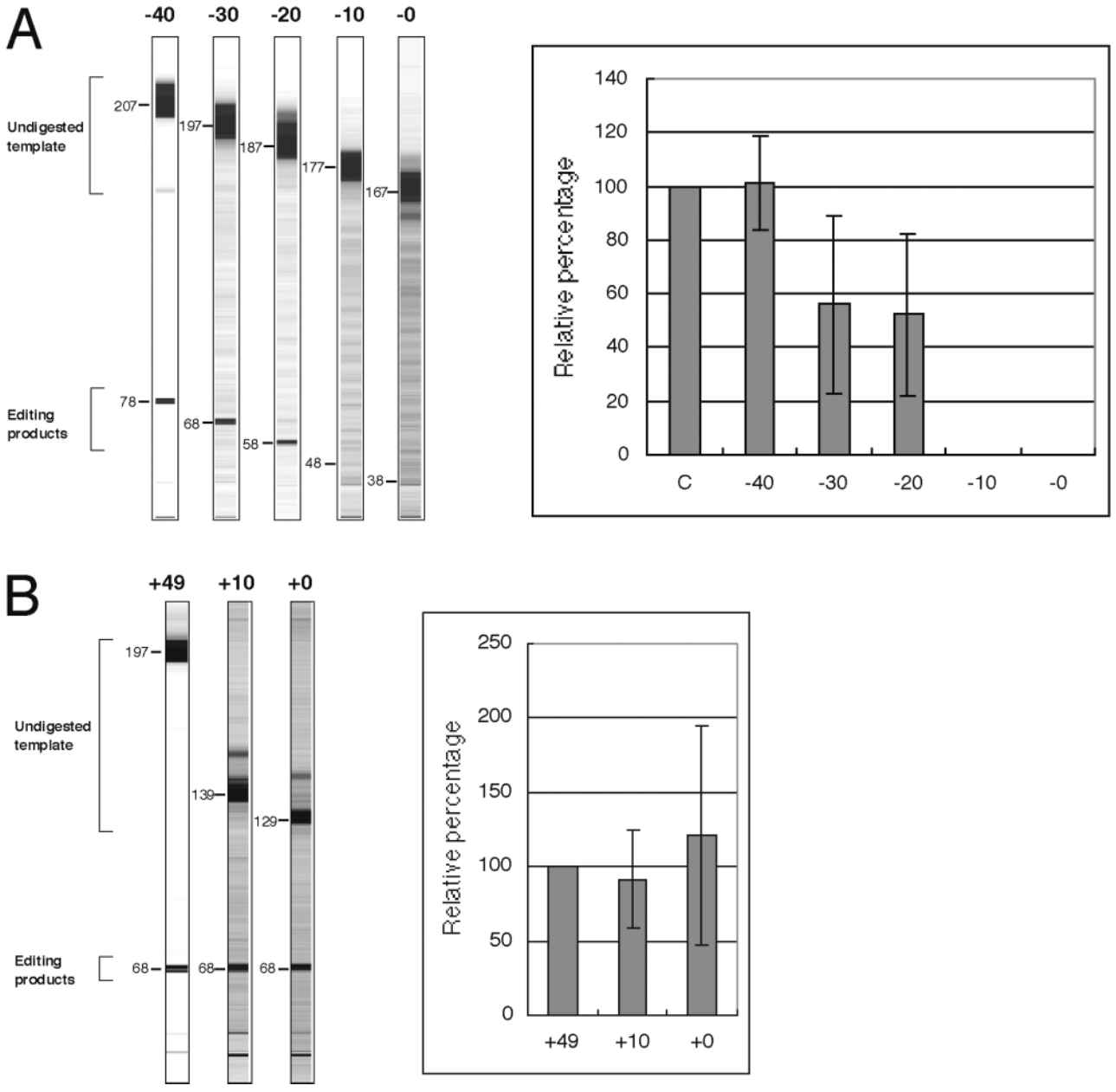

Figure 2. *In vitro* editing of deletion mutants shows 20 nt upstream to be sufficient to specify the editing site. (**A**) The 5′ deletion mutants reveal two levels of *cis*-sequences. The gel image of the TDG detection analysis is shown in the left panel. Sizes of unedited templates amplified as DNA fragments by RT–PCR between the Cy5 fluorescent dye labeled KS and the T3 primer and the predicted fragments resulting from cuts at the editing site are given in nucleotides for the respective deletion clones. Quantification of the respective editing efficiency (right panel) shows faithful editing with as little as 20 nt upstream of the edited C nucleotide. For full editing efficiency, however, 40 upstream nucleotides are required. In each of three experiments editing was quantified relative to the control template, the results were averaged and the standard deviation was calculated. (**B**) *In vitro* editing of 3′ deletion mutants suggests that the downstream nucleotides are probably not involved in marking the editing site. Gel image of a TDG analysis of the 3′ deletion mutants is shown on the left. DNA signals at 197, 139 and 129 nt correspond to the full-length RT–PCR fragments from atp9 + 49 (original clone length), atp9 + 10 and atp9 + 0, respectively, the DNA at 68 nt results from the fragments cleaved by TDG at the editing site. Quantification of the editing efficiency (right panel) shows correct editing even when all nucleotides downstream of the edited C nucleotide are substituted by bacterial sequences. Editing efficiencies are comparable in these deletion clones. The accidental sequence similarity and the high experimental variation have to be taken into account in the interpretation. In each of the five experiments editing was quantified relative to the control template, the results were averaged and the standard deviation was calculated.

Out of four assays with the mutant altered between −15 and −10 (M6) in different mitochondrial lysate preparations, we observed very low levels of editing and three times no editing only once. This result suggests that these nucleotides are crucially important, yet recognition and correct assignment of the nucleotide to be edited can occasionally occur without them. The sequence between nucleotides −10 and −5 was required absolutely to target this editing site, since we never see any editing in their respective mutant template (M7). The 5 nt immediately upstream of the edited C are less crucial, since

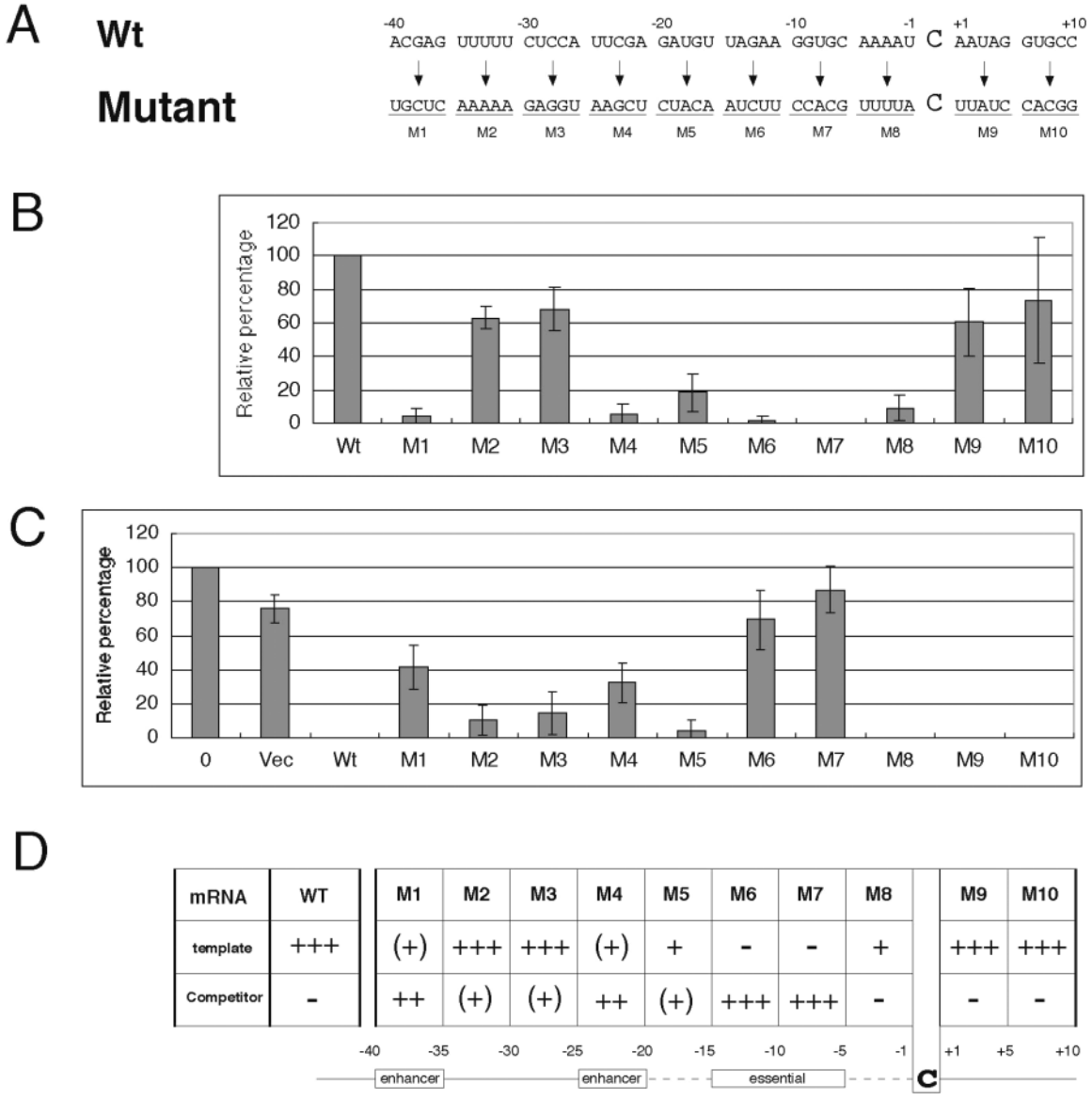

Figure 3. Mutant templates and competitors reveal distinct regions for recognition and efficiency of *in vitro* RNA editing. (**A**) Mutant *in vitro* editing templates/competitors were constructed by scanning mutagenesis with five consecutive nucleotides altered to their complement. The substituted sequence block is shown for each mutant construct M1–M10 underneath the wild-type sequence. (**B**) In each of four experiments, the respective editing efficiency was compared to editing of the co-analyzed wild-type sequence, the four results were averaged and the standard error was calculated. Mutant templates M1, M4 and M6 showed no clearly detectable editing in one or the other experiment, and yielded very little product in the respective other assays. (**C**) Mutant templates used as competitors further delineate the region upstream of the edited nucleotide sufficient for recognition of the template and for effective *in vitro* RNA editing. Mutant editing templates were added to the *in vitro* reaction in 1000-fold excess over the wild-type template. The respective editing efficiencies of three experiments were determined as the percentages of the editing of the wild-type sequence, the three results were averaged and the standard error was calculated. Mutant competitors M8, M9 and M10 showed no detectable editing in any of the assays, indicating that all essential and sufficient editing site determinants reside upstream of the edited nucleotide and can be completely titrated. (**D**) Comparison of the effects of mutant RNAs as templates and as competitors, respectively, delineates an essential recognition element and two sequence regions enhancing the reaction. The editing efficiencies in the individual experimental assays are categorized into full (+++), reduced (++), little (+), occasionally very little [(+)] and no detectable RNA editing activity (−). The complete lack of observed editing in mutants between nucleotide positions −15 and −5 defines an essential sequence region that cannot be substituted by any of the other surrounding sequence elements. In turn, these mutants have no discernible effect as competitors, suggesting that the other elements are recognized by distinct *trans*-acting factor(s) that are not titrated by the employed competitor concentration. Further details are discussed in the text.

RNA editing still occurs correctly in the respectively exchanged template RNA (M8), although with greatly reduced activity.

Mutations downstream of the editing site (M9 and M10) allow RNA editing to proceed at the correct nucleotide and affect the editing efficiency only mildly with a reduction of 30–40%, respectively, compared with the upstream mutations M2 and M3 (Figure 3B).

In summary, these assays with mutated templates resolve the initial classification of *cis*-regions by the deletion clones in more detail and allow tentative functional distinctions of the upstream elements. Two enhancing regions appear to be

located at −40/−35 and −25/−10, respectively, separated by less influential nucleotide positions. A promoting effect is also observed for the sequence immediately around the editing site itself. The essential recognition region of the to be edited nucleotide is located between −10 and −5 nucleotides upstream of the respective C, most probably stretching some nucleotides further upstream beyond the −10 position.

Competition with mutated templates suggests a limiting specificity factor

In the next series of experiments, the mutated RNAs are used as competitors of the wild-type template (Figure 3C). Competitors with mutations in non-essential regions will contain the wild-type versions of essential sequence elements and are thus expected to decrease or completely block editing in the monitored RNA. This is indeed observed: competition with mutations in non-essential regions M5, M8, M9 and M10 abolish detectable editing of the test substrate.

Conversely, mutant M7, in which the essential region between −10/−5 is eliminated, competes hardly at all with the template. Mutant M6 lacking the upstream adjacent region between −15/−10 similarly reduces editing only slightly. Surprisingly mutants M1–M4 inhibit the reaction to varying degrees, although all of them contain the essential region between −15 and −5. The different effects may reflect the individual contributing effects of these sequences observed with the mutants templates.

In control reactions the wild-type competitor completely blocks the reaction. The vector sequence alone reduces the editing process in the range of mutants M1 and M6, possibly due to some chance sequence similarities. These results confirm the distinction of separate *cis*-sequences, an essential region between −15 and −5, and enhancing regions at −40/−35 and −25/−15.

DISCUSSION

Here, we reported the analysis of *cis*-requirements for *in vitro* RNA editing in pea mitochondria that allows some functional conclusions about the recognition of an RNA editing site in plant mitochondria. Several *cis*-signals can be distinguished in the template by their respective influence on the reaction. This result suggests that either distinct protein (or RNA) molecules recognize these elements or one *trans*-factor attaches to different contact sites in the RNA.

The specific contribution of individual regions around (mostly upstream of) the investigated editing site are deduced from the relative *in vitro* editing reactions. However, we hesitate to interpret the precise percentages in these comparisons in fine details, since the variations between each individual experiment are in the range of the differences observed between individual constructs (compare the respective error bars in Figures 2 and 3). We thus restrict our interpretations to the five categories: full, reduced, little, occasionally very little and no detectable editing activity (Figure 3D).

All major determinants are located 5′ of the editing site

To investigate the extent of the *cis*-requirements, we tested the mutant templates processively deleted from the 5′ or 3′ terminus of the template (Figures 1 and 2). These deletions reveal an enhancer element in the region between −40/−30 and an essential recognition element between −20/−10. Deleting 3′ sequences has little influence on the overall editing activity, suggesting that no essential elements are located in this region. The somewhat enhanced reaction efficiency with complete substitution by bacterial sequences is hardly significant, but may be analogous to the observation made in a chloroplast RNA editing template (20), where the *in vivo* template may not be the optimal structure for (*in vitro*) editing. These results prove experimentally that the conclusions drawn from duplicated sequences in plant mitochondrial transcripts, where correct editing was observed when upstream sequences of ∼50 nt and downstream as little as 4 nt are conserved (e.g. 21).

An essential and two enhancing regions are separated by several nucleotides respectively

Mutation of nucleotides −40/−35 (M1 in Figure 3B and D) suppresses editing almost completely, suggesting that within these 5 nt important identities are located. Nucleotides downstream of this window from −35 to −25 before the editing site seem to be less involved in recognition. These little contributing 10 nt are followed by another important sequence between positions −25 and −15 (M4–M5). The competition experiments further define this region to be located mostly between −25 and −20 (Figure 3C and D; M4), because the mutant of this sequence M4 competes considerably less than the mutant of the downstream adjacent nucleotides M5. However, this sequence element probably does extend several nucleotides downstream, since this neighboring sequence appears to be important for recognition (mutant template M5 in Figure 3D), but not sufficient to compete for binding with the recognizing *trans*-factor (mutant competitor M5 in Figure 3D).

The adjacent less important nucleotides up to −15 lead up to the crucial recognition region around −10 (between −15 and −5). This essential region possibly reaches closer to the editing site into the −5 to −1 sequence, since mutation of this region (M8) drastically reduces its recognition as a template. However, as competitor this mutant M8 completely blocks the reaction, showing that these nucleotides are not sufficient to rescue recognition for the adjacent upstream element.

The sequences downstream of the editing site are clearly not relevant to define this editing site, since the respective mutants M9 and M10 show nearly wild-type levels of editing and as competitors fully suppress the editing reaction (Figure 4).

Intriguingly, the template mutated between −40 and −35 (which equals a −35 deletion clone) shows more than 90% reduction in editing (Figure 3D), while deletion up to −30 and even to −20 reduces editing only by ∼50%. This result may possibly suggest that the bacterial sequences moved up to −30 and −20, respectively, by chance contain nucleotides that can partially compensate for the missing −40/−35 sequence. Alternatively, the deletion up to −35 (caused by the inverted upstream pentanucleotide) and the further upstream bacterial sequences combine into an inhibitory sequence stretch or secondary structure, which lowers RNA editing activity. Such a chance similarity between bacterial and the template sequence in the −20 deletion clone (Figure 1B) may be the reason for its comparatively high activity (Figure 2A) and disguise the

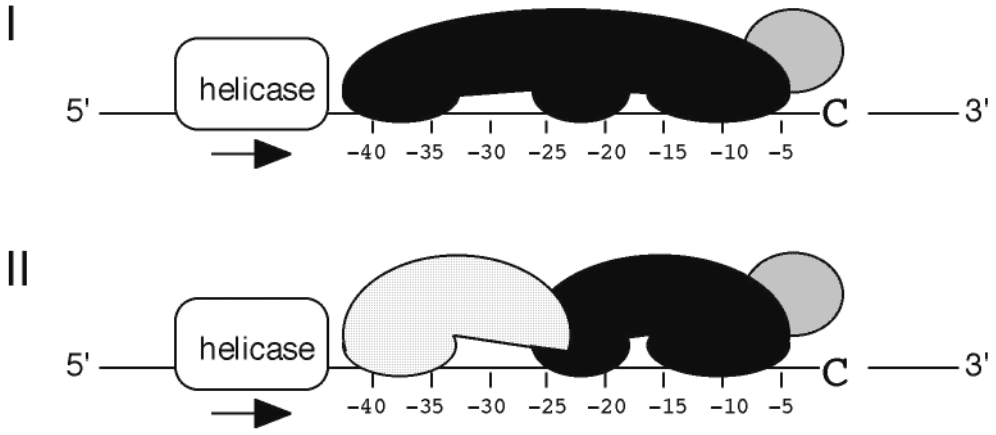

Figure 4. Models of *trans*-factors of RNA editing in plant mitochondria as deduced from the deletion, mutant and competition experiments are described in this report. The essential region and the two enhancing sequences are recognized by one *trans*-factor with distinct binding properties (model I; black body) or alternatively by at least two different *trans*-acting factors (model II; black factor and dotted cofactor). In both diagrams, the actual editing enzyme activity is drawn separately (hatched shape), but may also be an extension of the central *trans*-factor. The multiple coordinated factor model II is supported by the observation that binding at the essential region around −10 is titrated in the competition experiment, while the factor(s) binding to the upstream elements appear not to be out competed completely. The upstream elements between −40 and −35 and between −25 and −20 can each partially compensate the competition at the essential element between −15 and −5, suggesting that either two different *trans*-factors or one contacting these two binding sites can recruit and anchor the essential *trans*-factor binding at −15 to −5. A single *trans*-factor with distinct binding affinities at the three attachment regions could equally well explain the observations. The helicase either moves in to clear the RNA template before the editing complex binds or removes the complex after the actual editing is completed.

importance of the nucleotide identities between −25 and −20 (Figure 3B and D).

Distinct *trans*-factors in different stoichiometries or one factor with differential affinities?

The three separable *cis*-regions with different importance in the *in vitro* processivity of RNA editing suggest that each of them interacts differently with the *trans*-element(s) involved in identifying the site and catalyzing the reaction. This observation can be explained by two models equally consistent with the experimental information summarized here (Figure 4).

The first potential explanation for the observed behavior could be a single *trans*-acting (protein) factor, which extends its binding and recognition over the entire region between −40 and −5 nucleotide positions (Figure 4; model I). This *trans*-factor can contact different regions of the template, requiring the essential *cis*-element between −15 and −5 as major binding site. The non-essential enhancing elements then support this interaction and can partially substitute for the essential region. This model is supported by the experiment with mutant M1 as a competitor (Figure 3C and D): the observed (albeit lower) activity shows that this element may be able to partly substitute for −10 essential sequence. An analogous observation is made for the M2–M4 mutations, which can also partly rescue the competed essential element at −15/−5. These interpretations are of course only relevant if we exclude unpredicted structural effects of the deletions and mutations.

In the second alternative scenario, the specific reactions to individual competing sequence arrangements would be consistent with (at least) two different *trans*-factors contacting the RNA template at individual *cis*-elements (Figure 4; model II). The observation of little or no inhibition with the competing M6/M7 RNAs suggests that the two upstream elements at −40/−35 and at −25/−15 are recognized by *trans*-factor(s), which are not out-titrated by the 1000-fold excess of the respective added wild-type sequences. On the other hand, the putative *trans*-factor binding at −15/−5 seems to be present only in limited amounts, since it is only partially rescued by mutant competitors M1–M4 when compared to the complete inhibition of wild-type competitors.

In both scenarios, the NTP requirement may signal involvement of an RNA helicase as suggested from the previous biochemical analyses of *in vitro* RNA editing in pea mitochondria (7). The NTP requirement may be due to the initial binding of a *trans*-factor (protein), which the observed equally active dNTP substitution would suggest to be an RNA helicase. In this order of events, the RNA helicase would unwind and open secondary structures in the RNA template to allow firm contact with the other RNA editing complex proteins. The requirement for an RNA helicase as essential (co-)factor may however also signify a later step in the editing reaction, possibly the last, in which the attachment of a protein such as an RNA helicase may be required to dissociate the editing complex to allow its movement to the next site (23).

Similarities between RNA editing in plant mitochondria and chloroplasts

Here, the observed arrangement of various *cis*-elements for the first editing site in the *atp9* mRNA from pea mitochondria appears to be more complex than the *cis*-requirements of two chloroplast editing sites investigated in depth *in vitro* in the *psbE* and *petB* transcripts in tobacco and in pea, respectively (19). For the *psbE* editing site in tobacco (this site is not edited in pea), only one region between nucleotides −15 and −5 appears to be essential. Similarly, editing at the *petB* site requires one consecutive sequence stretch between −20 and −5, and possibly several downstream adjacent nucleotides in tobacco as well as in pea. This latter essential region, although larger than the *psbE* recognition site, is still only

half as large as the mitochondrial *atp9* editing recognition region identified here.

The differences observed between these *in vitro* results of site recognition in plastid and mitochondrial editing might reflect the differences between individual editing sites rather than a general interorganellar distinction, since mutational investigation of the editing site C259 in *cox2* transcripts in transfected mitochondria of wheat revealed a continuous specificity region covering only 22 nt from −16 to +6 around the edited nucleotide (11). For this site several downstream nucleotides appear to be essential, since complete substitution of the 3′ sequence even with that from another site did not recover activity. For another site investigated with this approach in wheat mitochondria, however, only upstream sequences appear to be necessary, analogous to the specificity requirements observed here for the *atp9* site in pea. These editing sites are thus specified by individually different *cis*-elements, which by extrapolation are recognized by unique *trans*-factors. Only sites with similar *cis*-sequence motifs could attract common factors, as observed in competition analyses in the transgenic chloroplasts (15,16).

For editing of the *psbE* site in chloroplasts, a two-step binding process for the identified p56 protein has been deduced from a detailed mutational analysis (20). The 56 kDa protein is proposed to bind initially upstream of the editing site and then to bend towards the C to be edited. For example, this second step may be hindered sterically by a G residue immediately upstream of the editing site. This process would be the equivalent of our single *trans*-factor model as depicted in Figure 4. Further experimentation is necessary to resolve these questions and to physically identify the *trans*-factors involved in RNA editing in plant chloroplasts and mitochondria.

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An *in-vitro* RNA editing system from cauliflower mitochondria: editing site recognition parameters can vary in different plant species.

An in vitro RNA editing system from cauliflower mitochondria: Editing site recognition parameters can vary in different plant species

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ABSTRACT

Most of the 400 RNA editing sites in flowering plant mitochondria are found in mRNAs. Consequently, the sequence vicinities of homologous sites are highly conserved between different species and are presumably recognized by likewise conserved *trans*-factors. To investigate the evolutionary adaptation to sequence variation, we have now analyzed the recognition elements of an editing site with divergent upstream sequences in the two species pea and cauliflower. This variation is tolerated at the site selected, because the upstream *cis*-elements reach into the 5′-UTR of the mRNA. To compare *cis*-recognition features in pea and cauliflower mitochondria, we developed a new in vitro RNA editing system for cauliflower. In vitro editing assays with deleted and mutated template RNAs show that the major recognition elements for both species are located within the conserved sequence. In cauliflower, however, the essential upstream nucleotides extend further upstream than they do in pea. In-depth analysis of single-nucleotide mutations reveals critical spacing of the editing site and the specific recognition elements, and shows that the +1 nucleotide identity is important in cauliflower, but not in pea.

Keywords: RNA editing; plant mitochondria; cauliflower in vitro editing; *atp9*

INTRODUCTION

In the 15 years since RNA editing was first recognized in plant mitochondria and chloroplast as a post-transcriptional process that alters mostly C-to-U nucleotide identities in mRNAs and tRNAs, progress toward elucidating the enzymes and the specificity recognition has been restricted mostly by the lack of efficient in vitro systems. In vivo analysis of transgenic chloroplasts has brought important insights into the structure and extension of *cis*-elements, but this approach is difficult to extent toward a biochemical characterization and the identification of the corresponding *trans*-factors (Chaudhuri et al. 1995; Bock et al. 1996, 1997; Chaudhuri and Maliga 1996; Reed et al. 2001; Chateigner-Boutin and Hanson 2002).

With the development of reliable in vitro activities for chloroplasts (Hirose and Sugiura 2001; Miyamoto et al. 2002, 2004) and also for pea mitochondria (Takenaka and Brennicke 2003; Takenaka et al. 2004) in the past few years, characterization of the *cis*-requirements at individual sites has accelerated considerably.

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For plant mitochondria, the in vitro RNA editing system from the pea has shown that for recognition by the RNA editing activity, only ~20 nucleotides are essential upstream, 40 are optimal, and basically none is necessary downstream of the first editing site in the *atp9* mRNA. Analysis of the *cis*-requirements by targeted mutations of the template and competition experiments have narrowed the sequence requirements for the site specificity to the region 5–20 nucleotides upstream of this site in the *atp9* mRNA (Takenaka et al. 2004).

Transfections of isolated wheat mitochondria with *cox2* mRNA (Farré and Araya 2001; Farré et al. 2001; Staudinger and Kempken 2003) and mutational analysis of two sites in this transcript showed that similarly 16–20 nucleotides upstream are required to define these sites (Choury et al. 2004). However, in addition to these upstream elements, one or more nucleotide positions downstream were found to be crucial for efficient editing. These sequence requirements suggest that individual recognition elements vary between different editing sites. Extensive mutational analysis revealed that the important nucleotide identities are different for the two sites, confirming the variation of *cis*-elements between individual RNA editing sites (Choury et al. 2004).

These two experimental approaches with a dicot, the pea, and a monocot, wheat, thus suggest that between

plants the recognition parameters of RNA editing sites are similar but can vary between individual sites. To investigate this inference in more detail, we have now assayed the *cis*-elements determining a given RNA editing site in two different plant species, cauliflower and pea, and compared the recognition parameters. Furthermore, we analyze the spacing requirements of the recognition elements and the edited nucleotides by specific insertion/deletion mutations.

RESULTS

The cauliflower in vitro mitochondrial extract

For a convenient source for mitochondria, we selected cauliflower inflorescences, since these offer several advantages to obtain large amounts of comparatively clean mitochondria from plants. These tissues contain few secondary plant compounds, which notoriously make biochemical and molecular analyses difficult. Furthermore, no chloroplasts differentiate in these pale white tissues, and few proplastids contaminate purification schemes of other organelles. Yet a third advantage is the economic source of material. Last not least, cauliflower (*Brassica oleracea*) is closely related to *Arabidopsis thaliana*, the model plant for which the complete genomic sequence and countless other data are readily available.

As detailed in the Materials and Methods section, we prepared an S-60 lysate from mitochondria purified from cauliflower inflorescences along the procedure adapted from the original protocol for tobacco chloroplasts (Hirose and Sugiura 2001). The cauliflower mitochondrial extract proved to be more active than the previously developed pea in vitro system from elongated hypocotyls (Fig. 1; Takenaka and Brennicke 2003). On average, 4%–7% of the template molecules are edited by the cauliflower system in comparison to 1.5%–3% in the pea lysate (Fig. 1). This may be due to the higher concentration of mitochondrial proteins consistently achieved with lysates from the cauliflower inflorescences (8.3 μg/μL on average) than in the pea (*Pisum sativum*), with ~1.9 μg/μL. The relationship is not linear, since the about five times higher protein content only yields a twofold increase in the editing activity.

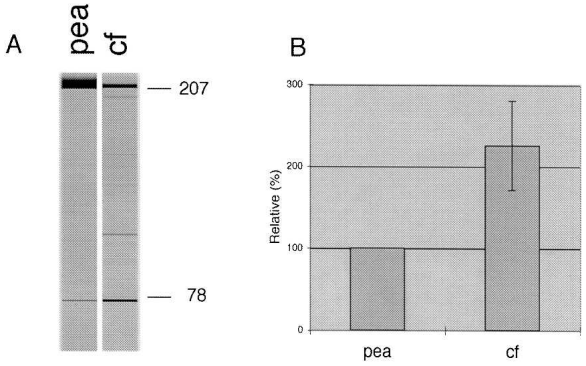

FIGURE 1. Comparison of the RNA editing lysates prepared from cauliflower and pea mitochondria. (*A*) Sample gel images of in vitro editing assays in the pea and cauliflower lysates. Numbers give the sizes of the full-length (207 nucleotides) and edited (78 nucleotides) RT-PCR-TDG products. (*B*) RNA editing activities in the two lysates as determined toward the pea *atp9* −40/+49 template. The average of three experiments is given with the respective standard error shown for the cauliflower activity (cf) relative to the pea, the latter taken as 100% in each set of experiments.

The cauliflower and pea mitochondrial *atp9* sequences diverge beyond 23 nucleotides upstream of the first RNA editing site

A disadvantage of any new system is the need to identify native sequences and confirm experimentally the presence of postulated RNA editing sites. This we did for the complete *atp9* gene in cauliflower and determined the RNA editing sites by genomic and cDNA analysis (data not shown). The complete sequence information and editing sites have been deposited in the European Molecular Biology Laboratory (EMBL)/GenBank databases (accession no. DQ102391). The sequence comparison with the previously analyzed pea *atp9* template (Takenaka et al. 2004) shows that the first RNA editing site in the open reading frame is conserved between these two species in the seventh codon from the pea AUG (Fig. 2). Upstream of this editing site, sequences diverge beyond nucleotide position −23 relative to the edited nucleotide. This sequence variation is possible because the editing site is close to the conserved AUG and the 5′-leader is not conserved between the two plants. Coding sequences in plant mitochondria are usually highly conserved between different species and thus do not allow much variation. Therefore, only an editing site located at the 5′-extremity of an open reading frame will display such natural sequence divergence in its upstream region and offers a choice of variable genuine wild-type templates from different plant species.

Both cauliflower and pea templates are recognized in the cauliflower lysate

Despite this sequence variation, both the homologous cauliflower and the heterologous pea templates are correctly recognized and edited in the mitochondrial lysate from cauliflower (Fig. 3). Little difference is seen in the efficiencies of the in vitro modification between the homologous and heterologous templates. This result suggests that for cauliflower, all essential upstream *cis*-recognition elements are contained within the 23 nucleotides conserved between both templates, with the potential participation of the scattered further upstream positions identical between pea and cauliflower (Fig. 2).

```
                -40         -30         -20         -10         -1  +1          +10         +49
P. sativum      ACGAGUUUUU CUCCAUUCGA GAUGUUAGAA GGUGCAAAAU C AAUAGGUGCC·····UAGGU
                |     ||  ||||  ||| ||||||||||| ||||||||||  ||||||||||      | |||
B. oleracea     GAGGAGAAUU AUCAACCCGA GAUGUUAGAA GGUGCAAAAU C AAUAGGUGCC·····UCGGU
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FIGURE 2. Sequence comparison between pea (*Pisum sativum*) and cauliflower (*Brassica oleracea*) mitochondria surrounding the first RNA editing site in the *atp9* coding region. Numbering is centered around this editing site. The native cauliflower and pea sequences deviate upstream of nucleotide −23, which is located in the 5′-untranslated leader region. This sequence variation between the two species allows a cross-wise comparison of the nucleotides necessary for site recognition. The AUG of the translational start in pea is boxed, the edited C is in large type, and identical nucleotides are indicated by the dashes between the two sequences. In cauliflower another AUG occurs in frame further upstream, which could theoretically be used (data not shown). The sequence between nucleotides +10 and +45 is not shown but is identical in the two plants. The complete sequence of the *atp9* gene in cauliflower is deposited in the databases (accession no. DQ102391).

Deletion templates show that the essential *cis*-recognition elements for cauliflower are located within the upstream 20 nucleotides, but efficient editing also requires specific downstream nucleotide(s)

To investigate the evolutionary conservation of *cis*-elements, and by extrapolation the *trans*-elements recognizing an editing site, we employed the pea mitochondrial *atp9* sequence in all of the following experiments, since this has been extensively examined in the native pea mitochondrial in vitro system (Takenaka et al. 2004).

Successive deletions of the template sequences in steps of 10 nucleotides show that a template RNA with 20 upstream native nucleotides is correctly recognized (Fig. 4A). Further deletions up to −10 or right up to the edited nucleotide do not allow editing any more, suggesting that the 20 nucleotides directly upstream of the edited nucleotide are necessary as well as sufficient for recognition of the site.

Conversely, substitution of all the downstream nucleotides by bacterial sequences still allows editing in vitro (Fig. 4B), but much of the activity is lost upon alteration of the 10 nucleotides immediately following the editing site. The lower editing activity in the complete 3′-substitution right up to the editing site is probably due to the effect of the identity of the adjacent nucleotide, which was investigated in detail through single nucleotide mutations (see below).

Scanning mutations and competitions suggest upstream and downstream extensions of the *cis*-recognition element in cauliflower versus pea

To investigate the necessary *cis*-sequences for recognition in more detail, the region surrounding the target editing site was mutagenized in steps of five nucleotides, which were exchanged for their respective complementary nucleotides. In the direct comparison of the relative activities of these templates in pea and cauliflower mitochondrial lysates (Fig. 5A), an overall similar pattern emerges, which corresponds to and extends the results of the deletion analysis.

Surprisingly the upstream enhancer element between nucleotides −41 and −35 of the pea template active in its native in vitro lysate (Takenaka et al. 2004) appears to serve a similar positive function in the cauliflower lysate, because its alteration in construct M1 effects a similar decrease in the editing activity in both species. This is particularly intriguing, since in the native cauliflower mitochondrial *atp9* sequence this element is not present (Fig. 2).

A second difference between the two plant species is the importance of the nucleotide identities between −21 and −15: While the pea lysate still shows ∼20% activity with this mutant M5, the cauliflower lysate does not accept this modified template at all. This observation suggests that in cauliflower mitochondria, the major *cis*-recognition element extends further into the region upstream of nucleotide −15 than in pea mitochondria.

A third difference is observed with mutant M9 altered just downstream of the editing site between nucleotides 0 and +6: While this template loses little of its activity in the pea lysate, the cauliflower extract has difficulties recognizing this sequence at all and shows hardly any activity.

Competition of the wild-type pea template with itself and the various mutants reveals the higher capacity for RNA editing of the cauliflower lysate: Even with the wild-

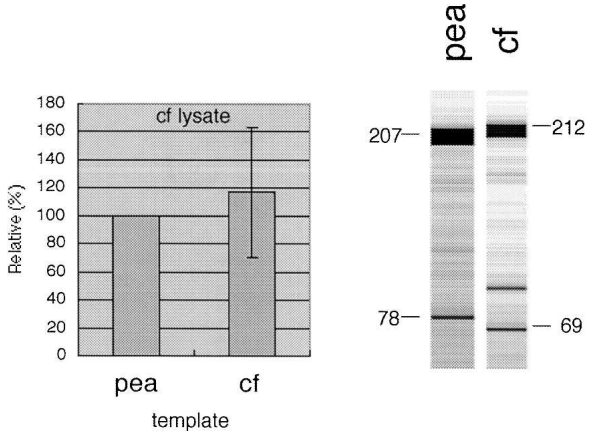

FIGURE 3. Pea and cauliflower templates are processed with similar efficiency in the cauliflower lysate. This observation suggests that in both plant species, the *cis*-elements for recognition of this editing site reside within the conserved 23 nucleotides (and possibly the few positions conserved further upstream). The cauliflower lysate activity appears to be slightly higher toward its cognate sequence, but the differences between the two templates are within the experimental variation. The average of four independent assays is shown with the activity toward the pea template taken as 100% in each assay. On the *right*, gel images are shown for representative assays of pea and cauliflower (cf) templates. Different cloning sites result in different respective TDG product sizes in pea (78) and cauliflower (69).

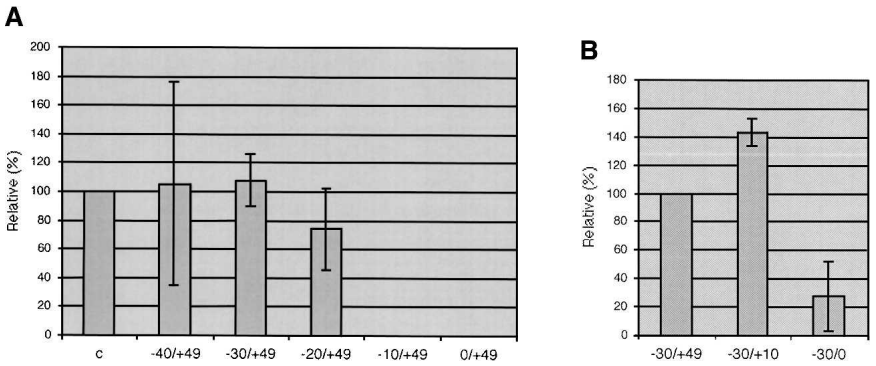

FIGURE 4. Delineation of the *cis*-recognition region by deletion clones in the cauliflower mitochondrial lysate. The sequences upstream and downstream of the monitored editing site were deleted in steps of 10 nucleotides; that is, they were in effect substituted by bacterial vector sequences. (*A*) Deletion of the 5′-sequence up to 20 upstream nucleotides (−20) still allows editing. Further deletion closer than −20 nucleotides from the editing site completely abolishes recognition of this editing site. Control was the pea template −40/+10; the deletions were all done+with the pea template to allow a direct comparison of the *cis*-elements. (*B*) Deleting the 3′-region has little effect up to +10 nucleotides, but the complete substitution up to the edited nucleotide (−30/0) shows substantial inhibition of the in vitro editing activity. The longest 3′-extension was used as standard, and the relative activities with the 3′-deleted templates are shown.

type sequence as competitor in 1000-fold excess, inhibition is not complete, but ~20% of residual editing activity remains (Fig. 5B). Therefore, all experiments with the mutated competitors must be accordingly interpreted; for example, the residual activities seen in the cauliflower lysate with the competing RNAs mutated between −6 and 0 (M8), 0 and +6 (M9), and +5 and +11 (M10) are comparable to the level of inhibition by the wild-type sequence. Their effects are thus identical to the observed effect in the pea lysate.

The differences between pea and cauliflower in the extent of the respective major *cis*-elements observed with the mutated templates are also reflected in the effects of the respective competitors: While in the pea the −20 to −16 mutant M5 as a competitor has a strong effect of ~90% reduction, in cauliflower only 40%–50% inhibition is seen. This difference reflects the greater extent of the essential *cis*-region, which, if covering a considerable part of these five nucleotides in the cauliflower, should inhibit less when mutated.

The absolute requirement of the nucleotides between five and 15 residues upstream of the editing site is confirmed by the absence of any significant effect on editing when the respectively mutated competitors are incubated with the reaction. The reduction observed is similar to the effect of added vector sequences in the control experiment (Fig. 5B). The relationship of the pea enhancer sequence between −41 and −35 to the major recognition element is similar in pea and cauliflower also in these competition experiments, their effect being considerably less than that of the next downstream mutants, which inhibit more strongly in both plants.

Mutants in the 3′ region of the editing site fully compete with the wild-type template in lysates from both plant species, showing that the upstream sequences are essential and limiting for RNA editing site recognition.

Point mutations reveal the importance of the +1 nucleotide in cauliflower

Since the exchange of the sequence downstream of the edited nucleotide by a bacterial sequence has a profound effect on the editing activity of the cauliflower lysate (Fig. 4B), we investigated the importance of the +1 nucleotide by mutating it through all four nucleotides (Fig. 6A). The effect of changing the wild-type adenosine to the other purine guanosine is similar to the effect of changing this nucleotide to any of the pyrimidines, showing that the adenosine identity is crucial at this position. This experiment thus focuses the effects of the respective substituted deletion template (Fig. 4) and the mutated pentanucleotide (Fig. 5) to this nucleotide position, which is changed in the former to a G and in the latter to a U.

Single nucleotide insertion/deletion shows a low tolerance toward the spacing between the *cis*-element and the edited nucleotide

To examine the importance of the spacing between the major *cis*-recognition element covering nucleotides −20 and −5 to the editing site, we altered its distance to the edited C by one nucleotide in each direction (Fig. 6B). Both alterations, insertion or deletion of one nucleotide, completely block RNA editing in vitro. This result suggests zero tolerance for the editing complex presumably assembled at the conserved *cis*-elements to reach the nucleotide to be edited. The wild-type configuration clearly represents the only allowed distance between the *cis*-recognition region and this editing site.

DISCUSSION

RNA editing sites can vary between individual plant species. One species may require a given C-to-U alteration to specify a conserved and presumably functional open reading frame, but this same site can be genomically encoded as a T in another species and thus may not require editing. Particularly, third codon positions, which do not alter the amino acid specified, may be edited in one plant but may remain an unedited C

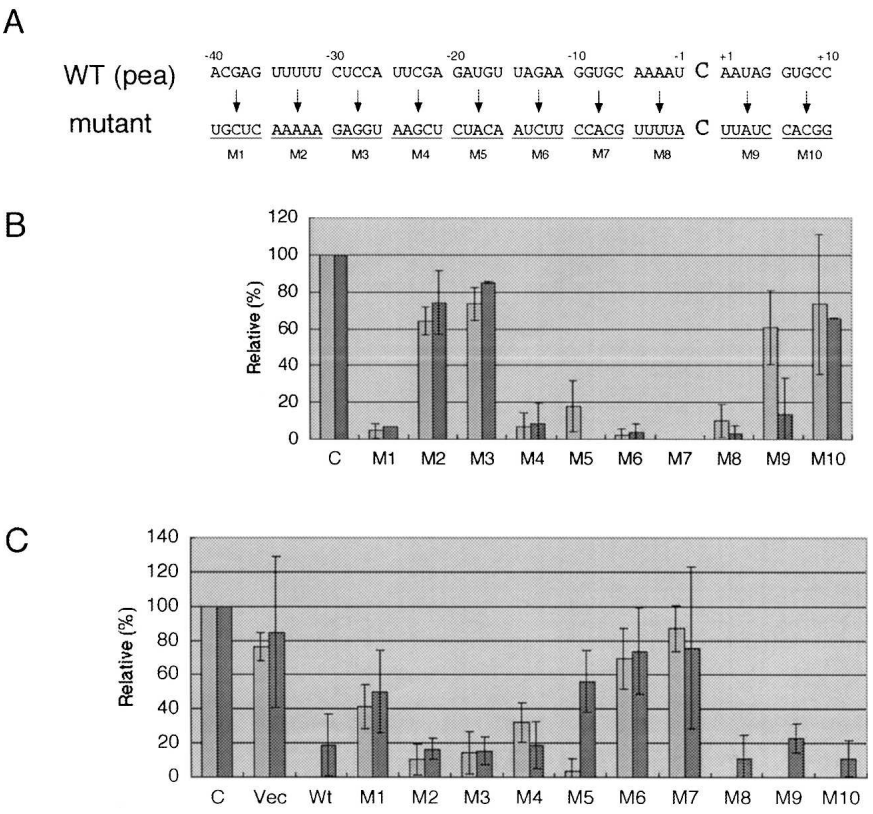

FIGURE 5. The effects of scanning mutations around the first *atp9* editing site as substrates and as competitors on in vitro RNA editing in pea and cauliflower mitochondrial lysates are compared. (*A*) The respective nucleotide quintet altered to its complementary sequence in each set of experiments for maximum effect and to maintain the G+C content is shown, and its designation is given *beneath* the mutated sequences. (*B*) The mutated pea templates are tested for their effectiveness in cauliflower (dark bars on the *right*) and pea (light bars on the *left*). Notable differences between the two species are observed toward mutants M5 and M9. (*C*) The wild-type pea template is competed with 1500-fold excess of the mutants M1–M10 from part *A* in the cauliflower lysate (dark bars; cf) and 1000-fold in the pea lysate assays, respectively (light bars; pea). Control template is the pea −40/+49 wild-type sequence without competitor. Vector sequences compete little, but the wild-type competitor suppresses recognition of the template completely in the pea lysate. Please note that this suppression is not complete in the more active cauliflower lysate, even though a 50% higher excess of competitor was used. The most striking difference between the lysates from these two plant species is seen with competitor M5. Further details are discussed in the text.

nucleotide in another species. The editing specificities thus appear to be quite variable between different plants, suggesting a certain adaptive flexibility within rather short evolutionary distances. By extrapolation, the *trans*-factors recognizing a novel or altered editing site should have changed and adapted to (or away from) the concomitant novel or altered *cis*-elements.

To gain further information and insight about this dynamic potential for creating or altering specificities, we have compared the *cis*-elements at a homologous editing site in the *atp9* mRNA in two different species of flowering plants, the pea and the cauliflower. We have chosen this site because the upstream sequences rapidly diverge between the two plants just upstream of the beginning of the respective coding regions, which is 23 nucleotides upstream of this editing site (Fig. 2).

The *cis*-recognition elements for an RNA editing site can vary between plant species

Deletion, substitutional mutation, and competition experiments suggest that the specific core nucleotide sequence necessary to address this first editing site in the *atp9* mRNA in plant mitochondria is slightly different in the cauliflower in vitro system in comparison to the pea. The 5′-requirement of the core-recognition region (in pea, −15 to −5) extends in cauliflower further 5′ into the −20 to −15 region. The 3′-side of this core element, which is necessary and sufficient to specify this editing site in the pea, appears to be similarly delineated in both species around five nucleotides upstream of the edited nucleotide.

In cauliflower, however, effective in vitro editing depends furthermore on the identity of the nucleotide immediately downstream of the edited C nucleotide. This is different in the pea, where the identity of the adjacent nucleotide at +1 does not influence the editing efficiency. These results thus suggest that the *cis*-elements have evolved between the two plant species, and it thus can be assumed that also the respective *trans*-elements have changed and presently differ between the pea and the cauliflower. Nevertheless, the basic mode of editing site specification has of necessity been conserved in evolution between these two flowering plants, since both definitely require this C-to-U alteration for a functional mitochondrial ATPase (Hernould et al. 1993; Zabaleta et al. 1996).

The enhancer region of the pea is absent in cauliflower

Compensating adaptative mutation of the *trans*-factors may have been influenced by the nucleotide sequence changes further upstream. Here, in the region between −40 and −35, the pea template contains an enhancer element (Takenaka et al. 2004), which increases the in vitro editing activity in the homologous system. The cauliflower sequence, however, is—beyond the usual chance similarities—completely different in this region.

Comparing the in vitro editing activities of the cauliflower lysate toward the pea and the cauliflower templates, respec-

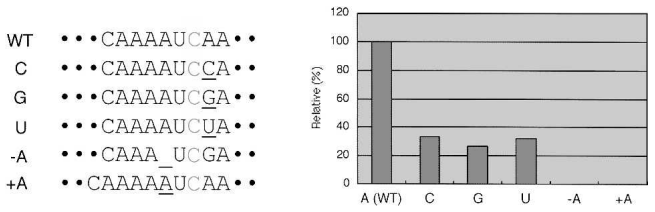

FIGURE 6. Importance of individual nucleotide alterations around the editing site. The influence of the identity of the first nucleotide downstream of the edited nucleotide was determined by mutating this position through all three alternative nucleotides. Any of these changes results in the loss of ~70% of the editing activity. All mutants were tested in four separate experiments, and the mean percentages of the wild-type editing activity were determined. The resulting standard error is indicated for each mutant. The tolerance of the in vitro editing activity for distance alterations between the upstream *cis*-recognition element and the edited nucleotide was investigated by deleting or inserting an adenosine nucleotide within the run of four As in the wild-type sequence. No activity was observed with either template, showing that the distance from the *cis*-element is crucial. Please note that in the deletion template a concomitant A-to-G change at the +1 position has occurred.

tively, shows that both are recognized with about equal affinities (Fig. 1). Even without the pea enhancer sequence in the cauliflower template, the in vitro editing complex in cauliflower thus appears to concentrate sufficient activity at this site for efficient editing. This is confirmed by the in vivo situation, since in the analysis of the steady-state cDNA from cauliflower mitochondria, only molecules derived from mRNAs fully edited at this site are observed (data not shown).

The extension of the recognized core-region itself in cauliflower does not appear to compensate for the altered upstream sequence, since deletion of the pea enhancer element diminishes editing in this template (Fig. 4A). Likewise, the altered binding right at and around the edited nucleotide does not seem to be sufficient to enhance assembly of the *trans*-factors and to ensure very efficient editing. Although the identity of nucleotide +1 seems to play a crucial role for editing and the enhanced binding of the A nucleotide seems to consequently enhance attachment and assembly of the *trans*-factors, these parameters are all present in the template in which the 5′ pea enhancer has been deleted and is less efficient in the in vitro system.

The equally enhancing effect of the divergent upstream sequences in pea and in cauliflower is evidenced by the lower in vitro editing activity in the −20 deletion construct in comparison to the full-length templates from either species. Thus the different upstream sequence in cauliflower effectively substitutes the pea enhancer and now attracts a (presumably different) *trans*-factor able to similarly enhance the binding of the RNA editing complex to achieve rapid and faithful editing. To search for a candidate *trans*-factor recruited from another function and therefore potentially connecting to a similar sequence elsewhere, the mitochondrial genomes of *Arabidopsis* and rape seed were scanned with the cauliflower −40/−30 sequence. However, outside of the homologous *atp9* upstream region, no overt similarities were detected that could donate an established *trans*-factor interaction to boost this RNA editing event.

The enhancer region from pea is addressed by a *trans*-factor conserved in cauliflower

It is surprising to find that deletion of the pea-specific enhancer element at −40/−35 also lowers the in vitro editing efficiency in the cauliflower mitochondrial lysate. This observation suggests that, even though cauliflower mitochondria do not encode this enhancer sequence of pea, they nevertheless still contain the potential to address this sequence motif through conserved *trans*-factor(s). Conservation of this *trans*-factor in cauliflower may be required if it has an additional other and essential function at another editing or processing site. A search of the genomic mitochondrial sequences from *Arabidopsis* and rape with the pea enhancer sequence did not reveal any striking similarity elsewhere in these genomes, but this search is difficult to conduct exhaustingly since the enhancer sequence itself is delimited to only about five nucleotides, too short to specify unique loci in the 350-kb genomes.

An analogous observation of a *trans*-factor conserved in evolution, although apparently not necessary any more, has been made in chloroplasts (Schmitz-Linneweber et al. 2001): Analysis of the allotetraploid tobacco *Nicotiana tabacum* has shown that the nuclear genome encodes a *trans*-factor necessary for recognition of an editing site that is not present in the *N. tabacum* chloroplast RNA. This chloroplast editing site is, however, found in spinach and in *Nicotiana tomentosiformis*, the paternal parent of *N. tabacum* (Schmitz-Linneweber et al. 2001). Again, one explanation could be a requirement of this *trans*-factor at another RNA processing event. More likely, this, in *N. tabacum*, superfluous factor just did not (yet) mutate in the relatively short evolutionary time between establishment of the line of *N. tabacum* from the cross between *Nicotiana sylvestris* and *N. tomentosiformis*.

Overall, the identification of RNA editing sites in plant mitochondria appears to be slowly adaptive, changing recognition parameters between different species and maintaining the capacity to recognize various elements. The *trans*-factors involved seem to act in a concert of several interconnecting proteins (and/or RNA?) that individually and together influence the overall efficiency at a given site. Meeting the challenge to identify and assign these *trans*-factors will be helped by the here-described novel in vitro system from cauliflower mitochondria.

MATERIALS AND METHODS

Preparation of plant mitochondrial extracts

RNA editing active mitochondrial lysates from pea seedlings (*Pisum sativum* L., var) were prepared as described (Takenaka and Brennicke

2003). Preparation of cauliflower lysates followed essentially the same protocol. About 2000 g fresh weight inflorescences from two to three heads of cauliflower (*Brassica oleracea* var. botrytis; purchased at local markets) were cut off from the stems. Cells were disrupted in a juice extractor, and the recovered 1 L of juice was diluted with 3× extraction buffer (3 × : 0.9 M mannitol, 90 mM Na-pyrophosphate, 6 mM EDTA, 2.4% PVP25 [w/v] with, added just before use, 0.9% BSA [w/v], 9 mM cysteine, 15 mM glycine, and 6 mM β-mercaptoethanol; pH 7.5 was adjusted with HCl). Mitochondria were enriched by several steps of differential centrifugation: Cell debris was pelleted by 10-min centrifugations each at increasing g-forces: 1100*g*, 2100*g*, and 3500*g*. Mitochondria were sedimented by 30 min at 11,300*g* and were resuspended in 1× extraction buffer. Subsequent purification on Percoll gradients was as described for the pea lysate. The mitochondrial fraction was diluted in 500 mL of wash buffer (1×: 0.3 M mannitol, 10 mM K-phosphate at pH 7.5, 1 mM EDTA with, added just before use, 0.1% BSA, 5 mM cysteine, and 15 mM glycine). Mitochondria were pelleted, resuspended in a small volume of wash buffer, and stored frozen. For lysate preparation 400 mg of isolated mitochondria were lysed in 1200 μL of lysis buffer (30 mM HEPES-KOH at pH 7.7, 10 mM Mg-acetate, 2 M KCl, 2 mM DTT, and 0.2% Triton X-100). After 30-min incubation on ice, the lysate was centrifuged at 60,000*g* for 20 min. The supernatant was recovered, and the KCl was removed by dialysis against 5 × 400 mL dialysis buffer (30 mM HEPES-KOH at pH 7.7, 3 mM Mg-acetate, 45 mM K-acetate, 30 mM ammonium acetate, and 10% glycerol) for a total of 5 h. All steps were carried out at 4°C. The resulting extract (~8 μg protein/μL) was rapidly frozen in liquid nitrogen.

Cloning and RNA substrates

DNA clones of the *atp9* coding region and flanking sequences were constructed in an adapted pBluescript SK^+ to allow run-off transcription of the editing template RNA as described (Takenaka and Brennicke 2003). Deletion clones were shortened by removing original mitochondrial sequences as indicated in the respective experiments. The outside bacterial anchors for PCR amplification accordingly moved closer into the editing sites. Coincidental nucleotide similarities between these and the substituted mitochondrial sequences were taken into consideration when evaluating nucleotide requirements for RNA editing. Cauliflower clones for sequence analyses were made from genomic mitochondrial DNA by PCR between primers derived from the respective *Arabidopsis thaliana* sequences and for cDNA analysis from mitochondrial RNA by RT-PCR, respectively.

In vitro RNA editing reactions

The in vitro RNA editing reactions were performed as described (Takenaka and Brennicke 2003). After incubation, template sequences were amplified by RT-PCR with one of the primers labeled with the Cy5 fluorophor. RNA editing activity was detected by mismatch analysis employing the TDG enzyme activity (thymine DNA glycosylase, Trevigen). The TDG treated fragments were separated, and the Cy5 fluorescence was scanned and displayed with an ALF express DNA sequencer (Amersham).

The efficiency of the in vitro RNA editing reaction was quantified by comparing the areas under the peaks of the cleaved and uncut DNA fragments. The ratio of cleaved (i.e., edited) fragment to uncut DNA was used to determine relative efficiencies of the investigated conditions in each experiment. To allow comparisons and to determine the variation between individual experiments, the ratios of cleaved to uncleaved fragments were displayed as percentages of the standard reaction conditions.

Generation of mutant substrates

The 5′-deletion mutants were constructed by inverted PCR from the cloned *atp9* sequences with primers −40, −30, −20, −10, and −0, respectively, on the one side and primer invertion1 on the other. The resulting fragments were digested with EcoRI to generate sticky ends in the primer contained EcoRI recognition site and were self-ligated. The 3′-deletion mutants were constructed by inverted PCR from clone atp9–30 with primer invertion2 and primers +10 and +0, respectively. The PCR fragments were digested with XbaI and self-ligated.

The mutant templates with defined sequence regions exchanged to their opposite sequence were constructed by introducing the respective complement pentanucleotide in primers M1-M10. PCR was performed on deletion clone atp9–40 with primer invertion1 and primers M1, M2, M3, M4, and M5, respectively, and in the second series with primer invertion2 and primers M6, M7, M8, M9, and M10, respectively. The resulting fragments were digested with EcoRI or XbaI, respectively, and self-ligated. The point mutations were introduced by the same procedure using the respectively altered primers. All mutants were confirmed by sequence analysis.

Competition assays

Wild-type competitor RNA was synthesized from the PCR product amplified with primers T7 and +10 from clone atp9–40. An entirely plasmid derived control RNA was synthesized from the PCR product amplified from pBluescriptIISK+ with T7 and SK primers. The mutant competitors were synthesized from the PCR products amplified from clones M1–M5 with the T7 primer and primer +10, and from clones M6, M7, M8, M9, and M10 with T7 and the respective mutant primers. One hundred attomoles of substrate and 1000 times (100 fmol) for pea and 1500 times (150 fmol) for cauliflower competitor RNA were first mixed and then incubated with the mitochondrial in vitro assay as described above.

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RNA editing sites in plant mitochondria can share *cis*-elements.

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RNA editing sites in plant mitochondria can share *cis*-elements

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Abstract RNA editing in flowering plant mitochondria alters numerous C nucleotides in a given mRNA molecule to U residues. To investigate whether neighbouring editing sites can influence each other we analyzed in vitro RNA editing of two sites spaced 30 nt apart. Deletion and competition experiments show that these two sites carry independent essential specificity determinants in the respective upstream 20–30 nucleotides. However, deletion of a an upstream sequence region promoting editing of the upstream site concomitantly decreases RNA editing of the second site 50–70 nucleotides downstream. This result suggests that supporting *cis*-/*trans*-interactions can be effective over larger distances and can affect more than one editing event.

Keywords: Plant mitochondria; RNA editing; Mitochondrial RNA

1. Introduction

RNA editing was first recognized in plant mitochondria and chloroplasts as a post-transcriptional process altering mostly C to U nucleotide identities in mRNAs and tRNAs. The more than 400 sites found in mitochondria in the mRNAs for only 53 genes imply that many sites are located relatively near each other [1]. This raises the question of whether these sites are addressed independently, collectively or consecutively.

Analysis of in vivo RNA editing in transgenic chloroplasts with individual gene fragments suggested that single sites can be edited faithfully, which is expected from their usually large distances from each other [2–6]. The development of reliable in vitro RNA editing activities for chloroplasts [7–9] and mitochondria [10,11] as well as in organello editing [12–15] in the past few years, has accelerated progress towards elucidating the *cis*-requirements. For plant mitochondria, in vitro RNA editing in pea lysates and in organello editing in wheat show that for some editing events only about 15–30 nucleotides are necessary upstream and very few or none downstream. These delineations of template requirements in a given mRNA template in plant mitochondria extend previous conclusions about the minimal recognition sequences from recombined transcript regions [16].

RNA editing on the template mRNA molecule appears to progress by site-by-site target recognition rather than a scanning process along the RNA molecule. This conclusion is based on the identification of cDNA clones edited at only some of the sites. The identity of these sites varies, which – supposing that these partially edited sites are editing intermediates – suggests that the editing activity attaches to the RNA molecules in numerous rounds at individual sites. Transfections of isolated mitochondria with *cox2* gene sequences also yielded partially edited mRNA molecules, in which several sites are not edited in all or some RNA molecules [12–15].

To gather more direct data about editing site recognition we have now investigated whether neighbouring editing sites can influence each other. The interdependence of two sites in the *atp9* mRNA separated by only 30 nucleotides was analyzed in an in vitro system from cauliflower mitochondria [17].

2. Materials and methods

2.1. Preparation of mitochondrial extracts

Heads of cauliflower were purchased at local markets. About 900 g of the top tissues of the inflorescences were harvested, manually chopped into small pieces and homogenized in a blender. Mitochondria where purified by differential centrifugation steps and a Percoll gradient [10]. Four-hundred milligrams of isolated mitochondria were lysed in 1200 μl extraction buffer [0.3 M HEPES-KOH, pH 7.7, 3 mM Mg-acetate, 2 M KCl and 2 mM dithiothreitol (DTT)] containing 0.2% Triton X-100. After 30 min incubation on ice, the lysate was centrifuged at 22 000 × *g* for 20 min. The supernatant was recovered and dialyzed against 5 × 100 ml dialysis buffer (30 mM HEPES-KOH, pH 7.7, 3 mM Mg-acetate, 45 mM K-acetate, 30 mM ammonium acetate and 10% glycerol) for a total of 5 h. All steps were carried out at 4 °C. The resulting extract (10–20 μg protein/μl) was rapidly frozen in liquid nitrogen. Mitochondrial lysates from pea seedlings (*Pisum sativum* L., var) were prepared as described [10].

2.2. RNA substrates

DNA clones (patp9) were constructed in an adapted pBluescript SK+ vector to allow run-off transcription of the editing template RNA as described [10]. Deletion clones were shortened by removing original mitochondrial sequences as indicated in the respective experiments. The outside bacterial anchors for PCR amplification accordingly moved closer to the editing sites. Coincidental nucleotide similarities between these and the substituted mitochondrial sequences as well as potential secondary structures were taken into consideration when evaluating nucleotide requirements for RNA editing.

2.3. In vitro RNA editing reactions

The in vitro RNA editing reactions were performed as described [10]. After incubation, template sequences were amplified by RT-PCR, the upstream primer labelled with the Cy5 fluorophor. RNA editing activity was detected by mismatch analysis employing the TDG enzyme activity (thymine DNA glycosylase, Trevigen). The TDG treated fragments were separated on 7.5% polyacrylamide gels containing 8 M urea at 900 V for about 400 min. The Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (Amersham).

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The RNA editing product percentages lie well within the range of the linear sensitivity of the TDG assay as determined experimentally and the relative amounts of cut fragments (3–7%) thus reflect the amount of the in vitro editing activity within this window (about 1–25% [10]).

The efficiency of the in vitro RNA editing reaction was quantified by comparing the areas under the peaks of the cleaved and uncut DNA fragments. The ratio of cleaved, i.e., edited, fragment to uncut DNA was used to determine relative efficiencies of the investigated conditions in each experiment. To allow comparisons and to determine the variation between individual experiments, the ratios of cleaved to uncleaved fragments were displayed as percentages of the standard reaction results.

2.4. Generation of mutant substrates

The 5′ deletion mutants were constructed by inverted PCR from patp9 with primers −40, −20, −10 and −0, respectively, on the one side and primer invertion1 on the other. The resulting fragments were digested with *Eco*RI to generate sticky ends in the primer contained *Eco*RI recognition site and were self-ligated.

2.5. Competition assays

Wild type competitor RNA was synthesized from the PCR product amplified with primers T7 and +10 from the different *atp9* deletion clones indicated in the figures. An entirely plasmid derived control RNA was synthesized from the PCR product amplified from pBluescriptIISK+ with T7 and SK primers. One hundred attomol of substrate and 1500 times (150 fmol) competitor RNA were first mixed and then incubated with the mitochondrial in vitro assay as described above.

3. Results

3.1. Detection of two adjacent editing sites in one template

In the plant *atp9* mRNA the first editing site in the open reading frame is located 19 nucleotides downstream of the AUG codon. As depicted in Fig. 1A, this site is followed by a second editing event 30 nucleotides further downstream. The alignment shows that the respective upstream nucleotides of the two edited nucleotides, which for the first site have been determined to harbour the recognition region [11], show no primary sequence similarity. When a homologous template RNA containing both RNA editing sites and 40 nucleotides upstream of the first site (covering all *cis*-determinants for this site) is incubated with the mitochondrial lysate from cauliflower, the first and the second site are edited in vitro (Fig. 1B).

3.2. Editing of the second site depends on a species-specific template

In the plant *atp9* mRNA the first editing site in the open reading frame is located close to the AUG and the upstream sequences in the 5′-UTR vary between species (Fig. 1A). To determine the influence of these species-specific sequences on the in vitro reaction we compared editing at the second site in template RNAs from cauliflower and pea, respectively (Fig. 2).

Surprisingly in vitro RNA editing with the heterologous pea *atp9* template is much less efficient than with the cauliflower RNA: only in about one-third of the assays with the pea template is the second site observed with confidence, while the first site is consistently detected.

3.3. Thirty nucleotides determine the second RNA editing site

In the homologous cauliflower *atp9* template the activity of the in vitro editing reaction at the second site is greatly diminished by deletion of the distant part of the first site recognition region, which harbours a sequence promoting editing at the

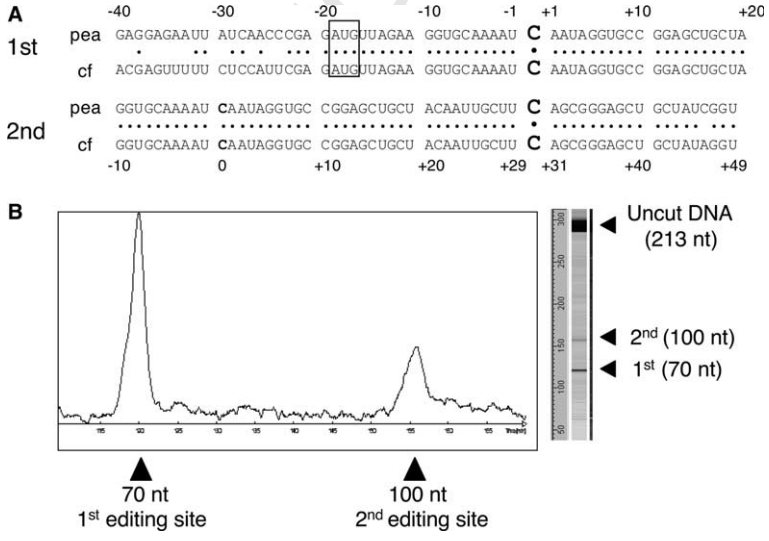

Fig. 1. Two neighbouring RNA editing sites are processed in an in vitro lysate from cauliflower mitochondria (*Brassica oleracea*). (A) Nucleotide sequence alignment of the first two editing sites (1st and 2nd) in the *atp9* mRNA reveals no sequence similarity around the sites. The edited C residues are shown by large bold letters, the upstream editing site (1st) of the top line pair is shown again in bold at the 0 position of the downstream site (2nd) in the lower part. In all experiments template RNAs are oriented and numbered relative to the upstream site, the first editing site in the *atp9* mRNA. For comparison of the templates used and to see the divergent sequence in the region around −40/−35, the native cauliflower sequence (cf) is aligned with the pea (*Pisum sativum*) sequence and nucleotides identical between the two are marked by bullets. The AUG codon is framed. (B) A sample gel tracing of a cauliflower template RNA containing both editing sites. The enlarged graph shows the section of the scan covering the two editing sites. The second site is detected usually at about 20–25% of the efficiency of the first site. The respective gel image for this tracing is shown on the right, gel conditions were 7.5% polyacrylamide gels containing 8 M urea run at 900 V for about 400 min. Numbering on the left of the gel as well as underneath the scan gives the respective running times in the gel in minutes. Sizes of the RT-PCR fragments are given in nucleotides for the uncut and the editing site-specific products, respectively. Unspecific bands in the background appear optically more prominent in the gel image than in the actual scan. This background probably results from the RT-PCR, TDG and denaturing steps during the procedure.

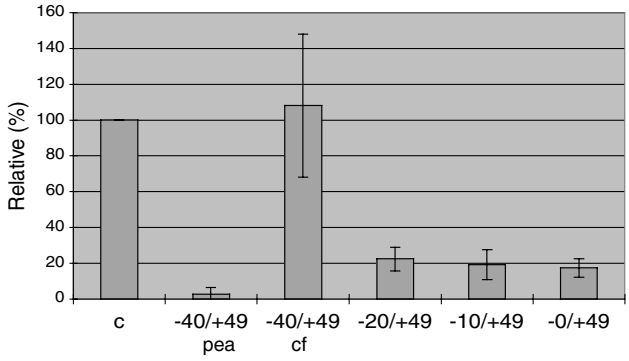

Fig. 2. Analysis of the *cis*-reqirements for in vitro RNA editing at the second site. The *atp9* sequences retained in the respective template RNAs are indicated underneath each column. All templates contain the second site at nucleotide +30 from the first site. In the three series of experiments summarized in this figure (five experiments for the cf −40/+49 template), editing at the first site in a pea template RNA covering −40/+49 is used as standard (column c). The deletion clones are derived from the pea template which is identical to the cauliflower sequence except for nucleotide +46. This difference 16 nucleotides downstream of the monitored second editing site is considered unlikely to be relevant for this editing event, since such downstream sequences are usually not involved in editing site definition.

first site (Fig. 2). Deletion of this element between nucleotides −40 and −20 relative to the first site results in a drop of the in vitro RNA editing efficiency at the second site by about 75%. A series of further deletion clones successively shortened in steps of 10 nucleotides from the 5′ end up to the first site has no further effect upon editing at the second site. The second editing site monitored here thus requires upstream only 30 nucleotides (or less) to sustain in vitro editing at a constant albeit low level. The heterologous pea template appears to contain editing–inhibitory sequences in the unique region between nucleotides −40 and −20 relative to the first site, since the deletion clones without this sequence are edited more efficiently (Fig. 2).

3.4. Only the cognate recognition sequences compete a given editing site

In vitro RNA editing at the first site is inhibited by competitor RNAs covering the first site sequences with the *cis*-recognition elements between −40 and +10 (Fig. 3B; −40/+10 cf). Downstream sequences as in the competitor −0/+49 have, as expected, little effect on the in vitro reaction, since the *cis*-elements for this site reside almost exclusively upstream [11].

The second editing site on the other hand is inhibited by competition with this latter −0/+49 RNA, further supporting the conclusion from the deletion templates described above that this region contains the *cis*-elements required for its recognition.

3.5. A cauliflower specific sequence supports editing over 70 nucleotides across the first editing site

To investigate whether the stimulating influence of the upstream element on in vitro editing of the second site is indeed species-specific, we first tested the effect of adding an excess of the pea upstream sequences covering nucleotides −40 to +10 relative to the first site (Fig. 3C; −40/+10 pea). The result of this experiment – no inhibition – allows three conclusions: Firstly, the pea distal element does not compete with the cauliflower sequence. Secondly, the *cis*-element of the second site does not seem to extend much further upstream beyond 20

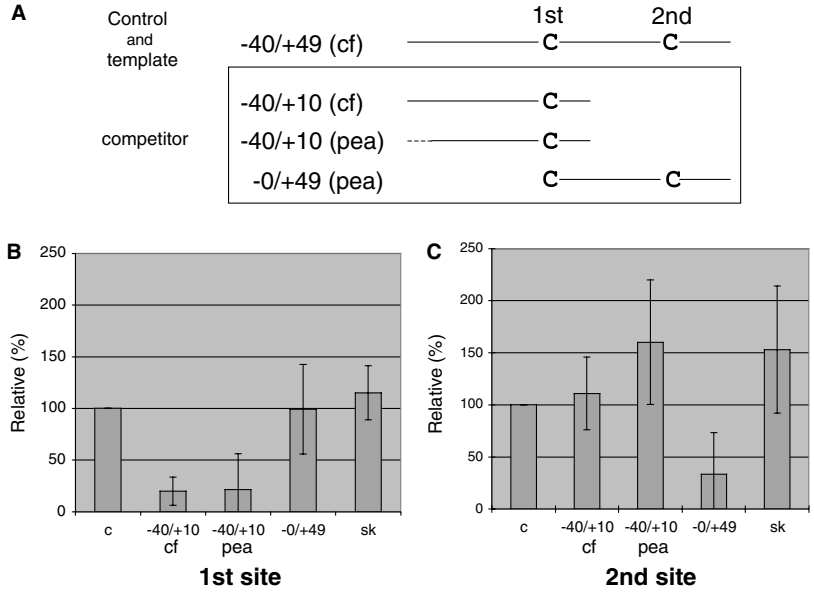

Fig. 3. Determination of competition for *trans*-factors between the respective core recognition sequences of the two neighbouring sites. (A) Scheme of the template *atp9* RNA from cauliflower (cf; top line), the competitors for the upstream site from cauliflower (second line) and from pea (third line), the dashed line indicating the different sequence in pea. The competitor for the downstream site (bottom line) differs only at nucleotide +46 between pea and cauliflower. The respective editing sites are indicated by a bold C. (B) The upstream site is monitored for effects by a 1500-fold excess of its upstream sequence (−40/+10) from cauliflower and pea, respectively, and of the downstream region (−0/+49), the latter containing the recognition sequence of the downstream site. The control c is run without competitor and the effect of a 1500-fold excess of a bacterial plasmid sequence is shown as sk. (C) The effect on in vitro editing of the downstream site by 1500-fold competitor excess of its own recognition sequence (−0/+49) and of the pea or cauliflower upstream site recognition sequences (−40/+10) is investigated.

nucleotides, since the nucleotides 20–30 nucleotides upstream are included in this competitor and do not interfere. Thirdly, the first site recognition sequence has no detrimental effect on editing of the second site.

The enhancement of the reaction upon addition of the pea competitor −40/+10 (Fig. 3C) reflects an unspecific RNA effect, possibly by binding inhibitory non-specific RNA binding proteins, since a similar observation is made with unrelated RNA derived from vector sequences (Fig. 3C; sk). The observation of a possibly editing–inhibitory sequence in the unique region in the heterologous pea template between nucleotides −40 and −20 further supports the importance of this distant region for editing at the second site (Fig. 2).

3.6. The trans-factor addressing the cauliflower species-specific long distance supporting sequence motif seems to be abundant

In a further competition experiment the abundance of the species-specific *trans*-factor interacting with the distal upstream element supporting in vitro editing of the second site was investigated by adding excess homologous cauliflower sequences (Fig. 3C; −40/+10 cf). Editing of the second site was not influenced although this competitor greatly reduced in vitro editing of the first site (Fig. 3B; −40/+10 cf). The result that one but not the other can be competed, suggests that the *trans*-factors promoting editing of the first and second sites, respectively, from this same distal region act or are distinct.

4. Discussion

4.1. Editing sites are addressed individually

The template RNA constructs were designed to monitor two neighbouring RNA editing sites in order to determine whether access to these sites by the editing activity is connected or whether contact is made individually. Deletion templates containing only one or the respective other site show that either of the two sites can be edited in the absence of the other. The distinct recognition elements for the two sites are separated by about 5–10 nucleotides and presumably targetted individually by specific *trans*-factors.

This observation furthermore implies that there is no overt order in the alteration of the various nucleotides in a given mRNA. An apparent hierarchy might become established by the effectiveness of individual specific *trans*-factors to attract the hit-and-run editing complex and result in the observed partially edited mRNAs in the mitochondrial steady state population.

4.2. Specific trans-factors recognize neighbouring editing sites

The *trans*-factors attracting the RNA editing complex to the respective nucleotide to be edited are different for these neighbouring sites. There is no sequence similarity between the essential *cis*-regions which cover 23 nucleotides for the upstream and up to 30 nucleotides for the downstream site (Fig. 1A). Experimentally, the cross-competition experiments show that only the cognate sequence can interfere with editing at either site (Fig. 3). The different down-shifting with equal amounts of competing RNA molecules likewise supports this conclusion that the specificity factors for these two sites in the *atp9* mRNA are distinct.

4.3. A species-specific editing-supporting sequence serves two sites

Efficient editing of the second site in the *atp9* mRNA is observed only with the −40/+49 cauliflower template RNA in the cauliflower in vitro lysate, but not with the pea template and with neither template in the pea system (Fig. 2 and data not shown). This template includes the region at nucleotides −40/−35, which was previously identified to increase editing at the upstream site [17]. When this region is deleted or altered as in the pea template, efficiency of the reaction drops dramatically (Fig. 2). The native cauliflower template thus appears to attract a *trans*-factor which can act over a distance of 50–70 nucleotides to boost the editing activity at the downstream site. This *cis*-enhancer region can thus function for both neighbouring editing sites.

The effect is species-specific, since the different pea supportive region (Fig. 1A) cannot substitute for the positive effect of the cauliflower element for the second site. The pea enhancer region does however increase editing of the first site in the cauliflower in vitro system [17], suggesting that this pea sequence attracts in the cauliflower lysate (a) different (for the first site positive) *trans*-agent(s). That in the cauliflower template two distinct *trans*-factors may interact with the cognate *cis*-region, is further supported by the result that the positive effect of this region can be titrated for the first site, but not for the second site. The nature and identity of these *trans*-factors remain to be solved to determine the differential binding properties.

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Partially edited RNAs are intermidiates of RNA editing in plant mitochondria.

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Partially edited RNAs are intermediates of RNA editing in plant mitochondria

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Summary

RNA editing in flowering plant mitochondria addresses several hundred specific C nucleotides in individual sequence contexts in mRNAs and tRNAs. Many of the *in vivo* steady state RNAs are edited at some sites but not at others. It is still unclear whether such incompletely edited RNAs can either be completed or are aborted. To learn more about the dynamics of the substrate recognition process, we investigated *in vitro* RNA editing at a locus in the *atp4* mRNA where three editing sites are clustered within four nucleotides. A single *cis*-element of about 20 nucleotides serves in the recognition of at least two sites. Competition with this sequence element suppresses *in vitro* editing. Surprisingly, unedited and edited competitors are equally effective. Experiments with partially pre-edited substrates indicate that indeed the editing status of a substrate RNA does not affect the binding affinity of the specificity factor(s). RNA molecules in which all editing sites are substituted by either A or G still compete, confirming that editing site recognition can occur independently of the actual editing site. These results show that incompletely edited mRNAs can be substrates for further rounds of RNA editing, resolving a long debated question.

Keywords: RNA editing, plant mitochondria, partially edited RNAs, *in vitro* RNA editing, atp4, cauliflower.

Introduction

RNA editing in plant mitochondria and chloroplasts is a post-transcriptional process altering mostly nucleotide identities from C to U in mRNAs and tRNAs. Previous *in vivo* and *in vitro* investigations found that for specific recognition by the RNA editing activity, often only about 20–40 nucleotides are necessary upstream and very few, if any, are necessary downstream of a given editing site (Bock *et al.*, 1996, 1997; Chaudhuri and Maliga, 1996; Choury *et al.*, 2004; Farré *et al.*, 2001; Hirose and Sugiura, 2001; Miyamoto *et al.*, 2004; Takenaka and Brennicke, 2003; Takenaka *et al.*, 2004).

The recent development of reliable *in vitro* activities for chloroplasts (Hirose and Sugiura, 2001; Miyamoto *et al.*, 2002) and mitochondria (Takenaka and Brennicke, 2003) has accelerated progress towards elucidating the details of *cis*-requirements, and has yielded further insights about the mode of editing site recognition.

For plant mitochondria, we have recently established *in vitro* RNA editing systems from pea shoots and from cauliflower inflorescences (Neuwirt *et al.*, 2005; Takenaka and Brennicke, 2003). Detailed analysis of the *cis*-requirements for site specificity showed that in some instances several elements can be distinguished in the *cis*-sequence of a given mRNA substrate (Neuwirt *et al.*, 2005). A basic essential region up to about 20 nucleotides upstream of an editing site in the *atp9* mRNA supports *in vitro* editing, albeit at a low level. A supporting region further upstream around the nucleotides from −35 to −40 enhances the *in vitro* RNA editing activity of this site.

Intriguingly the nucleotide identities in the immediate vicinity of the edited nucleotide have little influence on the specificity of recognition, and only moderately contribute to the efficiency of the reaction (Neuwirt *et al.*, 2005; Takenaka and Brennicke, 2003). The implied separation of recognition region and editing site may be bridged by an interconnected complex of several distinct proteins attaching to the different *cis*-elements in the RNA and the edited nucleotide respectively. Competition experiments suggest that the *trans*-factors for specificity can be titrated and are thus present in limiting quantities in plant mitochondria.

It is unclear, at present, how the editing machinery progresses along the substrate RNA. As in the steady state

plant mitochondrial mRNA population a considerable percentage of transcripts is edited at most but not all sites, individual editing sites are most likely addressed independently of each other. *In vitro* analysis of RNA editing sites spaced 30 nucleotides apart has shown that each site is indeed addressed individually and substantiated this model (van der Merwe *et al.*, 2006). This however raises the question whether already edited sites can be discriminated from unedited sites, and whether both are seen as potential targets by the editing complex.

It has been an open question for many years whether incompletely edited RNA molecules, which contain some sites edited and others not edited, are either intermediates that can still be recognized as substrates to complete this process or whether such molecules represent dead ends that have been aborted and will be rapidly degraded (Bock, 2001; Bonnard *et al.*, 1992; Wissinger *et al.*, 1992).

To investigate whether the editing machinery can make this distinction between incompletely edited and not edited substrates, we analysed a cluster of three editing sites within four nucleotides in the *atp4* mRNA in unedited and incompletely edited states. Interestingly, one set of *cis*-elements mediates editing at the two prominent sites *in vitro* (and probably at all three *in vivo*) in the *atp4* mRNA.

Results

Three RNA editing sites are clustered within four nucleotides

In the *atp4* mRNA in cauliflower mitochondria three RNA editing sites are located within four nucleotides, raising the question of how these sites are addressed (Figure 1). *In vivo* the first and the third site appear to be slightly more rapidly edited: of 38 cDNA clones sequenced, 37 are edited at all sites, whereas one clone is edited only at sites one and three (data not shown). This observation, although statistically not significant, nevertheless suggests that *in vivo* site two is edited somewhat slower than are the other two sites. In terms of information content, site two is a silent editing as soon as site three has been altered, and mRNAs either unedited or edited at site two specify the same protein (Figure 1a). Looking at other plant species, most, including dicots, monocots and gymnosperms, require editing at site one, whereas only dicots code edited C residues at sites two and three (Figure 1b).

Such *status quo* analyses do not allow to distinguish between editing intermediates and terminally aborted errors in this processing step. We thus designed the respective *in vitro* experiments detailed below to determine whether incompletely edited molecules can still serve as substrates.

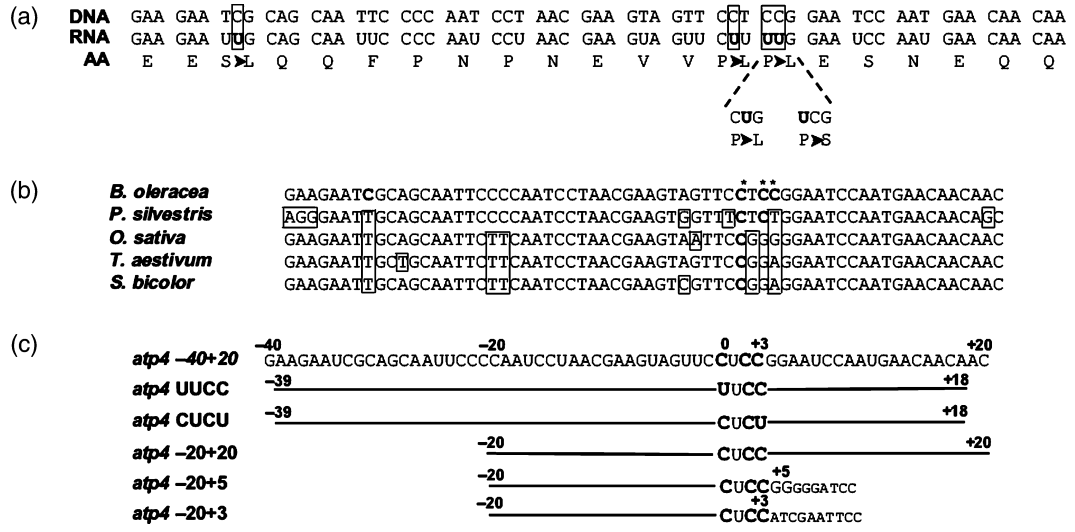

Figure 1. Three RNA editing sites are clustered within four nucleotides in the *atp4* coding sequence.
(a) Effect of RNA editing at the clustered sites on the encoded amino acid sequence. After editing the third site, the second site becomes a silent editing event, which does not change the amino acid specified. Uridines created by RNA editing are shown in bold and the resulting amino acid (AA) changes are indicated.
(b) Dicot plant species such as cauliflower (*Brassica oleracea*) contain a cluster of three editing sites, whereas monocots (*Oryza sativa*, *Triticum aestivum*, *Sorghum bicolor*) and gymnosperms (*Pinus silvestris*) require editing only at the first site. In the latter species editing at this site has not been experimentally shown. Nucleotide divergencies from the cauliflower sequence are boxed. The cluster of the three editing events in cauliflower is marked by asterisks. An additional upstream RNA editing event in cauliflower is indicated in bold and the consensus T at this position in other species is boxed.
(c) The initial substrate subcloned from the full length gene in cauliflower mitochondria contains 40 nucleotides upstream of the first edited C in the cluster, and 20 nucleotides downstream (*atp4 –40 + 20*; top line). Nucleotide positions are all numbered relative to the first editing site in the cluster, which is assigned position 0. Sequences of partially pre-edited RNA substrates as well as various deleted substrates examined for *in vitro* editing are depicted in the lines below. The three C-nucleotides edited *in vivo* as well as the respective edited U-moieties are shown in bold and larger type. Smaller fonts in the –20 + 5 and –20 + 3 deletion clones give the substituting bacterial sequences for comparison.

In vitro *the first and the third of the three sites are edited*

In the cauliflower *in vitro* editing system sites one and three are altered correctly. The second site is not edited *in vitro* in line with the *in vivo* observation that this site may be somewhat less accessible to the editing machinery. Sequence analysis of individually cloned *in vitro* editing products suggest that editing sites one and three are altered independently of each other: of 20 analysed clones three were edited at both sites, whereas two were altered only at site one, and one was edited only at site three (data not shown). It remains unclear why the second site is not edited *in vitro*. We assume that *in vitro* not all necessary *trans*-factors assemble correctly for all sites, as several editing sites in different mRNA substrates we tested are not addressed *in vitro* (Takenaka M., Neuwirt J., van der Merwe J.A., Verbitskiy D., Zehrmann, Universität Ulm, Germany, unpublished observations). Similar observations were made in the *in organello* assays, in which several sites are never altered although these assays are closer to the *in vivo* situation than the *in vitro* lysates (Choury *et al*., 2004).

Twenty nucleotides upstream of the first site and two nucleotides downstream of the last editing site are sufficient for in vitro *recognition of both editing targets*

In vitro analysis with various deletion substrates (Figure 1c) reveals that upstream of the first site only twenty nucleotides are required for correct editing at both sites (Figure 2). The efficiency of editing is however lower than with a substrate containing 40 nucleotides upstream. This result suggests that the sequence context further upstream of the immediate 20 nucleotides supports the attraction of the *trans*-acting editing protein(s).

In the downstream region five nucleotides beyond the first editing site (i.e. two nucleotides beyond the 3′-terminal site) are sufficient for effective editing at both target nucleotides. Removal of a further two nucleotide identities does not inhibit editing at site one, but abolishes alteration at the 3′-terminal site three, suggesting that these downstream moieties are necessary for this latter event. In the substrate these two nucleotides were not actually deleted, but the GG dinucleotide was substituted by AT residues from the vector. We thus conclude that the two adjacent 3′-nucleotide identities are essential for editing at this position, but do not influence recognition of the first site located three nucleotides upstream.

Incompletely edited substrates are recognized as well as unedited sequences

To investigate the influence of the processing status of individual editing sites on recognition of this region, we constructed substrates in which the first and third sites were

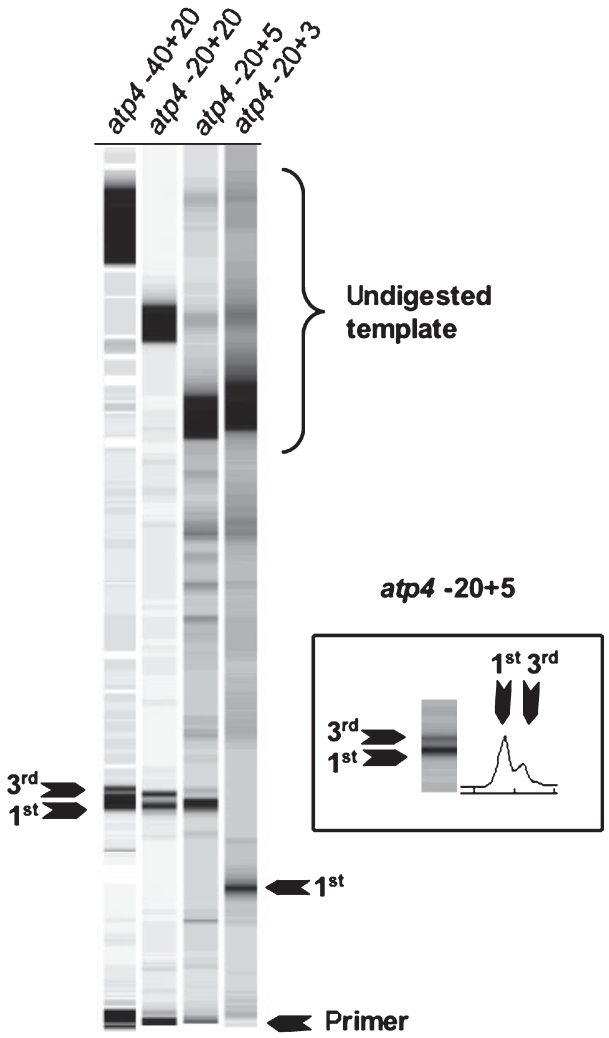

Figure 2. Determination of the minimal recognition site for the *atp4* editing site cluster. The longest substrate shown here (left lane, −40 + 20), covering 40 nucleotides upstream and 20 nucleotides downstream of the first editing site, is edited rather efficiently at both the first and third sites. The 5′-deleted substrate −20 + 20 with only 20 upstream nucleotides shows diminished editing activity at site 1. Deletion of downstream sequences in substrate −20 + 5 with only two nucleotides conserved downstream of the last site reduces the level of editing at both sites. Although only little *in vitro* editing product is seen at site three, the site as such is still recognized correctly. The inlet shows the gel image as well as the scan of a separate experiment with the −20 + 5 construct, in which the third site is more discernible. This 3′-most site is however not edited at all when the adjacent nucleotides downstream are substituted by different nucleotides in the −20 + 3 template (Figure 1c). The −40 + 20 and the −20 + 3 constructs are cloned in the *Sma*I site of the vector and the −20 + 3 TDG product is accordingly 20 nucleotides shorter. The −20 + 20 and −20 + 5 fragments are cloned into the *Eco*RV site of the vector, which is 18 nucleotides distant from the *Sma*I site. This results in TDG products with similar 5′-sequence extensions.

'pre-edited', i.e. already contained uridines at the respective positions. Both substrates are edited at the residual site with efficiencies comparable to the completely unedited substrate (Figure 3 and data not shown).

Figure 3. Analysis of *in vitro* RNA editing in unedited and partially pre-edited substrates. The influence of processed versus unprocessed editing sites on the editing efficiency at respective other sites targeted by the same *cis*-element is investigated in a comparison of editing in an unedited mRNA substrate (left-hand lane, *atp4* C**U**C**C**−40 + 20), in an RNA edited at site 1 (center lane, *atp4* **U**U**CC**−39 + 18) and in a substrate edited at site 3 (right-hand lane, *atp4* C**U**C**U**−39 + 18). The signal of the third editing site in the completely unedited substrate appears to be weaker as a result of the action of the TDG enzyme, which in this majority of doubly edited products will render the downstream site undetectable when having cut at the first site. The unedited substrate RNA covers 61 *atp4* nucleotides, and includes each of the partially pre-edited substrates 58 nucleotides respectively. The TDG products of the latter substrate RNAs are 1 nucleotide shorter than the product from the unedited substrate, hence the corresponding shift in mobility. In the *in vitro* reaction of course only the respective residual and not the pre-edited site of the two potentially processed editing sites within this *atp4* region can be monitored. Sites of *in vivo* RNA editing are highlighted in bold as unedited **C** as well as editing product **U**.

To further evaluate the respective binding affinities we tested the effect of incompletely pre-edited, entirely unedited and fully pre-edited RNAs in competition with one another. In a first set of experiments competition against a substrate pre-edited at the 5′-most site, site one, was analysed for editing at site three (Figure 4). All unedited, pre-edited and fully edited RNAs compete to comparable effect. In a second round of experiments a substrate pre-edited at site three, the 3′-most site, was assayed for the effect of the various competitors on editing at site one (Figure 5). Also at this site all fully and pre-edited competitors inhibit *in vitro* editing to an effect comparable with the non-edited RNA. These results suggest that firstly the specificity mediating *trans*-factors for these RNA editing sites are present in limiting quantities, and secondly that these factors cannot discriminate between unedited and edited substrate RNAs.

Binding of trans-factors is independent of the editing site(s)

To further analyse the influence of the editing site upon recognition of the site by the *trans*-factors, we substituted the four nucleotides containing the three potential editing sites in the *atp4* template by continuous stretches of four As and Gs, respectively, and tested the effect of these RNAs as competitors (Figure 6). Both sequences inhibited the *in vitro* reaction at a wild-type template, suggesting that the limiting *trans*-factors bind upstream of the editing sites independently of either the presence or the absence of the actual edited nucleotide(s).

Specificity of the editing site recognition region

The nucleotide sequence of the specificity region for the three *atp4* editing sites investigated here is unique, at least in the *Arabidopsis* mitochondrial genome, and is different from the sequence contexts at other editing sites. To investigate this specificity, and the possibility that common *trans*-factors might recognize different sequences through some undetected common denominator in the vicinities of the various editing sites, we tested the effect of this *atp4* specificity region on RNA editing at two different editing sites in the *atp9* mRNA (Figure 7). None of the fully, incompletely or unedited *atp4* elements have any detrimental effect on editing at either of the two *atp9* sites *in vitro*. This result extends observations from several previous assays in mitochondria (and in plastids), which similarly suggested that indeed unique *trans*-factors recognize and bind to the various sequence elements around the approximately 30 plastid-located editing sites and the >400 RNA editing sites in plant mitochondrial RNAs (Chaudhuri *et al.*, 1995; Hirose and Sugiura, 2001; Miyamoto *et al.*, 2002, 2004; van der Merwe *et al.*, 2006).

Discussion

Incompletely edited RNAs can be substrates for RNA editing

The observation of incompletely as well as fully edited mRNAs in the steady state RNA population of plant mitochondria about 15 years ago has immediately raised the question of whether these incompletely edited RNAs are either terminally abandoned errors or whether they represent intermediates, in which RNA editing can still be completed (Hiesel *et al.*, 1990; Schuster *et al.*, 1990). This question has so far not been resolved as there was no experimental assay available to follow the fate of such

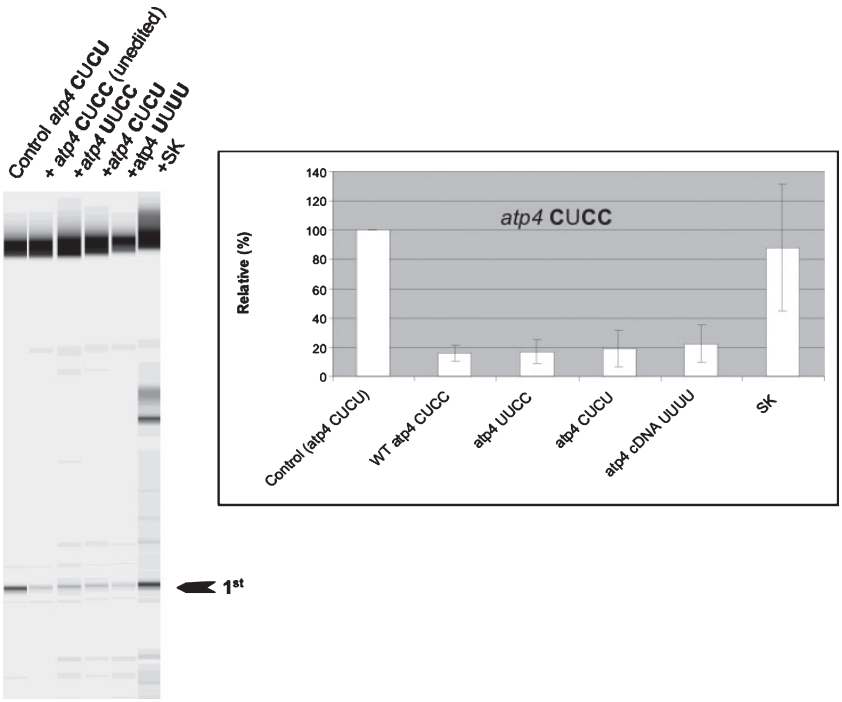

Figure 4. Competitor experiments of the first editing site with the *atp4* substrate region in various stages of RNA editing. The left-hand panel shows a representative gel image, the bar graph on the right summarizes the results of three independent experiments. To monitor editing only at the first site (arrowhead), the editing substrate was constructed to be an RNA in which the third site is already an edited **U** (*atp4* C**U**C**U**). The substrate reaction without competitor is run as a reference and set to 100%. The competitors (1000-fold) include an unedited sequence (*atp4* C**U**C**C**), partially pre-edited sequences altered at either the first site (*atp4* **UU**C**C**) or the third site (*atp4* C**U**C**U**), a fully pre-edited RNA with all three sites changed (*atp4* **UUU**C) and, as an unrelated control, a bacterial sequence (SK). RT-PCR products inadvertedly amplified from the bacterial competitor RNAs are detectable between the products from the full-length substrate and the TDG product after editing.

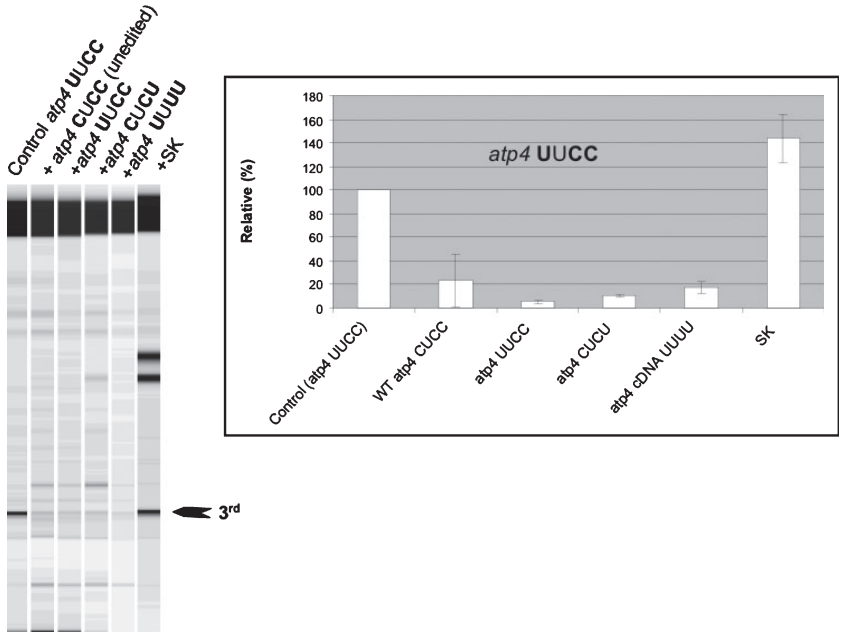

Figure 5. Competitor experiments for editing site three with the *atp4* substrate region in various stages of RNA editing. The left-hand panel shows a gel image, the bar graph on the right summarizes the results of three independent experiments. To monitor editing only at the third site, the editing substrate was constructed to be an RNA in which the first site is already a **U** (*atp4* **UU**C**C**). The substrate reaction without competitor is run as standard and set to 100%. Competitors (1000-fold) include an unedited sequence (*atp4* C**U**C**C**), partially pre-edited sequences altered at either the first site (*atp4* **UU**C**C**) or the third site (*atp4* C**U**C**U**), a fully pre-edited RNA with all three sites changed (**UUU**C) and, as unrelated control, a bacterial sequence (SK). RT-PCR products inadvertedly amplified from this bacterial competitor RNA are detectable between the products from the full-length substrate and from the TDG fragment after editing.

incompletely edited molecules. The development of reliable *in vitro* RNA editing systems has now allowed us to address this long-standing debate and to provide a clear answer.

In the cauliflower *atp4* mRNA the constellation of three closely spaced sites offers a unique opportunity to investigate the processivity of the RNA editing complex in plant mitochondria, and to test whether its binding affinities are influenced by the editing status of the RNA substrate. In the direct approach incompletely edited substrates show comparable *in vitro* RNA editing at the respective unedited sites. The indirect assay, in which incompletely and fully pre-edited RNAs are employed as competitors, corroborates this result, as these molecules compete as effectively as the completely unedited RNA.

These experiments now show that incompletely edited RNA molecules can clearly act as competent substrates, and

Figure 6. Competition between *atp4* RNAs, in which the four pyrimidines covering the three editing sites are substituted by either of the purines. To monitor editing only at the first site, the editing substrate was an RNA in which the third site is pre-edited to a **U** (*atp4* **U**U**CC**). The substrate reaction without competitor is run as standard and set to 100%. Competitors (1000-fold) are either one of the two purine sequences (*atp4* AAAA and *atp4* GGGG, respectively) or, as unrelated control, a bacterial sequence (SK). RT-PCR products inadvertedly amplified from this bacterial competitor RNA are detectable between the products from the full-length substrate and from the TDG fragment after editing.

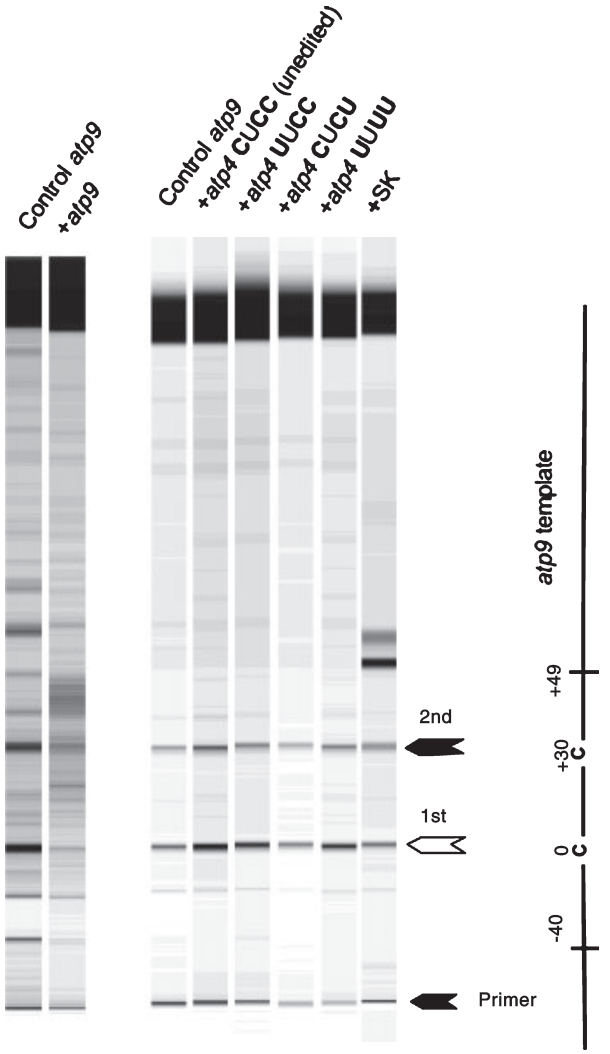

Figure 7. Heterologous competitor experiments to test the specificity of *trans*-factor binding. Two *atp9* editing sites located 30 nucleotides apart were monitored for the effect of 1000-fold competitors of the *atp4* substrate region in various stages of RNA editing. The locations of the two *atp9* RNA editing sites (first and second) are indicated in the schematic of substrate RNA (*atp9*) in the right-hand margin. The substrate reaction without competitor is run as a reference and control. Competition with the homologous RNA blocks RNA editing almost completely at each of the two sites, whereas heterologous RNA editing site sequences have little effect (quantification of the gel data not shown). Heterologous competitors include an unedited *atp4* sequence (*atp4* **C**U**CC**), partially pre-edited sequences altered at either the first site (*atp4* **UUCC**) or the third site (*atp4* **C**U**CU**), a fully pre-edited RNA with all three sites changed (**UUUU**) and, as an unspecific and unrelated control, a bacterial sequence (SK). RT-PCR products inadvertedly amplified from this bacterial competitor RNA are detectable between the products from the full-length substrate and the TDG product after editing.
Sequence data accession numbers: DQ202504.

thus have to be considered as intermediates in the processing step of RNA editing. This result furthermore makes it unlikely that these intermediates could give rise to a group of slightly variant proteins with modified properties in plant mitochondria (Grohmann *et al.*, 1994; Lu and Hanson, 1994; Phreaner *et al.*, 1996). The more probable scenario is that functional proteins will only be specified by fully edited RNAs as supported by protein sequence analysis (Grohmann *et al.*, 1994; Lu and Hanson, 1994).

Specificity determinants do not include the nucleotide(s) to be edited

The observation that the editing activity binds to unedited as well as edited RNA molecules suggests that the *trans*-acting specificity factors do not recognize the nucleotide to be edited. In the special situation of three clustered editing sites in the *atp4* mRNA, a single upstream *cis*-region acts as anchor for all these sites, *in vitro* at least for the two sites correctly addressed, as editing of both sites is lost upon the deletion of nucleotides closer than 20 nucleotides upstream (data not shown). The editing complex does however

recognize the identity of the nucleotides downstream of the last editing site for contact at this site, but must also keep the C, which is to be edited, accessible for contact by the actual editing enzyme(s). This latter conclusion is confirmed by the efficient competition of RNA molecules in which the four pyrimidines covering the three editing sites are substituted by either purine. These experiments suggest that the identity of the to-be-edited nucleotide has no influence on the recognition of the specific editing site by the *trans*-acting factor(s).

Two scenarios can alternatively explain these observations: In the first, the editing enzyme itself is not sequence-specific and is guided to an editing site by the separate specificity factor(s). These latter factors do not contact the nucleotide to be edited, but allow the enzyme to approach the RNA and to perform the editing reaction. The sequence-specific binding proteins can then additionally contact the downstream nucleotide(s), but must leave a gap for the approach of the enzyme. On the other hand the editing enzyme itself could have some steric preferences that require certain nucleotide identities in this downstream region. However, considering the large number of editing sites in plant mitochondria with very different downstream nucleotides, this latter constraint appears to be less likely. In the alternative scenario, instead of an assembly of several proteins with these distinct (partial) functions, a single *trans*-acting protein may contain a respective enzymatic from C to U deamination domain, and there may not be a separate editing enzyme. This single protein would have to recognize the specificity region, bind to the RNA without regard to the editing status of either this or other editing sites and attempt to alter the target nucleotide.

Site-specific distance requirements between the cis-recognition element and the nucleotide to be edited

In this region in the *atp4* RNA, two cytidines separated by two nucleotides are addressed from the same specificity region. This suggests that the editing complex assembled here has at least this three-nucleotide tolerance and a correspondingly low constraint on the distance from the specificity region. This flexibility is high in comparison to an editing site in the *atp9* RNA, which cannot be shifted by a single nucleotide in either direction (Neuwirt J. and Takenaka M., Universität Ulm, Germany, unpublished results). These observations suggest that not only different proteins assemble at the respective *atp9* and *atp4* editing sites, as predicted by the different sequences of the *cis*-elements, but also that these complexes have variant properties at least with respect to the flexibility of the outreach of the editing activity to the respective nucleotides to be edited.

The heterologous competition experiments between the *atp4* and *atp9* regions show that the *trans*-factors for these sites are indeed different and specific, as the heterologous *atp4* editing specificity region has little influence on editing at the respectively monitored sites in the *atp9* RNA. This observation supports the conclusion that although the specificity factor(s) are limiting in their abundance in plant mitochondria, the enzyme(s) performing the actual deaminating step from C to U are available in much higher concentrations. The alternative scenario is not excluded by these results, in which single protein moieties recognize and alter a given editing site, so that deletion of one would not affect the performance of the other RNA editing activities.

A single specificity factor targeting several editing sites in dicot plants and evolutionary aspects

Only dicot plants, such as cauliflower, genomically encode the three C-nucleotides found edited *in vivo* in the cauliflower *atp4* mRNA (Figure 1a,b). Monocot plants such as either rice or maize only code the first C, the other nucleotides have different identities and thus are not subject to editing. The gymnosperm *Pinus silvestris* does code for the first two Cs, but the third site is already genomically encoded as T. This makes the second editing event obsolete because it does not change the respective codon identity. Monocots and gymnosperms should thus both possess at least the *trans*-acting specificity factor to recognize the first editing site. This factor may have evolved in dicot plants to be able to recognize three sites. Alternatively, additional *trans*-factors may be guided to the editing complex, which could support editing at either one or the other additional sites. The observation of independent editing at sites 1 and 3 in our *in vitro* assays would be explained either by such additional co-factors or by slightly variant editing enzymes. A hypothetical co-factor for the central site in dicots could be present in very low quantities in our assays, which would explain why we do not observe this site edited *in vitro*. Alternatively, assuming only one specificity factor (plus the enzymatic moiety), editing at this second site may require previous editing at the other positions, sites one and three. To investigate this possibility we tested a substrate pre-edited at sites one and three in the *in vitro* assay (data not shown). However, we still did not observe any editing at site two suggesting steric hindrance in the artificial substrate RNA, the requirement for an additional co-factor, or a long temporal lag, which pushes effective editing at this site outside the range of the *in vitro* incubation time.

The scenario of only one specific *trans*-acting moiety targeting all three (two *in vitro*) editing sites, and thus coupling the editing events, is supported by the relative numbers of partially edited clones from the *in vitro* reaction (data not shown). The identified three clones edited at both sites one and three are, although few, still many more than predicted from a completely independent association of several additional factors, as only two clones edited at site one and one clone edited at site three were observed in the

total of twenty clones analysed. This distribution suggests that a bound *trans*-factor, which processes site one, is already in position and is thus more likely to also process site three (and vice versa). This is most likely explained by a single binding factor that can stretch to either or both sites.

The comparison of RNA editing in this region between the different plant species raises the question whether the editing systems in monocot and gymnosperm mitochondria can edit all the three sites found in dicot plants. Such an investigation could be pursued for example in the *in organello* RNA editing systems developed for wheat and maize mitochondria (Choury *et al.*, 2004; Farré *et al.*, 2001). If these sites are correctly processed in monocots, we could conclude that plant mitochondria have the potential for many more RNA editing sites than those that have been realized.

Experimental procedures

Preparation of mitochondrial extracts

Heads of cauliflower were purchased at local markets. About 900 g of the top tissues of the inflorescences were harvested, manually chopped into small pieces and homogenized in a blender. Mitochondria were purified by differential centrifugation steps and a PercoII gradient (Neuwirt *et al.*, 2005; Takenaka and Brennicke, 2003). Isolated mitochondria (400 mg) were lysed in 1200 μl extraction buffer [0.3 M HEPES–KOH, pH 7.7, 3 mM Mg-acetate, 2 M KCl and 2 mM dithiothreitol (DTT)] containing 0.2% Triton X-100. After a 30-min incubation on ice, the lysate was centrifuged at 22 000 ***g*** for 20 min. The supernatant was recovered and dialysed against 5 × 100 ml dialysis buffer (30 mM HEPES–KOH, pH 7.7, 3 mM Mg-acetate, 45 mM K-acetate, 30 mM ammonium acetate and 10% glycerol) for a total of 5 h. All steps were carried out at 4 °C. The resulting extract (10–20 μg protein μl^{-1}) was rapidly frozen in liquid nitrogen.

RNA substrates

Genomic as well as cDNA sequences for the *atp4* coding region in cauliflower were determined to identify RNA editing sites. The sequence data are deposited in the databases under accession number DQ202504. DNA clones (p*atp4*) were constructed in an adapted pBluescript SK^+ to allow run-off transcription of the editing substrate RNA as previously described (Neuwirt *et al.*, 2005; Takenaka and Brennicke, 2003). Substrate RNAs were synthesized from the T7 RNA polymerase promoter and thus contained vector sequences at the 5′-end. Similarly vector sequences border the 3′-end of the mitochondrial insert sequences (Figure 1c) up to the *Vsp*I site used for linearization of the template DNA. The bordering bacterial sequences were used for specific amplification of the substrate RNAs by RT-PCR after the *in vitro* assay. Mutant RNA competitors with purines instead of the native pyrimidines were synthesized from PCR products with respectively modified primer sequences.

Deletion clones were shortened by removing original mitochondrial sequences as indicated in the respective experiments. The 5′-deletion mutants were constructed by inverted PCR from p*atp4* with respective primers pairing to the −40 and −20 upstream sequences on the one side and primer invertion1 on the other. The resulting fragments were digested with *Eco*RI to generate sticky ends in the primer-contained *Eco*RI recognition site and were self-ligated. The outside bacterial anchors for PCR amplification moved accordingly closer to the editing sites. Coincidental nucleotide similarities between these and the substituted mitochondrial sequences, as well as potential secondary structures, were taken into consideration when evaluating nucleotide requirements for RNA editing.

In vitro *RNA editing reactions*

The *in vitro* RNA editing reactions were performed as described (Neuwirt *et al.*, 2005; Takenaka and Brennicke, 2003). After incubation, substrate sequences were amplified by RT-PCR, the upstream primer being labelled with the Cy5 fluorophor. RNA editing activity was detected by mismatch analysis employing the TDG enzyme activity (thymine DNA glycosylase; Trevigen, Gaithersburg, MD, USA). The TDG-treated fragments were separated and the Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (Amersham, Freiburg, Germany).

The efficiency of the *in vitro* RNA editing reaction was quantified by comparing the areas under the peaks of the cleaved and uncut DNA fragments. The ratio of cleaved, i.e. edited, fragment to uncut DNA was used to determine the relative efficiencies of the investigated conditions in each experiment. To allow comparisons and to determine the variation between individual experiments, the ratios of cleaved to uncleaved fragments were displayed as percentages of the standard reaction results with a co-treated substrate.

Competition assays

Competitor RNAs were synthesized by T7 RNA polymerase as run-off products from the PCR products amplified with primers T7 and respective primers matching the different *atp4* clones indicated in the figures. An entirely plasmid-derived control RNA was synthesized from the PCR product amplified from pBluescriptIISK+ with T7 and SK primers. Substrate (100 attomol) and 1000 times (100 fmol) competitor RNA were first mixed and then incubated with the mitochondrial *in vitro* assay as described above. In the competitor RNAs either the KS or the T3 sequences were deleted to avoid contaminating PCR amplifications of the monitored substrate RNAs.

Acknowledgements

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7.2 Curriculum vitae

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Place of birth:	Schlema/Germany	
Nationality:	German	
Marital status:	married to Abdul Fatai Alabi Alege	
Children:	Adam Ayoola Alege (* 16.09.2005)	
Education:	9. 1985 – 8. 1989	Primary school in Zschorlau
	9. 1989 – 8. 1991	Secondary school in Zschorlau
	9. 1991 – 6. 1997	Highschool (“Gymnasium”) in Aue
	June 1997	A-levels (with excellent; 1,3)
	9. 1997 – 8. 1998	Practical year in Denia/Spain
	9. 1998 – 4. 2002	study of biology at the University of Ulm/Germany (with excellent) main subjects: molecular biology, virology, microbiology and organic chemistry
	5. 2002 – 1. 2003	“Diploma work” in the Laboratory of PD Dr. Volker Knoop, department of molecular botany at the University of Ulm (with excellent, 1,3) **subject:** “RNA editing in lower land plants”
	2. 2004 till date	PhD with Prof. Dr. Axel Brennicke, department of molecular botany, University of Ulm **subject:** “Examination of *cis*- and *trans*-factors involved in RNA editing”
study related practical work:		2001 “Water company Baden-Würtemberg”. **subject**: “Microbiological screening of water samples for potentially infectious micro-organisms.”
languages:		German, English (business fluent), Spanish (basic knowledge)

7.3 List of Publications

Papers

Takenaka M, **Neuwirt J,** Brennicke A. Complex *cis*-elements determine an RNA editing site in pea mitochondria. Nucleic Acids Res. 2004 Aug 4;32(14):4137-44. Print 2004.

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Reviews

Takenaka M, van der Merwe JA, Verbitskiy D, **Neuwirt J,** Zehrmann A, Brennicke A. RNA editing in plant mitochondria. Version 4. 12. 2005, Review for Goeringer book, in press.

Posters

2004 Poster at the Botanikertakung in Braunschweig “Characterisation of *cis-, trans*-elements involved in RNA editing of *atp*9” **Neuwirt, J**.; van der Merwe, JA.; Brennicke, A and Takenaka, M.

2004 Poster at the Botanikertakung in Braunschweig: “Development of an *in-vitro* RNA editing system from cauliflower (*Brassica oleracea*) mitochondria and testing for the involvement of trans acting RNA's in RNA editing” van der Merwe, JA; **Neuwirt, J**; Brennicke, A. and Takenaka, M.

2005 Poster at the RNA Editing Gordon research conference in Ventura: "*In-vitro* analysis of *cis*-and *trans*-elements for RNA editing in plant mitochondria" Takenaka, M; **Neuwirt, J**; van der Merwe, JA; Verbitskiy, D and Brennicke, A.

2005 Poster at the "International congress on plant mitochondria biology" in Obernai/France: "*In-vitro* analysis of *cis*- and *trans*-elements for RNA editing in pea and cauliflower mitochondria" Takenaka, M; **Neuwirt, J**; van der Merwe, JA; Verbitskiy, D and Brennicke, A.

2005 Poster at the 3rd Japanese-German joint symposium: "*Cis*- and *trans*-requirements for RNA editing in plant mitochondria." Takenaka, M; **Neuwirt, J**; van der Merwe, JA; Verbitskiy, D and Brennicke, A.

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Erklärung

Hiermit versichere ich, dass ich vorliegende Arbeit selbstständig angefertigt habe und keine anderen Hilfsmittel, als die angegebenen benutzt, sowie wörtlich und inhaltlich übernommene Stellen als solche kenntlich gemacht habe.

Ulm, im Juli 2006

Julia Neuwirt