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Development of a cauliflower *in vitro* editing system and analysis of various *cis*-and-*trans*-factors

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1 Summary

The mechanism(s) behind RNA editing in higher plants is not yet understood.To gain further insight, the work reported in this thesis has improved the current mitochondrial *in vitro* RNA editing system (in mitochondria) and adapted it to another, convenient plant species, cauliflower. This will allow the identification of *trans*-factors, as cauliflower is closely related to the model plant *Arabidopsis thaliana*.

Furthermore, the *cis*-requirements for the second editing site in the *atp9* mRNA construct were analysed and interesting differences were observed between cauliflower and pea. Sequence differences 70 nucleotides upstream of the 2nd editing site influence the processing of this site. This site is only edited in the cauliflower RNA, since the sequence 70 nucleotides upstream in the pea RNA suppresses this activity. The cauliflower sequence seems to contain an 'enhancer' sequence in this region for this editing site, while the pea sequence 70 nucleotides upstream supresses *in vitro* editing.

Since elucidating *trans*-factors responsible for RNA editing in plant mitochondria was one of the main aims of this study, the effect of several potential *trans*-acting factors was tested in the *in vitro* editing system. For example, external RNAs had a positive effect on the editing reaction which however seems to be non-specific. Another set of experiments investigated if a transaminase is involved in RNA editing. An important co-enzyme in transamination reactions is pyrodoxal-5'-phosphat. Which inhibited the *in vitro* editing reaction, whereas an enhancing effect would be expected if a transaminase was involved.

In vitro editing assays in chloroplasts as well as in mitochondria require the addition of NTPs or dNTPs. It was thought that the editing complex needs these nucleotides as an energy source. This study showed,however, that the NTP/dNTP requirement can be replaced by NADH and NADPH, both substrates of the glutamate dehydrogenase (GDH) enzyme. A specific inhibitor of GDH, phosphinothricin has an analogous effect, suggesting that the GDH has an inhibitory effect on RNA editing *in vitro.* This implies that RNA editing in plant mitochondria does not require the addition of an external energy source in the form of NTPs/dNTPs.

In a genetic approach to *identify trans*-factors, I screened 97 different editing sites in three ecotypes of *Arabidopsis,* for differences in RNA. An ecotype specific variance was found in the 1st editing site of the *atp4* construct, which is fully edited in both C24 and Columbia ecotypes, but only partially (50%) in Landsberg *erecta*. Reciprocal crosses and investigation of F2-phenotypes suggests that this recessive trait originates in the nuclear genome.

Zusammenfassung

Das RNA Editing in den Organellen höherer Pflanzen ist bisher noch nicht verstanden. Im Rahmen dieser Arbeit habe ich das mitochondriale *in vitro* Editing System verbessert und für die Spezies Blumenkohl aufgebaut. Blumenkohl ist ein naher Verwandter der Modellpflanze Ackerschmalwand (*Arabidopsis thaliana*), so dass *trans*-Faktoren leichter identifiziert werden können.

Weiterhin habe ich die *cis*-Elemente der zweiten Editing-Stelle der *atp9* mRNA ermittelt und einige interessante Unterschiede zwischen Blumenkohl und Erbse festgestellt. Sequenzunterschiede 40 Nukleotide 5` der ersten Editing-Stelle beeinflussen diese zweite Editing-Stelle. Diese Stelle wird *in vitro* nur in der Blumenkohl RNA editiert, während die Sequenz 70 Nukleotide 5' in der Erbsen RNA die Reaktion unterdrückt. In der Blumenkohlsequenz scheint in dieser Region damit eine 'Verstärkersequenz' zu liegen, in der Erbsensequenz dagegen eine 'Hemmsequenz'.

Da eines der wesentlichen Untersuchungsziele dieser Studie die Identifizierung von *trans*-Faktoren für das RNA Editing in pflanzlichen Mitochondrien war, wurden mehrere mögliche *trans*-agierende Faktoren im *in vitro* Editing-System getestet. Zum einen wurde die Zugabe von externen RNAs getestet. Diese hatten einen positiven Effekt auf die Editingreaktion, der jedoch unspezifisch zu sein scheint. Des weiteren wurde untersucht, ob an der Deaminierung des editierten C ein Enzym aus der Gruppe der Transaminasen beteiligt ist. Ein wichtiges Coenzym solcher Transaminierungsreaktionen ist Pyrodoxal-5`-phosphat. Dieses Coenzym zeigte in der *in vitro* Editingreaktion einen hemmenden Effekt, wohingegen bei Beteiligung einer Transaminase ein fördernder Effekt zu erwarten gewesen wäre.

In vitro Editingassays in Chloroplasten und Mitochondrien erfordern die Zugabe von NTPs oder dNTPs. Bislang wurde angenommen, dass diese Nukleotide als Energiequelle benötigt werden. Die Ergebnisse dieser Arbeit zeigen aber, dass NTP/dNTP durch NADH und NADPH ersetzt werden kann. Einen ähnlichen Effekt wie diese Substrate des Glutamatdehydrogenaseenzyms (GDH) hat auch der spezifische Inhibitor der GDH, Phosphinothricin. Daraus folgt, dass GDH das *in vitro* RNA Editing inhibiert und dass das RNA Editing in pflanzlichen Mitochondrien keine externe Energiequelle benötigt.

In einem genetischen Ansatz zur Identifizierung von *trans*-Faktoren habe ich ferner 97 verschiedene Editingstellen in drei Ökotypen von *Arabidopsis* auf Unterschiede im *in vivo* Editing untersucht. Eine Ökotyp-spezifische Varianz findet sich in der ersten Editingstelle der *atp4* mRNA, die in den C24 und Columbia Ökotypen vollständig, im Landsberg *erecta* (Ler) Ökotyp aber nur teilweise (50%) editiert ist. Reziproke Kreuzungen und Untersuchungen des F2 Phänotyps deuten an, dass diese rezessive Eigenschaft im Genom des Zellkerns kodiert ist.

2 Introduction

2.1 RNA editing: an overview

RNA editing is the process of altering a RNA sequence from that originally encoded by the genome. RNA editing is found in very diverse species of eukaryotes, in mammals, marsupials, trypanosomes, slime molds and plants. RNA editing also occurs in a few retro-viruses such as: measles, Sendai, mumps, parainfluenza and Ebola viruses. RNA editing *per se* is rather extravagant and costly, since without exception all of the RNA editing events described would be rendered unnecessary if the sequence of the mature mRNA was encoded in the genome, as it is in bacteria. RNA editing was observed for the first time in the mitochondrion of trypanosomes, where the insertion and to a lesser extent the deletion of uridine (U) residues takes place in mitochondrial mRNAs (Benne *et al*. 1986, Stuart *et al*. 2005). A few years later it was also discovered in the mitochondria (Covello and Gray 1989; Gualberto *et al*. 1989; Hiesel *et al*. 1989) and plastids (Hoch *et al*. 1991) of plants. Despite the extensive editing of mRNAs in plant and protozoa organelles, only few instances of RNA editing are known in mammalian systems such as apolipoprotein B and the glutamate-gated channel mRNAs (Wedekind *et al*. 2003).

2.2 Methods and effects of RNA editing

The evolutionary distances between the different organisms in which editing has been observed suggests independent origins of the different editing mechanisms. While one level of classification can be based upon such a phylogenetic scheme, another grouping could reflect the mechanisms involved.

RNA editing can occur by either deletions or insertions, or by modification of the single stranded RNA molecule. The insertions are not specified in the gene sequence from which the RNA has been transcribed. In editing processes found in mitochondria of the *Trypanosoma* parasites and of the slime mold *Physarum*, the mature mRNA will thus be longer than the respective primary transcript (Benne *et al*. 1986, Mahendran *et al*. 1991, Arts and Benne 1996, Alfonso *et al*. 1997) In

trypanosomes some mature mRNAs have more nucleotides inserted than are encoded by the gene. In these cases the translated mRNA may thus be more than twice as long as the actual gene in the DNA. Insertional RNA editing also includes the non-templated co-transcriptional insertion of nucleotides in certain viruses, which is specified by the surrounding sequence (Cattaneo 1991). Other borderline cases are the 3' polyA additions which complete UAA translational stop codons and some tRNAs in animal mitochondria.

A second class of RNA editing occurs when the nucleotide is changed from one entity to another, the two most prevalent changes inducing deaminations of C to U and A to I (Fig2.1). Less widely distributed changes are U to C, G to A and U to A conversions, the latter being found in mammals such as mice and humans.

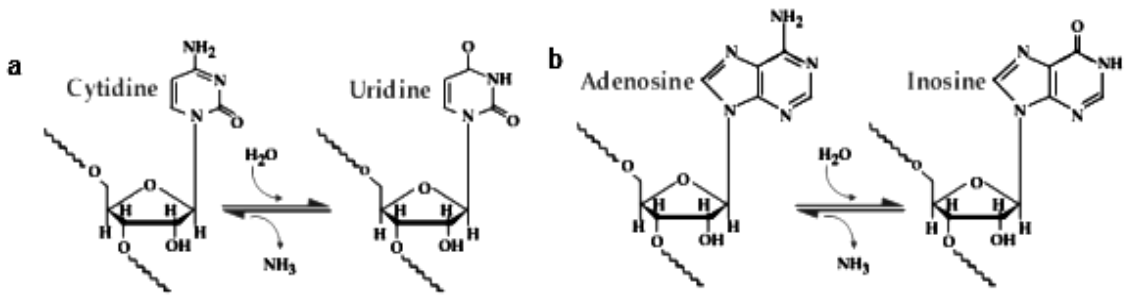

Figure 2.1 The deamination reactions from (a) cytidine to uridine and from (b) adenosine to inosine

The question thus arises, whether there is a hidden biological significance of RNA editing. Such an agenda might include a regulatory control of gene expression at the post-transcriptional level, for example, the introduction of an AUG translational start codon that could rapidly provide an mRNA for translation, without requiring the complete *de novo* synthesis of the transcript. Another possible advantage of RNA editing could be the potential of synthesizing two or more distinct products from a single gene as exemplified by the viral editing systems or in the tissue-specific apoB editing, where two lipid carrier proteins with different properties are derived from one and the same genomic coding region (Greeve *et al*. 1991, Mehta and Driscoll 1998). The adenosine (A) to inosine (I) editing in the GluR receptor channel mRNAs results in increasing the family of variant

proteins available for the assembly of these ion channels and allows fine-tuning of the chemo-electric potential transmission properties (Lomeli *et al*. 1994, Feng *et al*. 2005).

In plant mitochondria, partially edited mRNAs are found in polysomal fractions of and are apparently translated into a family of variant proteins (Mulligan 2004). However, only one type of protein sequence appears to be incorporated into the polypeptide complexes of the respiratory chain. These integrated polypeptide sequences generally correspond to the protein specified by the fully edited mRNA (excluding the sporadic editing events) and not to any of the intermediates (Phreaner *et al*. 1996, Mulligan 2004;). The polypeptide sequences predicted from the fully edited mRNA are better conserved with the homologs in other organisms than the proteins encoded by the genomic sequences or by any of the editing intermediates and may thus have been selected for their conserved physiological and biochemical functionality. Indeed it is unlikely that polypeptides synthesized from unedited RNAs would function properly, such proteins will rather impair the efficiency of mitochondrial respiration. This has been investigated by importing an ATP9 protein synthesized from an (unedited) genomic mitochondrial sequence inserted into the nuclear genome of tobacco. This protein indeed disturbs mitochondrial function, presumably by competition with the 'correct' endogenous native mitochondrially encoded proteins. The resulting dysfunction manifests as problems in pollen development and leads to male sterility (Hernould *et al*. 1993, Zabaleta *et al*. 1996).

Even if the coding capacity of a gene could be increased by using partially edited mRNAs as templates to produce protein variants without increasing the number of genes in plant mitochondria, this presumed advantage may be illusionary as additional polypeptides are required for the editing process. RNA editing thus appears to be a biologically selfish process, which has become established (by chance) and now has to be perpetuated.

On the other hand, the mitochondrial genome may have welcomed the establishment of RNA editing as a means to protect its genome and genes against transfer to the nucleus. Since there is by all appearances no editing in the nucleus/cytoplasm of plants, a mitochondrial gene requiring editing could not immediately become functional upon transfer to the nuclear genome. A

successful transfer would require the respective coding region to be reverse transcribed from a fully edited mitochondrial mRNA molecule. The resulting cDNA sequence could then code for the correct protein sequence once inserted and activated in the nuclear genome.

2.3 RNA editing in trypanosomes

Most trypanosomatid mitochondrial mRNAs undergo RNA editing by which precursor mRNA (pre-mRNA) sequences are changed, often extensively, by the insertion and less frequently the deletion of uridine nucleotides (Us). The edited mRNAs are translated into components of the oxidative phosphorylation system including subunits of the respiratory complexes. Trypanosomes have evolved a complex system to ensure the fidelity of the number of nucleotides which have to be inserted as well as the precise location of the editing events in the transcript.

In trypanosomatid RNA editing, the location of the correct insertion site and the precise number of inserted Us are specified by the so-called 'guide RNAs'. These are small RNA molecules, which contain a short anti-sense sequence which can base-pair with the unedited primary transcript downstream of a site where uridines are to be inserted (Fig. 2.2). As a result of such base-pairing the guide RNA is anchored to the transcript, the anchor forming a perfect match of 10-15 nucleotides between guide RNA and transcript RNA. This double stranded region and the specific structure of the guide RNA are enveloped in a large multi-protein complex, the 'editosome', which performs the actual editing. This editosome contains several enzymatic activities, which begin to open the transcript RNA chain at the first mismatched nucleotide and start inserting uridines. These will base-pair with the next nucleotides in the guide RNA and U insertion in the transcript will continue as long as A (or G) in the guide RNA is matched. Insertion of U into the transcript RNA molecule will stop when C or U is encountered in the guide, which can base-pair with the next original nucleotide in the transcript RNA.

This mechanism requires certain properties of the editing process. Firstly, insertion or deletion of Us progresses from the 3' to the 5' end of the primary RNA molecule. Secondly, a single guide RNA will not cover all of the editing sites in an extensively altered primary transcript and several

guide molecules will be required. Thirdly, in frequently edited transcripts an anchor site for a guide RNA in the 5' region of the transcript will sometimes only be created by the downstream editing events, thus resulting in a chain of guiding alignment events proceeding in the 3' to 5' direction along the primary transcript eventually generating the mature mRNA.

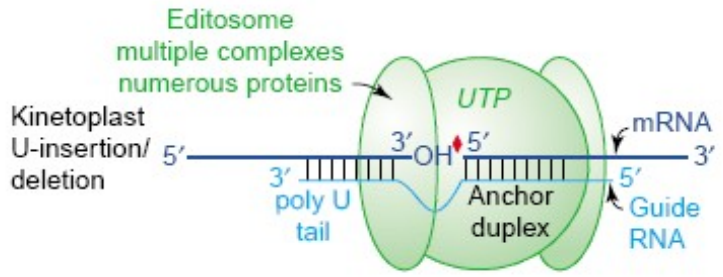

Figure 2.2 The editing process in Trypanosomes (Wedekind *et al*. 2003)

2.4 RNA editing in animals

RNA editing in mammals is a rare event that predominately occurs in nuclear-encoded mRNAs and differs from the editing discussed above in Trypanosomes.

The single C to U editing in the apolipoprotein B (apoB) mRNA generates a stop codon, which results in the translation of a truncated protein (Powell *et al*. 1987; Chen *et al*. 1987). This RNA editing thus results in the expression of two different proteins, apoB48 and apoB100, from one single gene and is regulated tissue-specifically. Mammalian small intestine edits almost all of the apoB mRNAs resulting in the truncated apoB48 protein. In some mammals e.g. mice, apoB mRNA is also edited in the liver, but in humans and most other species, the mRNA is not edited and the full length apoB100 is produced in this organ (Greeve *et al*. 1991; Nakamuta *et al*. 1996).

The protein APOBEC-1 (apoB editing catalytic subunit 1) is responsible for editing apoB mRNA (Teng *et al*. 1993). This enzyme contains a zinc-dependent deaminase domain that is conserved among cytidine deaminases (Mian *et al*. 1998) and is essential for cytidine deamination.

Site-specific editing requires APOBEC-1 homodimerization (Oka *et al*. 1997) and an interaction with at least one of two other RNA-binding proteins. These 65-kDa and 64-kDa proteins known as

ACF65 (APOBEC-1 complementation factor) and ACF64, respectively (Fig. 2.3) bind with high affinity (Dance *et al*. 2002; Mehta and Driscoll 2002) to an RNA sequence element known as the mooring sequence located 3' of the edited cytidine (Smith 1993), and together with APOBEC-1, form the minimal editosome.

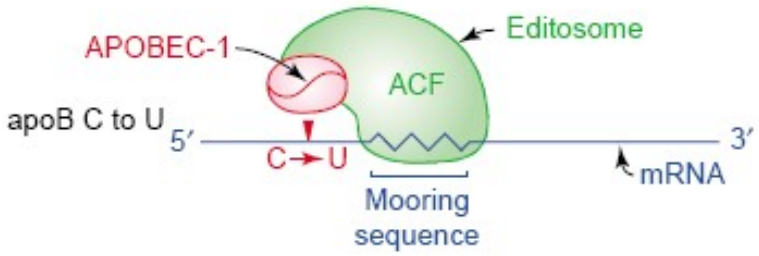

Figure 2.3 The editing process involving the apobec-1 editosome (Wedekind *et al*. 2003)

2.5 RNA editing in plants: plastids and mitochondria

RNA editing sites in flowering plant mitochondria are almost exclusively C to U changes, in which the C in the pre-mRNA is deaminated to a U moiety (Fig. 2.1). The more than 400 RNA editing sites are mostly found in coding regions of mRNAs and occur less frequently in introns and other non-translated regions. In some cases RNA editing in tRNA molecules restores essential base-pairings (Bonnard *et al.* 1992). In these instances only complete RNA editing allows correct folding and further maturation and processing of the tRNA precursors. For as yet unclear reasons the ribosomal RNAs in plant organelles appear to undergo little if any, RNA editing. In flowering plant species U to C conversions are very rare events. In *Arabidopsis thaliana*, *Brassica napus* and *Oryza sativa* none have been identified (Giegé and Brennicke 1999; Handa 2003; Notsu *et al*. 2002), while only one each have been reported in the mitochondrial RNAs of the spermatophyte species wheat and *Oenothera* (Gualberto *et al*. 1990; Schuster *et al*. 1990). In non-flowering plants the reverse reaction, the amination of U to C, is much more common and can reach the level of the C to U editing events, a prime example being the hornworts (Malek *et al*. 1996; Knoop 2004). The only land plant in which no editing has been observed at all is the liverwort *Marchantia polymorpha* (Oda *et al*. 1992) and closely related species of the Marchantiales (Malek *et al*. 1996).

Green algae, including the closest relatives to the land plants of the order of the Charales, do not show any RNA editing (Turmel *et al*. 2003). A possible date of when RNA editing in plant mitochondria was established is thus in the early evolution of land plants, since the Marchantiales are now considered to branch near the very root of the land plant tree (Malek *et al*. 1996; Knoop 2004). Intriguing and with far-reaching consequences for the possible biochemical reactions is the observation of frequent U to C aminations among the mosses and ferns, for example in hornwort species and in the lycopodiopsida (Knoop 2004; Groth-Malonek and Knoop 2005). From previously characterized cytidine deaminases and cytidine synthases it is difficult to envisage a single enzyme performing both reactions. The asymmetric distribution of editing sites between coding and non-coding regions in RNAs suggests that there has to be some selective pressure on maintaining an editing site, which will otherwise be lost. The necessity to produce functional proteins with certain amino acid sequences may contribute to this selective pressure in open reading frames, where an U at a given editing position may be required for a specific codon identity. In introns RNA editing is in some cases a prerequisite for proper folding of essential secondary structures and thus efficient intron excision (Carillo and Bonen 1997), suggesting that the requirement of a functional protein product exercises evolutionary pressure on this editing event to occur.

The present-day requirement for RNA editing has most likely evolved after the establishment of the biochemical potential to post-transcriptionally modify certain nucleotide positions. This scenario was first clearly outlined in the review by Covello and Gray (1993), in which the authors suggest that as a first step a deaminating enzyme mutated to also accept polynucleotide templates and that from there on the DNA was free to mutate in certain positions. Once one such mutation had occurred which required a compensating correction in the RNA, RNA editing had become established and needed to be functionally maintained. This scenario was aptly named the bureaucracy model (Mulligan 2004), in analogy to the various processes in administrations which introduce often unnecessary complexities with the sole aim of making themselves indispensable.

Experiments with *in vitro* and *in organello* assays following the fate of the sugar-phosphate backbone as well as the nucleotide base have shown that both are retained in the editing process (Rajasekhar and Mulligan 1993; Yu and Schuster 1995). These observations exclude nucleotide excision and base exchange as possible reactions and leave deamination or transamination to result in the C to U change seen in flowering plant mitochondria.

Transamination reactions are common in the various pathways of amino acid biosynthesis, where they require a limited number of compounds as amino group acceptors. Even though the role of a transaminase could not be excluded, it does seem highly unlikely considering the results obtained from several *in vitro* experiments. The transamination reaction requires amino group acceptors for example alpha-ketoglutarate or oxaloacetate both of which are small molecules, likely to be dialysed out of the lysate used in most of the *in vitro* reactions. These molecules have also been added to the *in vitro* reaction without any noticeable effects (M.Takenaka unpublished).

This leaves a modified cytidine deaminase as the most likely candidate for the enzyme responsible for editing. In the fully sequenced nuclear genome of *Arabidpsis thaliana* a family of 9 such genes has been described. At least one of these isoforms is targeted to the mitochondria (Faivre-Nitschke *et al*. 1999). This enzyme could be involved in the RNA editing reaction, while others have to perform the classic role of cytidine deaminase in the nucleotide biosynthesis pathway from cytidine towards uridine.

However, there are two arguments against the participation of such a straightforward cytidine deaminase. The first is based on experimental evidence, which suggests that no accessible zinc ions are required for the reaction. While all classic cytidine deaminases contain reaction-centre bound zinc and can be inactivated by specific zinc chelators such as 1,10 o-phenanthroline, the *in vitro* RNA editing reaction in plant mitochondrial extracts is not inhibited by addition of this compound (Takenaka and Brennicke 2003) . The bacterial cytidine deaminases as well as the apo-B cytidine deaminase are both completely blocked by addition of this chelator (Carter 1998). Similarly, in chloroplast *in vitro* assays the zinc-chelator has some detrimental effect on the editing reaction (Hegemann *et al*. 2005). The unaffected plant mitochondrial enzyme must have evolved

along a different track to protect or to not require the central zinc ion. Alternatively a completely different deaminating enzyme with an entirely distinct evolutionary origin is involved.

The second point against the involvement of such cytidine deaminases derives from the 'reverse' editing events of U to C frequently observed in ferns and mosses. Cytidine deaminases are from energetic considerations unlikely to be able to catalyse the reverse reaction of adding an amino group to a U residue to generate a C. Of course, a second unrelated enzyme could be responsible for these reverse reactions, the most parsimonious solution would however be a single enzyme system able to perform both reactions. In the hornwort and related plant species this would be a variant protein, which is more prone to catalyse the U to C reaction, while the distinctly evolved enzyme in flowering plants is less suited to facilitate this reaction.

2.6 Aims of this study

This study aims to improve the understanding we currently have concerning RNA editing in plant mitochondria. At the start of this work very little was known about the recognition of specific editing sites. It was unclear how a site is specified by the surrounding sequence and how many nucleotides are required for successful editing. It was clear that there is no simple common motif discernible that could distinguish a C to be edited from a C not to be edited.

The establishment of an *in vitro* RNA editing assay (Takenaka and Brennicke 2003), had shown that 173 nucleotides upstream (5'), and 49 nucleotides downstream (3') are sufficient for successful editing of the *pea* atp9 mRNA *in vitro*. Subsequent studies by Takenaka *et* al. (2004) reduced the requirements to 40 nucleotides upstream (5') and determined that, for this site at least, no downstream sequence was necessary for site recognition.

The above mentioned *in vitro* reaction had a sensitivity of 1-2% and one of my aims was to optimise the system, and maybe to develop it for other plant systems as well. I also set out to identify more editing sites and to characterise their *cis*-and *trans* factors. The main goal of the study was, however, to identify the specific *trans*-factors that are responsible for editing in plant mitochondria.

3 Materials and Methods

Materials

3.1 Plant material

Pea seedlings (*Pisum sativum* L.) were grown at 25 °C in the dark for 6 days. The etiolated shoots were harvested and mitochondria extracted as discussed below. Cauliflower heads (*Brassica oleracea* var. botrytis) were purchased at local markets and were left in water overnight at 4 °C to remove unwanted bugs and to adjust the temperature. The inflorescences were used in the subsequent mitochondrial extraction. Thale cress (*Arabidopsis thaliana*), hereafter referred to as *Arabidopsis* for clarity, cell suspension cultures were maintained throughout the study, the cells were used on the fourth day after, having been sub cultured. Three ecotypes (*Landsberg erecta,C24* and *Columbia*) were grown on soil at 21 °C and 61% humidity under long day conditions (16 h light, 8 h darkness).

3.2 Consumables

Commercially available chemicals from the companies, Duchefa Biochemie B.V. (Haarlem, Netherlands), 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) and MP Biomedicals GmbH (Eschwege, Germany), were used in subsequent analyses. All buffers were prepared using doubly distilled water.

3.3 Enzymes

The enzymes used 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).

3.4 Oligonucleotides

The oligonucleotides used during the subsequent studies were supplied by the following companies: Biomers (Ulm, Germany) and Invitrogen GmbH (Karlsruhe, Germany). Sequences are listed in Appendix 1.

3.5 Devices

The equipment used is common for a Molecular Biological laboratory. The PCR was performed using a GeneAmp® PCR System 9700 from Applied Biosystems. Sequencing reactions were performed using an ALF express-Sequencer from Amersham Bioscience (GE).

General Methods

3.6 Standard methods in molecular biology

All molecular biology methods, e.g. extraction of nucleic acids, enzymatic treatment of nucleic acids and electrophoresis, were performed according to the standard protocols commonly available (Sambrook & Russell, 2001). Standard PCR reactions were performed using 10 µM of each primer in a 50 µl reaction. Buffer and Taq Polymerase BioTherm were provided by Genecraft GmbH (Lüdinghausen, Germany). *Escherichia coli* (*E. 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-deoxy sequencing the T7 polymerase kit form Amersham Pharmacia (Freiburg) was used. cDNA-synthesis was performed using the Stratascript reverse transcriptase kit from Stratagene (Heidelberg) according to the protocol. The recovery of plasmid DNA and purifying fragments from agarose gels were performed using the GFX^{TM} PCR and gel extraction purification kit from Amersham.

3.7 The extraction of plant mitochondria

Mitochondria were isolated from pea shoots using the following method: The etiolated shoots were harvested and placed into a kitchen blender (Waring) with 2-3 times (fresh weight/volume) extraction buffer [0.3 M D-mannitol, 30 mM MOPS, 1 mM EGTA, adjust pH to 7.8 with KOH; 0.1 % BSA* (w/v), 2 mM dithiothreitol (DTT)*]. Several bursts at high speed for about 10 seconds each, chopped the shoots into small fragments and lysed most of the cells, this mixture was pressed through four layers of cheese, and one layer of Mira cloth. This was followed by several consecutive differential centrifugation steps of: 10 min at 600 x g, 10 min at 1,300 x g and 10 min at 2,100 x g. After each step the supernatants were transferred into a new centrifugation vessel. The mitochondria were pelleted at 7,000 x g for 30 min and the pellet was re suspended in no more than 6 ml of wash buffer [0.25 M sucrose, 20 mM Tris-HCl, 5 mM EDTA, 15 % glycerin (v/v), adjust pH to 8.0 with KOH; 1 mM DTT*] the suspension was layered on top of preformed Percoll (GE Healthcare) gradients in steps of 13,21,and 45 % Percoll in a 2 X gradient buffer [0.5 M sucrose, 20 mM MOPS, 2 mM EGTA, adjust pH to 7.2 with KOH; 0.4 % BSA*, 4 mM DTT*]. After centrifugation at 70,000 x g in a swing-out rotor for 45-50 minutes the mitochondria were removed from the border between the 21 and 45 % Percoll layers using a pipette. The creamy yellowish interface was diluted to a volume of about 400 ml in wash buffer. The mitochondria were again pelleted by centrifugation at 9,000 x g for 30 min. The mitochondria were then transferred to an Eppendorf tube, pelleted at 12,500 rpm in a standard table top centrifuge for 20 min after which the buffer was removed. After the final centrifugation step the mitochondrial pellet was weighed and quick-frozen in liquid nitrogen and stored at –80 °C.

Mitochondria from cauliflower inflorescences were isolated using the same method as described above, but using different buffers as well as a juice extractor in stead of a blender. The extraction buffer was diluted using the juice to a final concentration of: [0.3 M mannitol, 30 mM sodium pyrophosphate ($Na_4P_2O_7$), 2 mM EDTA, adjust pH to 7.5 with HCl; 0.8 % PVP 25* (w/v), 0.3 % BSA*, 3 mM cysteine*, 5 mM glycine*, 2 mM ß-mercaptoethanol*]. The wash buffer used for

cauliflower contained: 0.3 M mannitol, 10 mM K_2PO_4, 1 mM EDTA, 0.1 % BSA*, 5 mM cysteine*, 15 mM glycine* pH 7.5 whereas the 2 X gradient buffer consisted of: 0.6 M Sacharose, 20 mM K_2PO_4, 2 mM EDTA and 1% BSA*.

* Ingredients added just before use, all buffers were used cold (4 °C).

3.8 Preparation of mitochondrial extracts

Four hundred mg of purified 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(v/v). After incubating the lysate on ice for 30 min, with occasional mixing, the lysate was centrifuged at 60,000 x g for 20 min. The supernatant was recovered and dialysed for 1 h against 400 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), this was repeated 5 times. All steps were carried out at 4 °C. The resulting extract (10-20 µg protein/µl) was flash frozen in liquid nitrogen and stored at -80 °C. Stored under these conditions the lysate was stable for at least 3 months.

3.9 RNA substrates

The genes of interest were cloned into the multiple cloning site of the adapted pBluescript SK^+ (pvec) vector, flanked by the T7 (upstream) and T3 (downstream) primer binding sites. The vector was treated with restriction enzymes (PvuII or VspI) and purified. Run off transcription was performed using T7 RNA polymerase (Fermentas). The synthesized RNA molecule contains the editing sites of interest, flanked by bacterial sequences to allow specific amplification from these bacterial sequences against the background of internal mRNAs.

3.10 *In-vitro* RNA editing reactions

The *in-vitro* RNA editing reactions 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 aM (100 x 10^{-18} mol) mRNA substrate, and 6 µl mitochondrial extract. After incubation at 28 °C for 4 h, the substrate mRNA was extracted using the Nucleospin RNA extraction kit (Machery-Nagel). Variations of individual concentrations and additions of various other compounds are indicated in the respective figure legends.

3.11 Detection of the RNA editing activity by mismatch analysis

The cDNA was synthesized from the substrate mRNA using the reverse transcriptase enzyme (Stratagene) from the T3 primer site. The subsequent PCR reactions were performed with 0.1 units of taq polemerase (GeneCraft) using 10 mM of a Cy5 labelled 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 re-annealing 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 1 h. After reannealing, the resulting heteroduplexes were treated with 0.2 units of the enzyme TDG (thymine DNA glycosylase (Trevigen). The TDG-treated fragments were denatured for 5 min at 95 °C in alkali buffer (300 mM NaOH, 90% formamide and 0.2% bromophenol blue), and the single stranded DNA was separated on a 6 M urea 6% PAGE. The Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (Amersham Biosciences). 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. When detecting two or more editing sites in one template, inherent experimental detection shifts have to be considered. 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 (Fig 3.1).

UV cross linking of labelled template to RNA binding Proteins

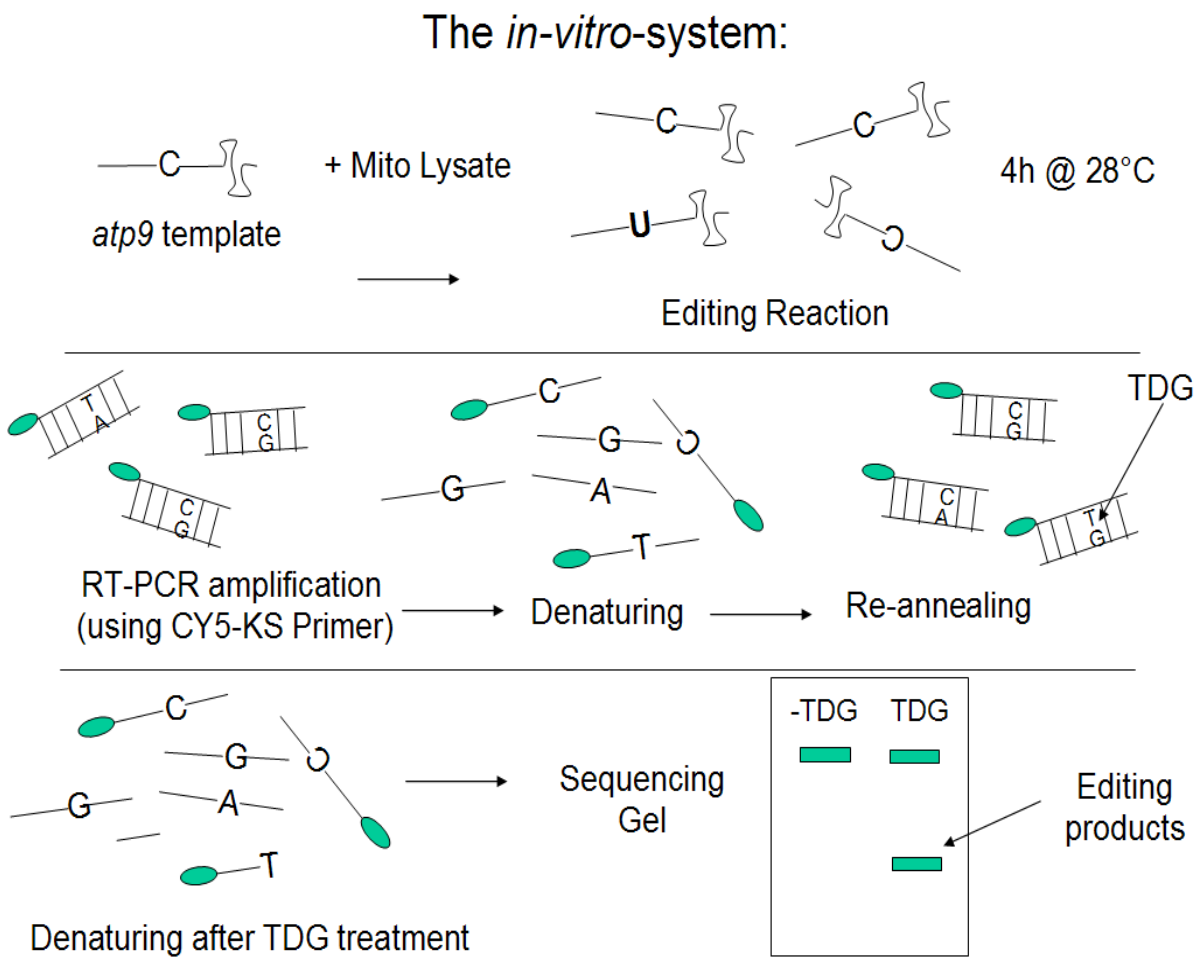

Figure 3.1 A schematic overview of the *in vitro* reaction illustrating the mismatch analysis.

The *atp9* RNA template was radioactively labelled using ^{32}P labelled C dNTPs. The method used was a modified method implemented by Hirose & Sugiara (2001). The labelled template was incubated in the mitochondrial lysate for 30 min at 28 °C. The reaction mixture was irradiated with UV light (254 nM, 1,8 $J.cm^{-2}$) using an UV Strata linker 1800 (Stratagene). The reaction was followed by a RNase A treatment at 37 °C for 30 min, after which the proteins (bound to the radio labelled template) were precipitated by 50% acetone. The proteins were then dissolved in SDS-PAGE loading buffer and separated on a 12% polyacrylamide gel containing 0.1% SDS.

3.12 Isolating specific RNA binding proteins using glass beads

The isolation of specific RNA binding proteins was performed by modifying the method developed by Liu & Sun (2005) to suit our templates and *in vitro* system. Common glass powder (150-212 µM, Sigma) was cleaned thoroughly and dried. The washed beads were treated using a 1:1 (v/v) solution of 3'-aminopropyltriethoxysilane (Sigma) in acetone for 30 min, after which the treated beads were washed with acetone and dried at 50 °C.

The empty pVec vector was digested using the SmaI restriction enzyme. This enzyme was chosen as the insert of interest was cloned into this restriction site (Fig 3.2a). The linear vector was treated with calf intestine alkaline phosphatase (CIAP) to remove the 5' phosphate groups. The linear vector was digested using a second restriction enzyme NdeI. The newly created 76 bp fragment, containing a 5' phosphate group, was separated on a 2% agarose gel and purified using the freeze and squeeze method and eluted in 100 µl of elution buffer (0.1 M MES pH 6.0 and 0.5 M NaCl). The eluted fragment was treated (activated) with 0.4 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Sigma) and 1 mg of N-hydroxysuccinimide (Fluka), for 30 min at room temperature. The reaction was stopped by adding 2 µL of ß-mercaptoethanol. The DNA was precipitated, washed and redissolved in elution buffer.

The activated DNA was mixed with 0.5 g of aminosilanized glass beads and allowed to react for 2 h at room temperature with gentle shaking. The beads were washed thoroughly with TE buffer.

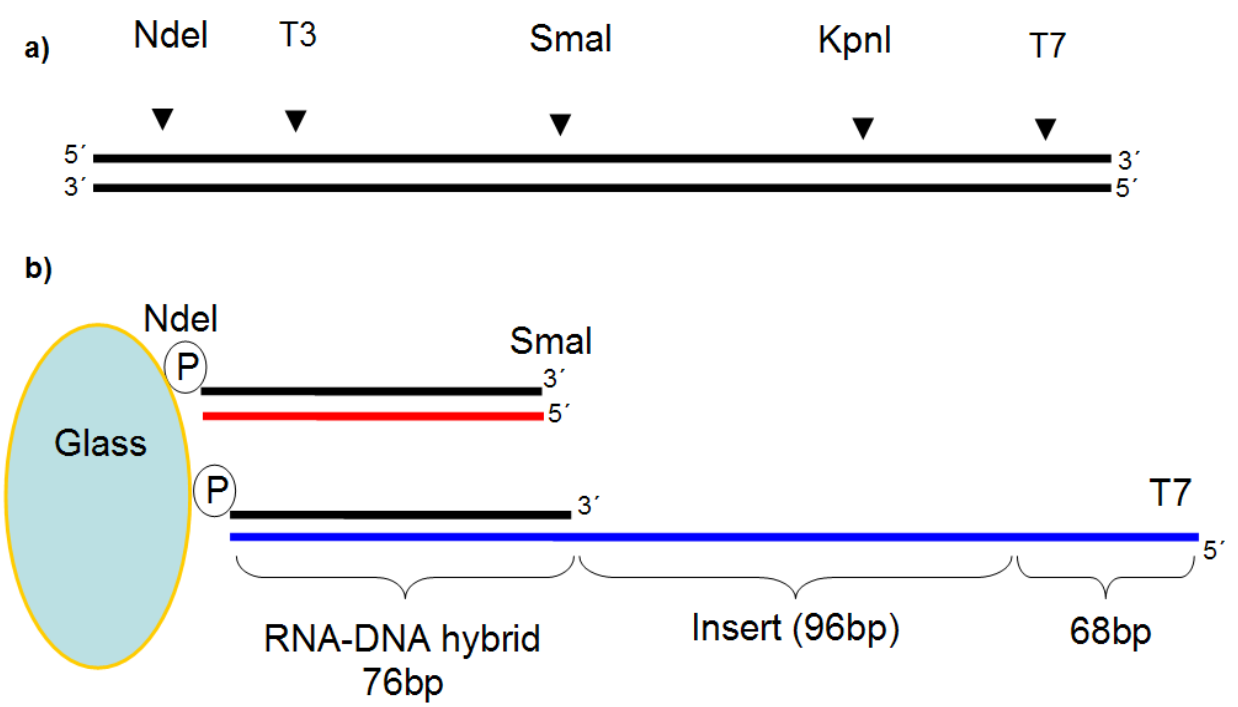

Figure 3.2 Schematic overview of the method using glass beads to recover RNA binding proteins. a: Positions of the different restriction and promoter sites in the empty vector. b: Graphic representation of the method.

The RNA templates were constructed as discussed above, using the NdeI restriction enzyme, precipitated and redissolved in annealing buffer (90 mM NaCl, 2 mM EDTA, 10 mM HEPES, pH 7.8). After the run off transcription a complementary sequence remains that is attached to the glass beads. The template will form a DNA-RNA hybrid with the DNA 'arm' attached to the glass bead (Fig 3.2b).

The RNA template was incubated with 0.5 g glass beads at 70 °C for 10 min and then cooled slowly to 37 °C and incubated for 2 h with light shaking. After the annealing step the mixture was cooled further to 4 °C and washed 3 times using annealing buffer. The washed beads were stored at 4 °C or frozen and kept at -20 °C. The annealing efficiency was checked by repeating the experiment with radioactively labelled RNA and monitoring the amount of radioactivity remaining on the glass beads after the washing steps. It was found that approximately 80% of the RNA template attaches to the glass beads.

4 Results

4.1 Screening for *cis*-elements *in silico*

The fact that there are so many editing sites (441) in *Arabidopsis* mitochondria raises the possibility that some of these editing sites share *cis/trans* elements. A database was created, with the help of Dr. Neuwirt, containing the sequences of all known mitochondrial editing sites in *Arabidopsis,* 100 bases upstream (5') and 20 bases down stream (3') from the edited C. This database was run through a programme (developed by Mr. Reimer) to detect any similarities in sequences between the different editing sites. The results of this screen yielded surprisingly few 'matches'. Recent algorithms and RNA editing prediction programmes have been very successful in predicting editing sites (Mower 2005). These programmes focus on the translated protein sequences rather than primary RNA sequences, which was not possible at the time we conducted our screens, as the amount of comparable protein data was limited to one or two species at the most.

4.2 Optimising the pea *in vitro* system

The *in vitro* editing system developed by Dr. Mizuki Takenaka has a detection efficiency of between 1 and 2% (Takenaka and Brennicke 2003). The fact that the system was developed using pea, also complicated the search for genes and proteins, as the pea mitochondrial genome has not been sequenced yet. These and other difficulties made us explore other possibilities concerning the *in vitro* system. The obvious candidate was *Arabidopsis thaliana* seeing that all three genomes have already been sequenced and that it has been used as a model organism for the past 20 years. Using the above mentioned mitochondrial extraction methods (with some modifications) mitochondria were extracted from *Arabidopsis* cell suspension cultures. The resulting lysate was used in the *in vitro* system. Unfortunately the system did not show any activity using the *Arabidopsis* lysate.

Other alternatives were explored including the monocot *Oryza sativa* (rice) and the dicot cauliflower. The rice experiments were performed by Dr. Julia Neuwirt and like the *Arabidopsis* experiments the lysate was not active in the *in vitro* system. Only the cauliflower mitochondrial lysate showed *in vitro* editing activity. Working with cauliflower has many advantages, the main one being that it belongs to the Brassicaceae family and is therefore closely related *to Arabidopsis.* Other advantages include the availability of material and the low amounts of contaminating chloroplasts found in the white inflorescences.

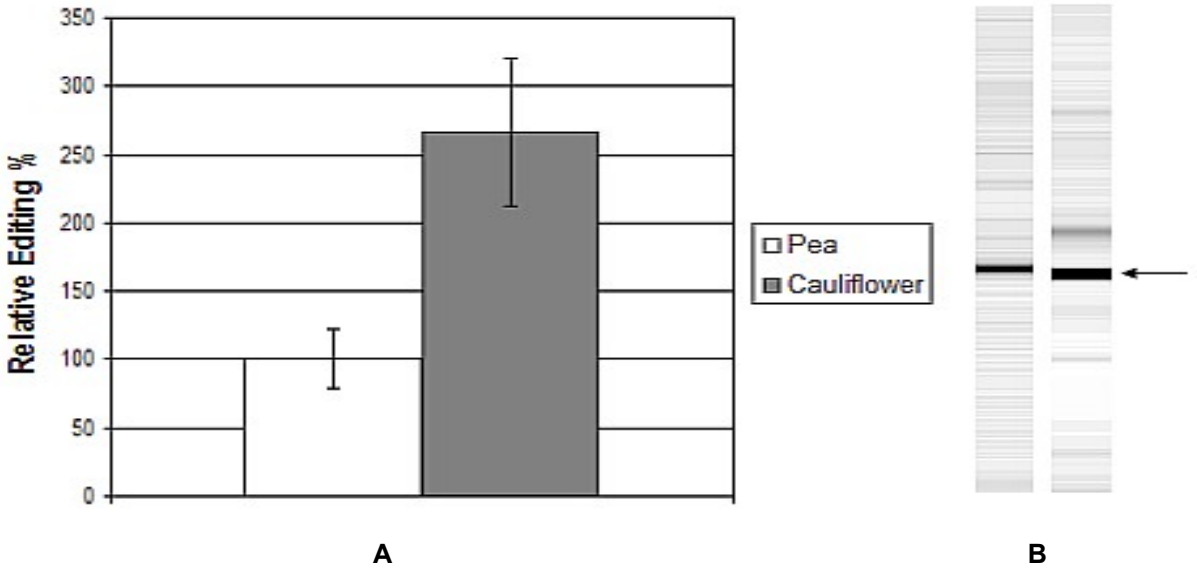

Figure 4.1 The relative percentage of *in vitro* RNA editing with either the pea or the cauliflower lysate (A). In figure B the differences can be seen on the sequencing gel with the arrow indicating the editing 'event'. The left lane is the reaction with the pea lysate, the right lane with cauliflower.

Using a mitochondrial lysate extracted from cauliflower inflorescences the efficiency of the *in vitro* reaction increased by more than 2 fold (Fig 4.1A). This could be due to the higher protein concentration measured in the cauliflower lysate (Fig 4.2A). The relationship between editing efficiency and protein concentration does not seem to be linear, as the editing efficiency increases by roughly two and a half times in the cauliflower lysate, whilst the protein concentration is roughly 7 fold that of the pea lysate.

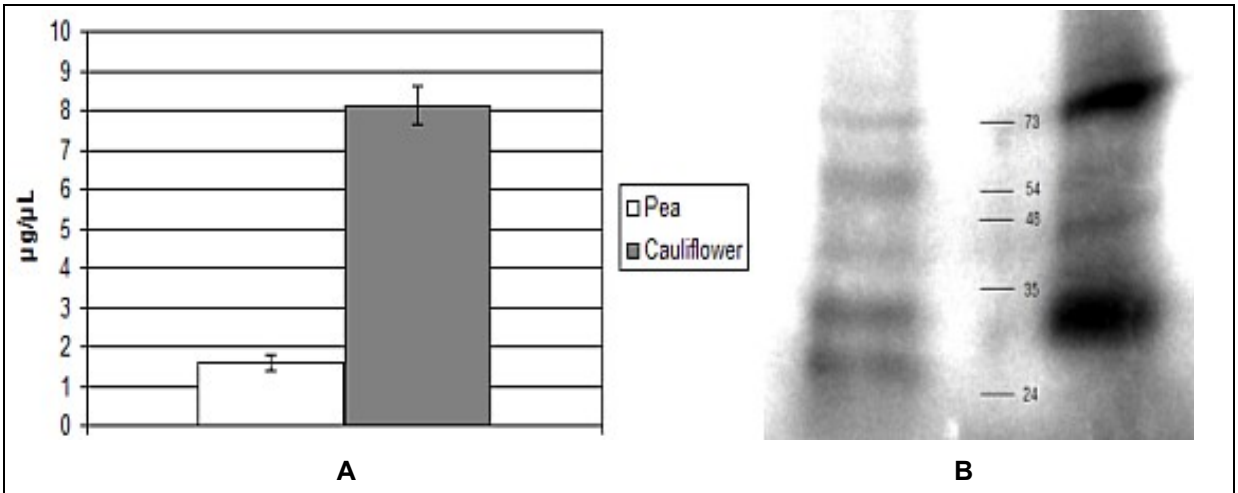

Figure 4.2 The difference in protein concentration is roughly 7 fold in the cauliflower lysate (A). This was verified after UV-cross link experiments (B). A ^{32}P labelled *atp9* template was incubated with pea (left lane) and cauliflower (right lane) lysates and then cross linked using UV light. After a RNase treatment and acetone precipitation the proteins were loaded on a 12% SDS-PAGE. The marker depicting molecular weight in KDa.

4.3 Characterising *cis*-elements in the 2nd editing site of *atp9*

In most flowering plant *atp9* mRNA constructs, the first editing site in the open reading frame is located 19 nucleotides downstream from the AUG start codon. As depicted in Fig 4.3A this site is followed by a second editing event 30 nucleotides further downstream in both cauliflower and pea. 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 (Takenaka *et al.* 2004), 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. 4.3B).

Interestingly the editing efficiency of the 2nd site differs between the cauliflower and pea templates. Whereas editing of the 2nd site is observed almost every time using the cauliflower template, it is only reliably observed about one-third of the time, and then only at an extremely low level, with the pea template. This lead us to investigate the upstream sequences especially in the 5' UTR region.

As shown in Fig 4.3A, there is a difference of 11 nucleotides in the 5' UTR region upstream from the AUG initiation codon.

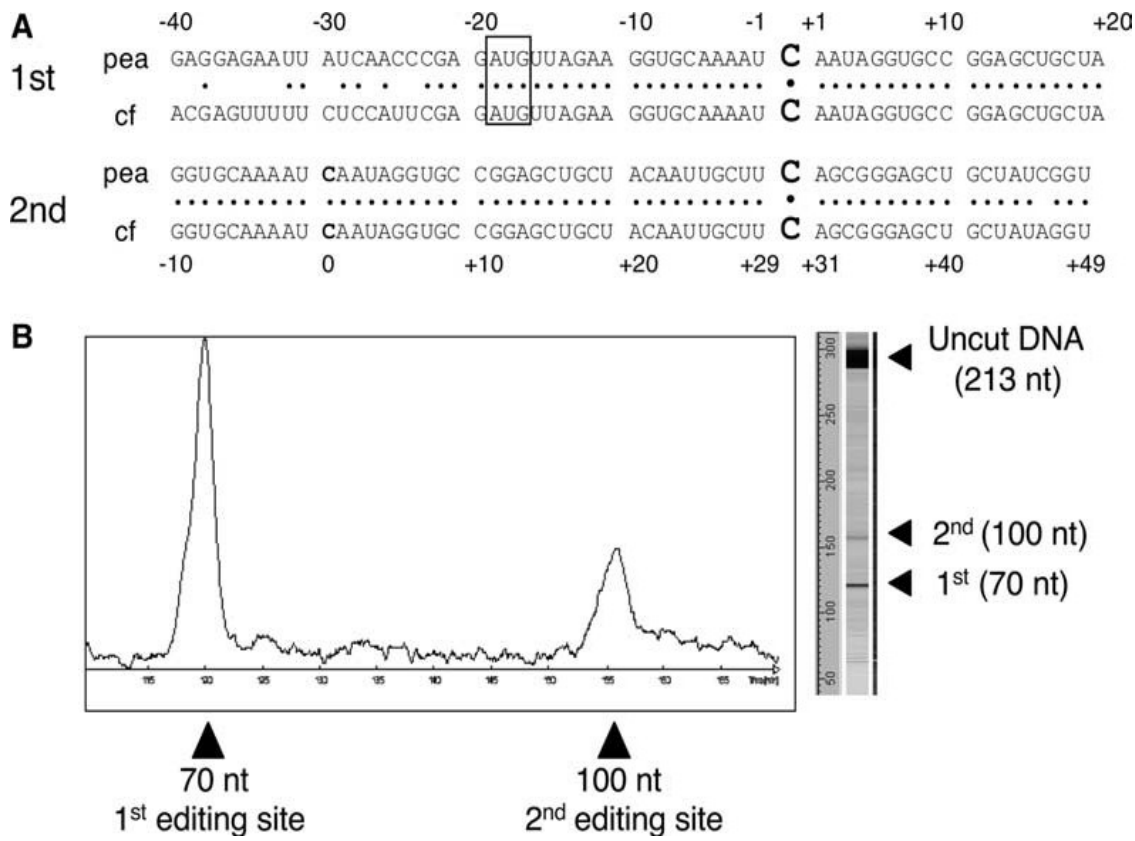

Figure 4.3 Sequence alignment between pea and cauliflower templates (A), shows no sequence similarity between the 1st and 2nd sites. The edited C is shown by large bold letters, the upstream editing site (1st) of the top line pair is shown again in bold at the 0 position of the alignment of the downstream (2nd) site. The AUG codon is framed. (B) A sample gel tracing of a cauliflower template RNA containing both editing sites. The 2nd site is usually detected at about 20-25% of the efficiency of the 1st site. The respective gel image of this tracing is shown on the right. Numbering at the left of the gel and underneath the scan give the respective running time in minutes. Sizes of the RT-PCR fragments are given in nucleotides for the uncut and the editing site specific products respectively.

Surprisingly the *in vitro* editing of the 2nd site is greatly diminished by the deletion of a distant part of the 1st site recognition region. The deletion of the sequence between -40 and -20 relative to the 1st site, results in a drop of the editing efficiency of the 2nd site by as much as 75% (Fig 4.4). A series of further deletion clones successively shortened in steps by 10 nucleotides from the 5' end

up to the 1st site, has no further effect upon editing at the 2nd site. The 2nd site requires a minimum of 30 nucleotides (or less) upstream to maintain *in vitro* editing at a constant, albeit low level.

This result suggests that the pea sequence contains a suppressor sequence, acting on the 2nd editing site, upstream of the recognition sequence of the 1st site. This observation is interesting as the recognition sites of the 1st editing site can be found in the 40 base pairs upstream of this editing site. This extent of *cis*-specificity determinants is supported by this experiment, which shows that the 30 base pairs upstream are essential for the editing of the 2nd site, but the fact that nucleotides 70 base pairs upstream in the cauliflower template has such an enhancing effect is unheard of in plant mitochondria (van der Merwe *et al.* 2006).

To further clarify this peculiar behaviour, competition experiments were performed using a different set of competitors (Fig 4.5A). This work was done in cooperation with Dr. Neuwirt and the results were compared for consistency.

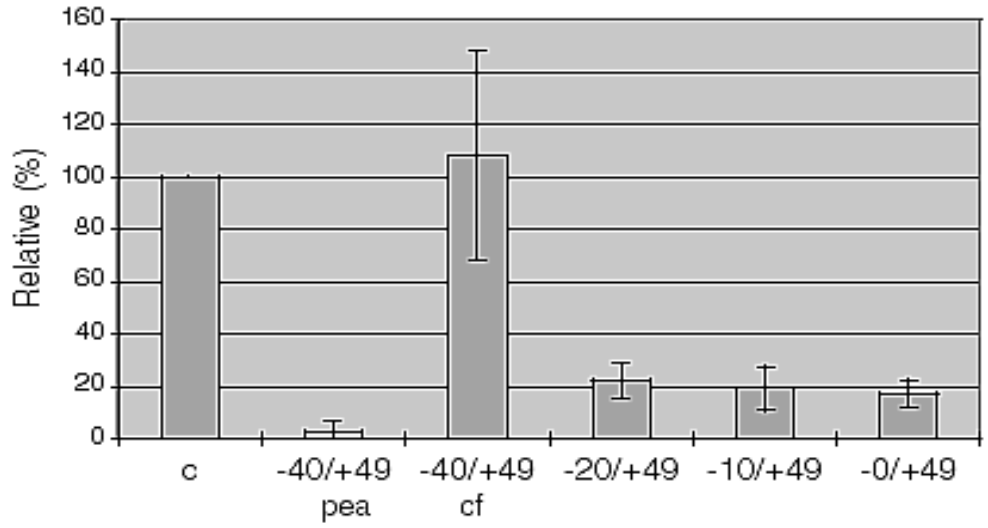

Figure 4.4 Analysis of the *cis*-requirements for *in vitro* RNA editing at the 2nd site. All templates contain the 2nd editing site at position +30 from the 1st site. The control template is the 1st site using pea template in a pea lysate. The deletion clones are derived from the pea template which is identical to the cauliflower sequence except for nucleotide +46.

Competitor RNAs were mixed with the *in vitro* cocktail at a concentration of 1,500 fold that of the template (150 fmol). Two different competitors for the -40/+10 region were used to determine if the

11 base pair difference in the 5'UTR between cauliflower and pea does have an effect on *in vitro* editing. It was confirmed (Fig 4.5B) that the 1st editing site was inhibited by -40/+10 competitors from both cauliflower and pea as described by Takenaka *et al.* 2004. Similarly the downstream competitor had little effect on the 1st site, as the *cis*-elements for this site lie almost exclusively upstream. The 2nd editing site on the other hand is inhibited by the 0/+40 inhibitor further verifying the fact that essential *cis*-elements lie in a similar region, namely 30-40 base pairs upstream from the editing site (Fig 4.4). The sk and 'non competing' competitors are shown to enhance the editing reaction. This will be discussed later in this chapter.

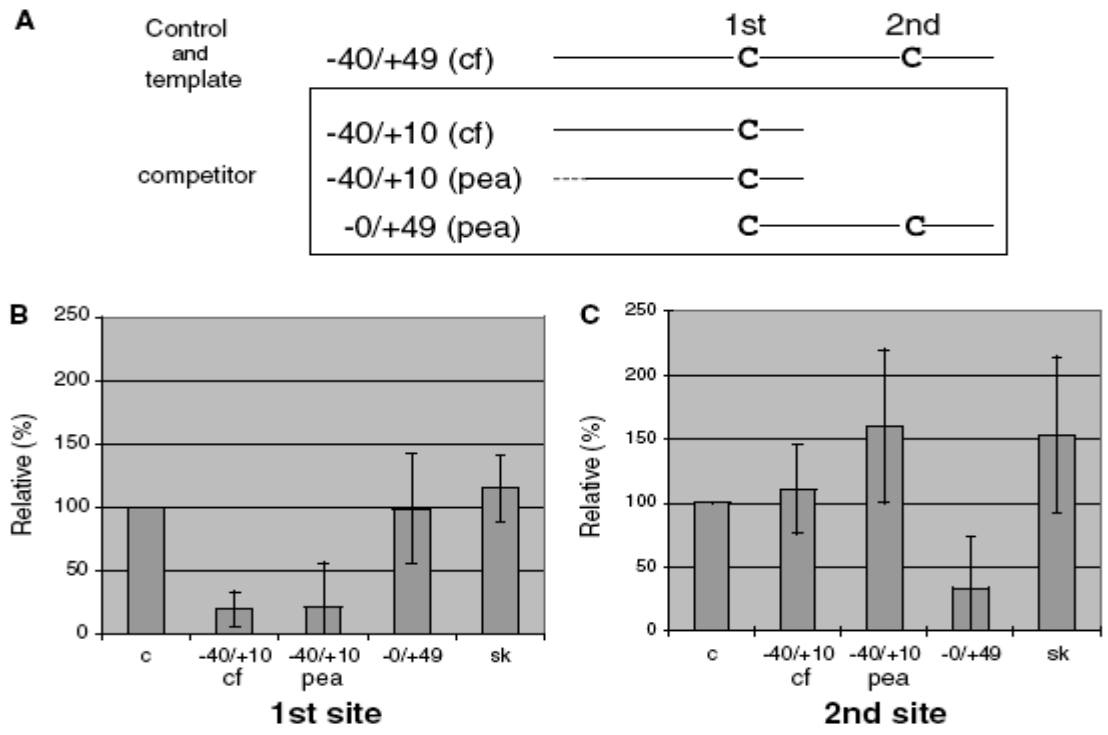

Figure 4.5 A: Diagram of the template *atp9* RNA from cauliflower (cf: top line), the competitors for the upstream site from cauliflower (2nd line) and from pea (3rd line), the dashed line indicating the different sequence in pea. The competitor for the downstream site (bottom line) differs only at nucleotide +46 between pea and cauliflower. The respective editing sites are indicated by a bold C. B: The 1st editing site is monitored using a 1,500 fold excess of the respective competitors. The control (c) is run without competitor and the effect of 1,500 fold bacterial plasmid sequence is shown as sk. C: The 2nd editing site is monitored using a 1,500 fold excess of the respective competitors, the same controls as for the 1st site were employed also for the 2nd.

From these results one can stipulate that these two neighbouring editing sites contain different *cis*-elements, and most probably require different *trans*-elements. These also seem to be different between the species investigated. The fact that the 2nd editing site is edited only when using cauliflower template, points to a *cis*-element 70 nucleotides upstream which is absent in the pea template. The *trans*-factor associated with this area also seems to be available in abundance as it did not influence the editing of the 2nd site, while inhibiting the editing of the 1st site markedly. This is another conformation that these two editing sites are edited independently.

4.4 Investigating possible *trans*-acting elements

Is RNA involved in RNA editing in higher plants?

Since guide RNAs play such an important role in RNA editing in Trypanosomes, I decided to test the effect different RNAs have on the *in vitro* reaction. The *in vitro* reaction was performed as per normal, but different concentrations of RNA was added to the reaction. As seen in Fig 4.6 the added RNA increased the *in vitro* editing quite dramatically.

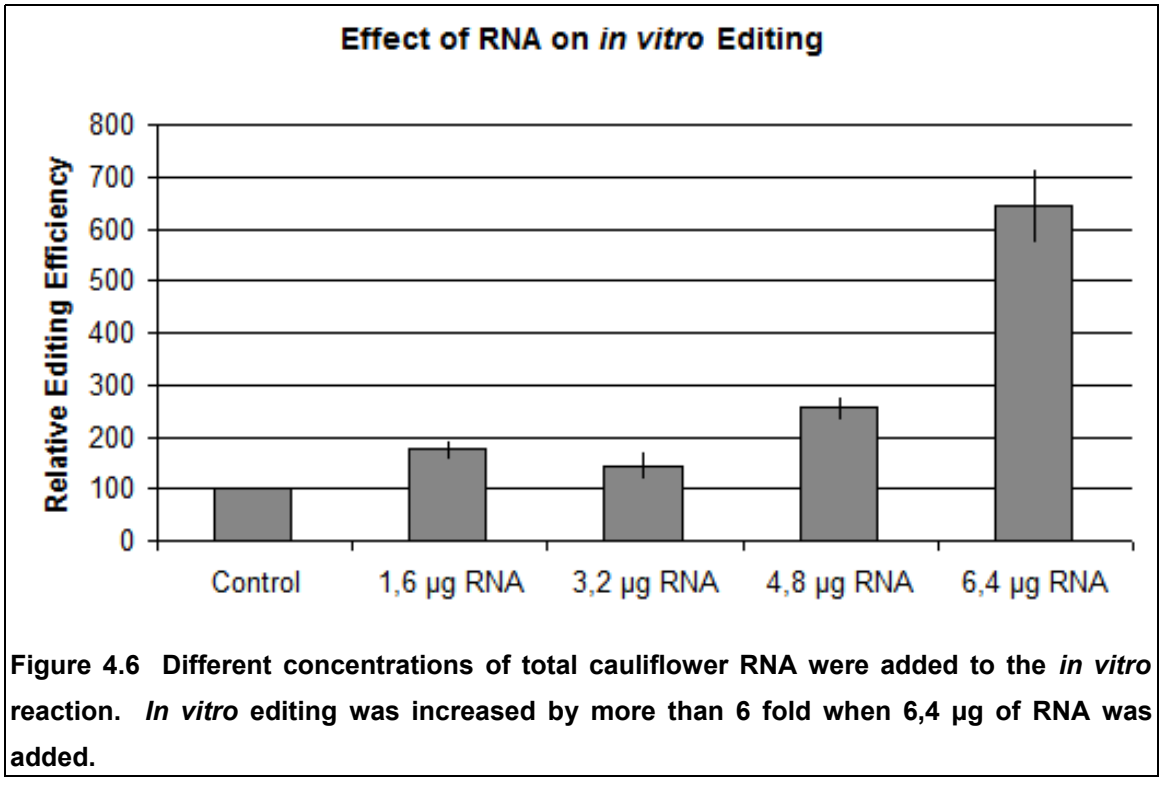

Figure 4.6 Different concentrations of total cauliflower RNA were added to the *in vitro* reaction. *In vitro* editing was increased by more than 6 fold when 6,4 µg of RNA was added.

RNA preparations from different organisms were also tested. Total RNA from bacteria had an enhancing effect, whereas yeast RNA had no noticeable effect (Fig 4.7). Poly G, U and C RNAs were also tested in the *in vitro* reactions. Poly As were not tested as it's well known that poly A sequences are seen as degradation signals in mitochondria (Lupold *et al.*1999) and chloroplasts (Kudla *et al*. 1996). The mechanism of this degradation is still not known in full but it's different from the mechanism found in the nucleus where poly adenylation serves to stabilise mRNAs (for review see: Gagliardi *et al*.2004). As seen in Fig 4.7, poly G and U seem to inhibit the *in vitro* reaction. Poly C has no visible effect on the reaction. Mitochondrial RNA seemed to work better than total RNA at first, but after several repeats the values where inside the standard deviation observed over 3 independent experiments (results not shown). tRNA was also added to see if secondary or tertiary complex RNAs could influence the *in vitro* reaction, but there was no notable increase in the *in vitro* activity (results not shown).

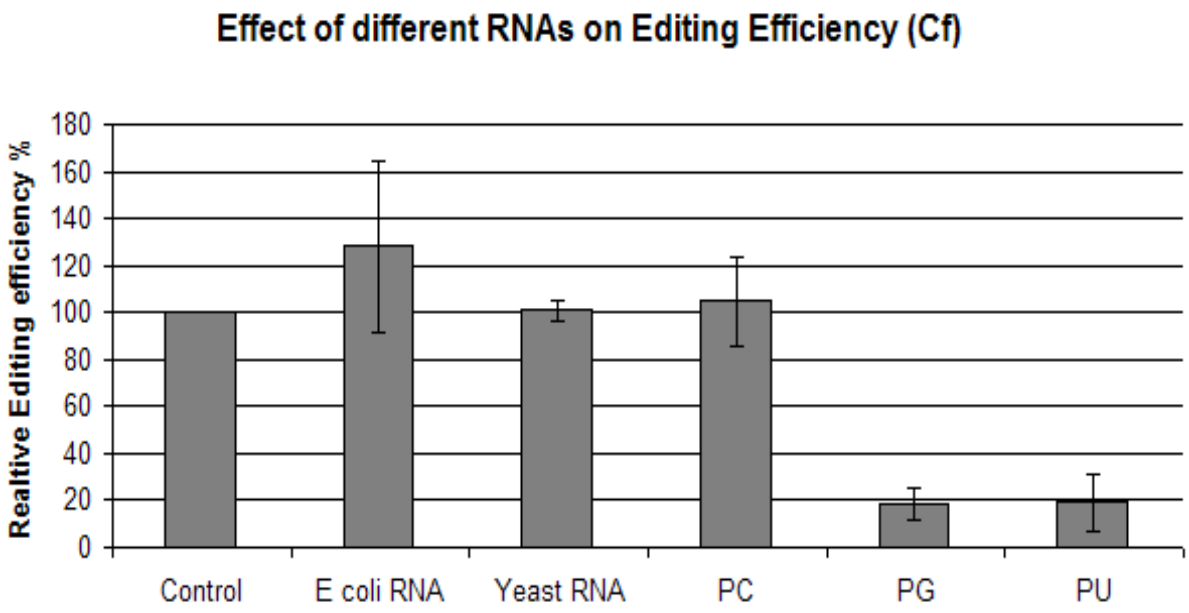

Figure 4.7 The addition of *E. coli* RNA has a slight enhancing effect on *in vitro* editing, whereas yeast seem to have no effect at all. The addition of poly C RNA (20-mers) also has little effect on the editing reaction. The addition of poly G (20-mers) and poly U RNAs (20-mers) however, inhibits the reaction severely.

These results lead us to believe that RNA could play an integral part in the RNA editing mechanism in higher plant mitochondria. To explore these possibilities a set of experiments where designed using the enzyme Micrococcal nuclease (MN). Micrococcal nuclease (S7 nuclease) is a relatively non-specific endo-exonuclease that digests single-stranded and double-stranded nucleic acids, but is more active on single-stranded substrates. Cleavage of DNA or RNA occurs preferentially at AT or AU-rich regions yielding mononucleotides and oligonucleotides with terminal 3'-phosphates. This enzyme is calcium (Ca^{2+}) dependent and the reaction can be stopped by adding a chelating reagent such as EGTA.

The *in vitro* lysates were incubated with 300u of MN (Fermentas) and 0,5mM $CaCl_2$ for 30 minutes to destroy any nucleic acids present. The MN was then deactivated using 0,5mM EGTA and the RNA template added. The initial results looked very promising Fig 4.8. It seemed that the RNA contained in the lysate was essential for the *in vitro* reaction to work. When total pea RNA was added after the MN was deactivated the *in vitro* reaction started working again. We explored the possibility that the MN,$Ca^{2+}$and EGTA complex could also inhibit the reaction. We therefore mixed these reagents beforehand and added this mixture to the reaction. A slight inhibitory effect was noted, that disappeared at the addition of external total pea RNA. To test if the addition of the calcium chloride or EGTA was inhibitory to the reaction, these reagents where added separately as well, and indeed, as can be seen in Fig 4.8, there was a slight inhibitory effect. We concluded from these and the RNA results (discussed earlier) that the RNA present in the lysate was actively involved in the editing reactions, as adding 'foreign' total RNA after deactivating the MN seemed to re-activate the *in vitro* reaction.

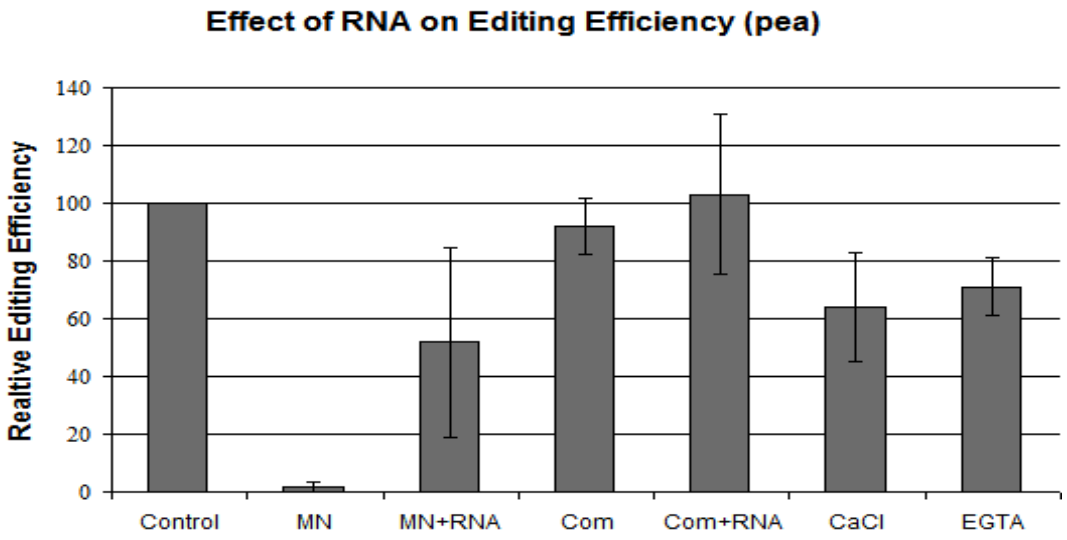

Figure 4.8 The reaction incubated with micrococcal nuclease and $CaCl_2$ after adding the template RNA (MN) totally inhibits the RNA editing *in vitro* reaction. After deactivating the the MN and adding external total RNA to the reaction, the reaction could be re-activated to a certain extent (MN+RNA). $CaCl_2$, MN and EGTA were mixed in a separate tube before hand and then added to the *in vitro* reaction to see if this complex influences the reaction (Com). This complex has a slight effect on the *in vitro* reaction. Addition of total RNA to the complex restored the activity to the levels of the control reaction (Com+RNA). The reaction was also incubated with only $CaCl_2$ or EGTA to see if these reagents alone might inhibit the reaction ($CaCl_2$ and EGTA respectively).

These experiments were repeated using the cauliflower system but unfortunately the results were not reproducible. This could be due to the fact that the results obtained in the pea experiments were masked by the fact that we were working at the bottom end of the detectable editing range (1-2%), whereas with the cauliflower system we were working at a more 'stable' level (2-5%). The idea that RNA could play a role in pea and not in cauliflower could not be discarded but is extremely unlikely.

The involvement of a transaminase?

The transaminases are enzymes that catalyse the transfer of an amino group from a donor molecule to a recipient molecule. The donor molecule is usually an amino acid while the recipient is an alpha-2 keto acid. Since an amino group is 'removed' during RNA editing, transaminase enzymes could play a central role in this reaction (Fig 2.1).

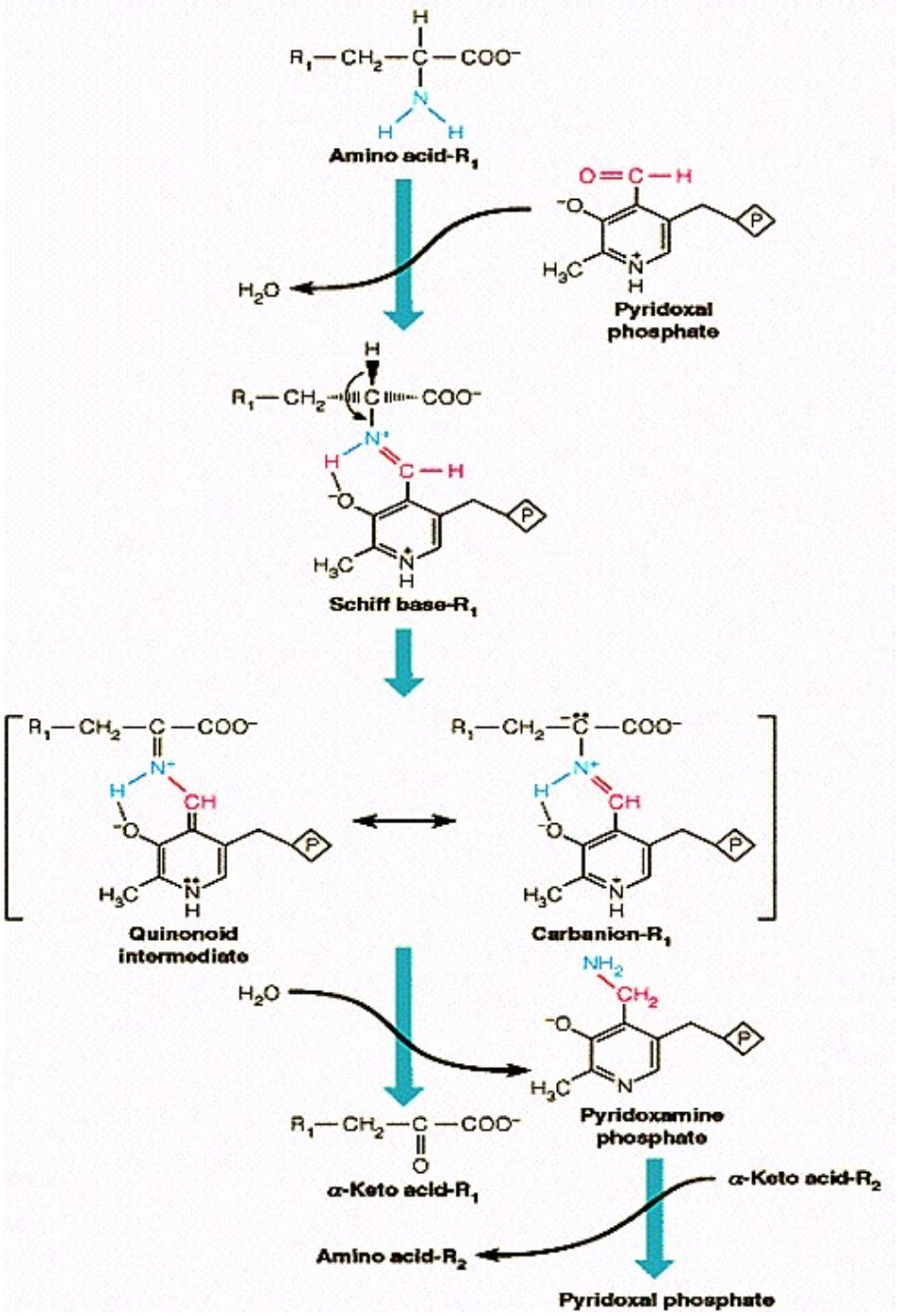

Figure 4.9 The aldehyde group of PLP forms a Schiff base linkage with the ε-amino group of a specific lysine group of the aminotransferase enzyme. The α-amino group of the amino acid substrate displaces the ε-amino group of the active-site lysine residue. The resulting aldimine becomes deprotonated to become a quinoid intermediate, which in turn accepts a proton at a different position to become a ketimine. The resulting ketimine is hydrolysed so that the amino group remains on the complex.

Pyridoxal-5'-phosphate (PLP) is a co-enzyme that facilitates many enzymatic reactions. It is the active form of vitamin B_6 which comprises three natural organic compounds, pyridoxal, pyridoxamine and pyridoxine.PLP acts as a co-enzyme in many transamination reactions, and in some decarboxylation and deamination reactions of amino acids (Fig 4.9). Adding PLP to the *in vitro* reaction, can potentially give an indication if a transamination reaction could be responsible for the editing. In theory the addition of an important co-enzyme should increase the *in vitro* activity. This however was not the case, as the reaction was inhibited by more than 40% (results not shown). PLP also requires an available hydrogen molecule to form a Schiff-base, but cytidine does not have an available hydrogen (Fig 2.1a). In summary it seems highly improbable that the editing enzyme is a transaminase, based on these results.

Energy requirements of the *in vitro reaction*

It is well known that the *in vitro* editing reactions in chloroplasts and mitochondria require an energy source (Takenaka and Brennicke 2003; Hirose and Sugiura 2001). In the *in vitro* system developed for mitochondria by Dr. Takenaka, the energy requirements seemed to be accomplished by the addition of relatively high concentrations of NTPs/dNTPs (15 mM), whereas the optimal NTP concentration in the chloroplast system was lower, at 5 mM (Hegeman *et al.* 2005). After various cross-linking experiments and identification assays of proteins associated with labelled template RNA the protein, glutamate dehydrogenase (GDH) was identified to associate with target RNAs (Takenaka *et al.* under review).

GDH catalyses the reversible oxidative deamination of glutamate to α-ketoglutarate and ammonia (Fig 4.10). These enzymes are very diverse and can be divided into four distinct classes. GDH-1 and GDH-2 are small hexameric enzymes with a broad taxonomic distribution that utilize either NAD^+or $NADP^+$ as a co-enzyme and are mostly associated with ammonia assimilation (Andersson *et al.* 2003). A class of larger 115 kDa GDHs (GDH3), that have previously been found only in fungi and protists, is responsible for glutamate catabolism. Finally, enzymes of a fourth class, GDH-4, have been recently discovered in Eubacteria that are approximately 180 kDa in size and are NAD^+ specific (Minambres *et al*. 2000). We decided to test the cofactors of the GDH enzyme

in the *in vitro* system, to see if we could link the presence of GDH to the deamination of the cytdine residues. The most prominent and essential cofactors of the normal GDH catalysed reactions are NADH and NADP for the deamination and amination reactions, respectively (Fig 4.10). NAD, NADP and their respective reduced forms NADH and NADPH were individually added to the *in vitro* editing reaction. The assay also contained the suboptimal concentration of 10 mM ATP to be able to observe either enhancing or detrimental effects. NADH as well as NADPH enhanced the *in vitro* reaction while the oxidized dinucleotides NAD and NADP had no effect (data not shown).

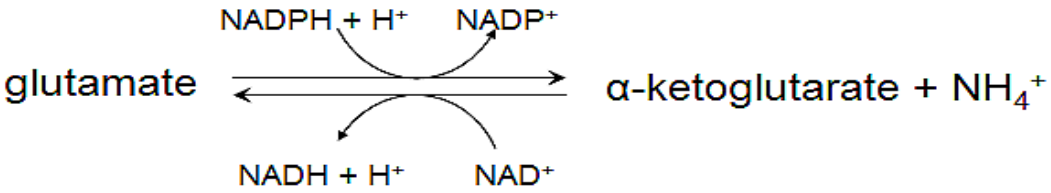

Figure 4.10 The glutamate dehydrogenase utilises both nicotinamide nucleotide cofactors; NAD^+ in the direction of nitrogen liberation and $NADP^+$ for nitrogen incorporation. In the forward reaction as shown above, glutamate dehydrogenase provides an oxidisable carbon source used for the production of energy as well as a reduced electron carrier, NADH. In the reverse reaction glutamate dehydrogenase is important in converting free ammonia and α-ketoglutarate to glutamate.

Next we tested if the NADH and NADPH could replace the NTPs as 'energy source' in the *in vitro* reaction. Both NADH and NADPH were able to replace the NTP requirement, while NAD and NADP did not support the in vitro RNA editing reaction (Fig 4.11A). These results prompted us to investigate GDH a little closer, trying to block the enzyme by using a specific inhibitor.

DL-phosphinothricin (PPT or commercially known as BASTA) is a specific inhibitor of GDH (Leason *et al.* 1982; Fraser *et al*. 1984). This compound binds irreversibly to the active site of GDH and totally inhibits the enzymatic function. Adding phosphinothricin to the *in vitro* reaction did indeed recover the editing activity, without the addition of NTPs (Fig 4.11B).

From these results we concluded that the GDH-specific inhibitor phosphinothricin can substitute the requirement for NTP in the *in vitro* RNA editing reaction This result shows that NTPs solely serve to keep the GDH protein(s) away from the template and that no added energy is required for the RNA editing reaction *per se*. The equivalent capability of NADH and the other reduced

dinucleotide NADPH suggests that the site of action of these compounds is the GDH itself. Whether the added NTPs and dNTPs also bind to the GDH can not be concluded by these results.

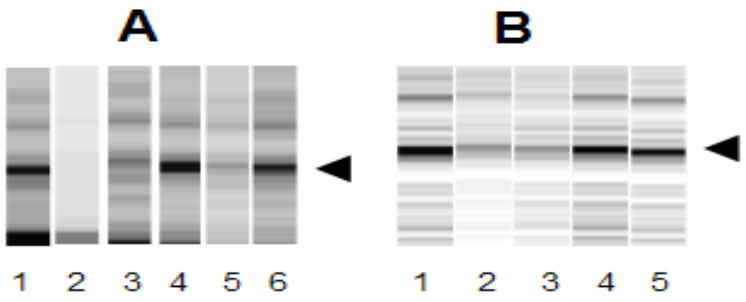

Figure 4.11 Gel representations of the *in vitro* reactions. These assays monitor the first editing site in an *atp4* template RNA. The arrow head on both sides indicates the editing 'events'. Lanes 1 and 2 are the same in both gels, where 1 is the control of 15 mM CTP, and 2 the negative control containing no NTPs. A: Lanes 3 and 5 contain the probes with 15 mM NAD,and NADP respectively. Lanes 4 and 6 were loaded with 15 mM NADH and NADPH respectively. B: Lanes 3-5 were loaded with phosphinothricin, containing 5, 10 and 15 mM respectively.

Differences in editing sites between different *Arabidopsis* ecotypes

It has been proposed by Bentolila *et al.* (2005) that differences in editing frequencies could exist between the different *Arabidopsis* ecotypes. They found that the 24th editing site in the *ccb206* gene was edited at different levels, in the ecotypes Landsberg *erecta* (Ler) and Columbia (Col). I found similar results with the *atp4* gene, where the 1st editing site is edited to only 50% in Ler and 100% in Col and C24 (Fig 4.12).

Unlike the site in *ccb206* the one in *atp4* seems to be recessive. This can be concluded from the obeservation that the reciprocal F1 generations Col x Ler and Ler x Col both edit this site to 100%, identical to the Col parent plants. This also indicates that the *trans*-factor is encoded in the nucleus as there is no maternal effect. To test if the trait was recessive in Ler, F2 crosses were performed, and plants analysed using the mismatch analysis discussed earlier (3.11). A reverse primer was designed with two A's on its 5' end. These two A's correspond to adjacent editing sites that are always edited 100%. RNA was extracted from the F2 plants and reverse transcription was performed using this modified primer.

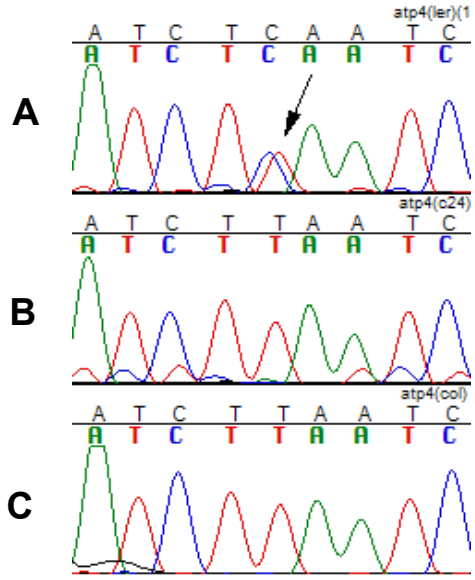

Fig 4.12 The direct sequencing results of the *atp4* gene in 3 different ecotypes of *Arabidopsis thaliana.* The 1st editing site in position 89 is only partially edited in Ler (A), see arrow, whereas it is completely edited in the other ecotypes C24 (B) and Col (C). All other editing sites in the sequence were edited completely, serving as an internal genomic DNA contamination control. The F1 generations of crosses between Col and Ler were also sequenced and the editing was found to be always complete (results not shown). Both repricocal crosses were tested and found to have complete editing of the site of interest.

The modification in the primer should guarantee that only edited RNA molecules are transcribed and subsequently amplified in the PCR reaction. The forward primer was labelled with Cy5 to enable subsequent detection on the sequencing gel. After the PCR reaction the probes were treated the same way as in the editing reaction (3.11). Partially edited molecules should appear at their given size in the sequencing gel (Fig 4.13). Sixty F2 plants have been screened in this way, with 17 positive plants found to date. The positive plants are then sequenced to confirm their status as plants carrying the recessive allele.

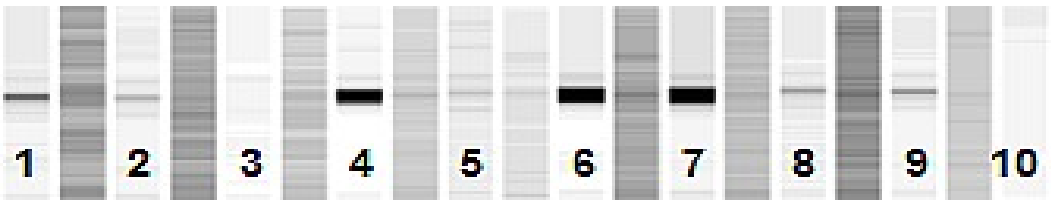

Figure 4.13 Ten F2 plants screened for the extent *of in vitro* editing of the 1st editing site in the *atp4* gene. Every 2nd lane was loaded to prevent over loading contamination from neighbouring wells. Lanes 4, 6 and 7 show partial editing, which was confirmed by sequencing of the PCR products.

Since detecting this ecotype specific difference in RNA editing, all the editing sites in the mitochondrial genome have been screened in these three ecotypes, and together with Dipl. Biol. A. Zehrmann 6 differences in editing levels in 5 different genes have been found.

5 Discussion and Conclusions

Determination of *cis* elements *in silico*

With the host of gene and protein information available currently, programmes have been developed to predict RNA editing events, based on the conservation of protein sequences between different species. These programmes, such as PREP-mt (Mower 2005), predict RNA editing events using complex algorithms and have become relatively accurate. These programmes could be useful in determining the editing sites of the many mitochondrial sequences where the editing status is not known, without the necessity to sequence cDNA clones. They do not however, improve our understanding of the mechanisms of RNA editing.

Development of the cauliflower *in vitro* editing system

The development of an *in vitro* RNA editing system by Takenaka and Brennicke in 2003 was an important step in studying the requirements needed for RNA editing. The system processes mRNA transcripts successfully, but at a very low efficiency of 1-2%. The system was developed for pea which at the time had not been sequenced yet. Since the lysate prepared from the *Arabidopsis* mitochondrial lysate showed no *in vitro* activity, cauliflower was investigated for activity.

Cauliflower offers several technical advantages and is also closely related to *Arabidopsis* which will make the subsequent protein identification easier. Mitochondria are extracted from the white inflorescences, since these offer several advantages to obtain large amounts of comparatively clean mitochondria. These tissues contain few secondary plant compounds, which notoriously make biochemical and molecular analyses difficult. Furthermore no chloroplasts differentiate in these pale white tissues, decreasing the probability of contaminating the mitochondrial extract. Cauliflower is also economically viable, as it can be purchased when needed.

The mitochondrial lysate from cauliflower is more 'active' than the pea lysate with the editing efficiency increased by more than 2 fold that of the pea lysate (Fig 4.1). This is probably due to the

higher protein content observed in the cauliflower lysate. The protein concentration in the cauliflower lysate is almost 7 fold that found in the pea lysate (Fig 4.2). The increase in editing efficiency is therefore not linear when correlated with the protein concentration. This could be due to the loss of an essential *trans*-factor during the mitochondrial extraction process, alternatively to *cis* factors at the specific editing site investigated. As Dr. Verbitskiy has shown, some modified templates can reach an *in vitro* editing efficiency in excess of 50% (personal communication). Introduction of the cauliflower lysate has enabled us to identify more editing sites that are edited *in vitro.* The increased efficiency also increases the range of feasible experiments, since many possibilities were excluded by the low efficiency of the pea system. The *improved in* vitro reaction, however, makes it more difficult to inhibit the reaction by specific competitors, as the concentration of competitors needs to be increased dramatically.

***Cis*-elements for the 2nd editing site in the atp9 mRNA transcript.**

In the cauliflower *in vitro* editing system, the 2nd editing site in the *atp9* mRNA was observed to be correctly recognised. This was the firstt time that two editing sites were reliably detected in one mitochondrial mRNA transcript *in vitro.* This second editing site is located 30 nucleotides downstream from the first, and is conserved in both cauliflower and pea (Fig 4.4A). The interpretation of the experiments in 4.3 leaves a few possibilities open for discussion.

The two neighbouring sites seem to be edited independently from each other. This is rather unexpected, as it implies two different sets of *cis/trans*-factors being employed in close proximity to each other. Each site is addressed separately without being influenced by the editing state of the other. This also lead us to believe that there is no specific order or direction of editing concerning these two sites. Furthermore RNA editing most probably occurs in a hit-and-run mechanism, rather than a linear progressive scanning with the editosome sliding along the transcript. This does not exclude that one of the sites might be preferred by the editosome *in vivo*, due to for example more abundant *trans*-factors.

Efficient editing of the second site in the *atp9* mRNA is observed only with the homologous, native -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. The cauliflower template includes a region at nucleotides -35/40, which was previously identified to increase editing at the upstream (first) site (Neuwirt *et al.* 2005). When this region is deleted or altered to that in the pea template, efficiency of the reaction decreases dramatically (Fig. 4.4). 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 (second) site. This *cis*-enhancer region can thus function for both neighbouring editing sites. The effect seems to be species-specific, as the different pea supportive regions (Fig. 4.3A) cannot substitute for the positive effect of the cauliflower element for the second site (Neuwirt *et al.* 2005). The pea enhancer region does however increase editing of the first site in the cauliflower *in vitro* system, suggesting that this pea sequence attracts different (for the first site positive) *trans*-factor(s) in the cauliflower lysate. In the cauliflower template two distinct *trans*-factors may interact with the cognate *cis*-region, an idea which 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 their presumed differential binding properties.

Is external RNA involved in RNA editing in plant mitochondria?

The addition of external RNAs dramatically increases the efficiency of the RNA *in vitro* activity, in both pea and cauliflower systems. This helped to discover more sites susceptible to *in vitro* RNA editing. The reason(s) as to why the addition of external RNAs increases the efficiency of the RNA editing reaction is still not clear. Evidence suggests that it is a non-specific effect, as almost all added RNAs had a positive effect, regardless of their origin, plants or bacteria. Theoretically the addition of copious amounts of RNA could serve to engage RNase proteins, and thus protect the template RNA, thereby leaving more template molecules available to the editing machinery. It could also have the effect of attaching to non-specific RNA-binding proteins, and keeping the template free of such 'interfering' non-specific RNA-binding proteins, and increase access of the

editing complex to the template RNA. Even though there are some differences between the effects of external RNA in the pea and cauliflower systems, it is highly unlikely that these two plants employ different mechanisms to edit their mRNAs. The difference observed is probably due to the difference in editing efficiencies in the two systems.

The involvement of a deaminase or transaminase?

The deamination of cytidine to uridine typical for RNA editing in higher plant mitochondria and chloroplasts could be achieved by direct deamination reactions such as those catalysed by cytidine deaminases. The most well known example of a cytidine deaminase modified to perform RNA editing is found in several mammals, where the mRNA of the apolipoprotein B is post-transciptionally modified. Takenaka and Brennicke (2003) tested the possibility of such a cytidine deaminase being involved in RNA editing in plant mitochondria. They did this by adding Zn^{2+}-chelators to the RNA editing *in vitro* system. Such chelators can completely block the classic cytidine deaminases, which require Zn^{2+} ions to deaminate cytidine. However, in the plant extract these chelators had no noticable effect on the *in vitro* editing, which suggests that a classic deamination reaction is not responsible for RNA editing in higher plant mitochondria. These experiments do not completely exclude the involvement of a classic cytidine deaminase, as Zn^{2+} ions typically bound in such an enzyme, might not be accessible to the chelators.

Another group of enzymes that could facilitate in the deamination of cytidine is the class of transaminases (Fig 4.9). Takenaka and Brennicke (2003) tested this by adding several NH_2 acceptors to their *in vitro* reaction, expecting an increase in editing activity. There was no effect noted, although it could be possible that the transaminase responsible for editing uses a different amino group acceptor than was tested *in vitro*. In this work I tested this possibility by adding pyridoxal-5'-phosphate (PLP), an essential co-enzyme in many transaminase reactions. The *in vitro* reaction was surprisingly inhibited by this substance, and taken in context with the previous results, does not support the theory that transmainases are involved in RNA editing *in vitro*.

Energy requirements of the *in vitro reaction*

The requirement for added NTP in the *in vitro* RNA editing reaction has been well documented (Takenaka and Brennicke 2003; Hirose and Sugiara 2001). No RNA editing product with uridine in the relevant position of RNA editing is detectable if no NTP is added. It was thought that the addition of NTPs was needed for the energy requirements of the editing enzyme/complex (editosome). Takenaka and Brennicke (2003), however, found that the NTPs could be substituted by dNTPs, without a noticeable loss of *in vitro* editing activity. This could point to the involvement on an RNA helicase, which can use both NTPs as well as dNTPs as an energy source (Okanami *et* al. 1998). This work determined that no external energy source is required for the *in vitro* editing template RNA in plant mitochondria.

After identifying GDH as a protein associating to radioactively labelled RNA constructs, it was shown that this enzyme inhibits the *in vitro* editing in our system (Takenaka *et al.* 2007). It is now clear that there is no external energy requirement for the *in vitro* editing of mRNA constructs (4.4). The addition of NTPs seems to inhibit the non-specific binding of GDH to the RNA construct and so to increase the editing efficiency. This mode of action was confirmed by the addition of a GDH specific inhibitor, phosphinothricin, which encourages *in vitro* editing in the same way as NTPs (Fig. 4.11B). Similar results were obtained when testing NADH and NADPH, both reduced forms of the GDH co-factors NAD^+ and NADP, which were able to replace the NTPs as 'energy' source (Fig 4.11A).

It is still not clear if dNTPs have a similar effect on GDH, and it would be interesting to test this hypothesis. It is possible that the dNTPs could be an energy source for an RNA helicase, that slides down the RNA molecules and removes unspecific RNA-binding proteins, including the GDH (Fairman *et al.* 2004). The helicase could also unwind secondary structures to make the editing sites more accessible to the editosome. The fact that the GDH enzyme inhibits the RNA editing reaction *in vitro* does not exclude the possibility of an RNA helicase being involved.

Differences in editing sites between different *Arabidopsis* ecotypes

The final approach used in this work, was to screen RNA editing sites in the protein coding regions in three different ecotypes of *Arabidopsis thaliana.* An anomaly was found in the 1st editing site of the a*tp4* mRNA in the Landsberg *erecta.* In both other ecotypes investigated, Columbia (Col) and C24, the site was edited to 100%, while it was only partially (50%) edited in Ler (Fig. 4.12). Analysing the F1 generations Col x Ler, and Ler x Col, resulted in 100% editing in both crosses, which could indicate a recessive trait on a single allele. These crosses also confirmed that the trait is encoded in the nuclear genome, as no maternal effect was observed. A further 60 F2 plants (self crossed F1 plants) were analysed, of which 17 showed the partial editing phenotype. An additional 35 plants showing this phenotype are needed to continue to map the genes responsible for these phenotypes.

After narrowing down the locus that causes these phenotypes, a list of potential proteins can be drawn up and investigated to see if they are either directly or indirectly responsible for RNA editing in higher plant mitochondria. Knock out/down mutants could then be investigated to see if different degrees of the phenotypes are indeed caused by these genes. This will be the first nuclear gene identified for RNA editing in plant mitochondria.

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7 Appendix

7.1 Publications

Neuwirt, J., Takenaka M., van der Merwe J.A. and Brennicke A. (2005) An in vitro editing system from cauliflower mitochondria: Editing site parameters can vary in different plant species. RNA 11: 1563-1570

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

JULIA NEUWIRT, MIZUKI TAKENAKA, JOHANNES A. VAN DER MERWE and AXEL BRENNICKE

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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

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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.

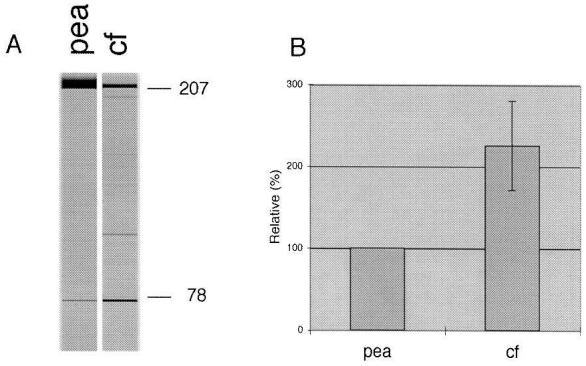

FIGURE 1. Comparison of the RNA editing lysates prepared from cauliflower and pea mitochondria. (*A*) Sample gel images of in vitro editing assays in the pea and cauliflower lysates. Numbers give the sizes of the full-length (207 nucleotides) and edited (78 nucleotides) RT-PCR-TDG products. (*B*) RNA 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).

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                -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-

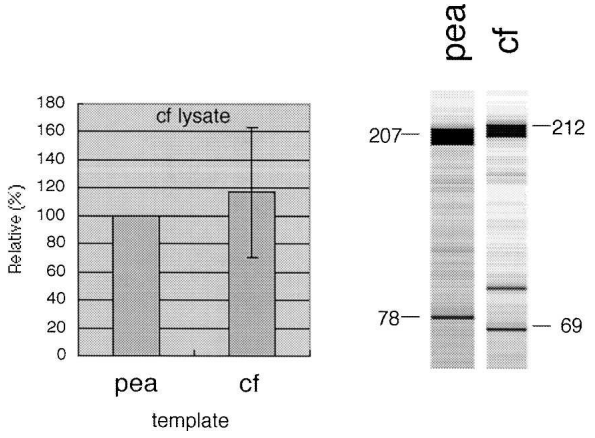

FIGURE 3. Pea and cauliflower templates are processed with similar efficiency in the cauliflower lysate. This observation suggests that in both plant species, the *cis*-elements for recognition of this editing site reside within the conserved 23 nucleotides (and possibly the few positions conserved further 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).

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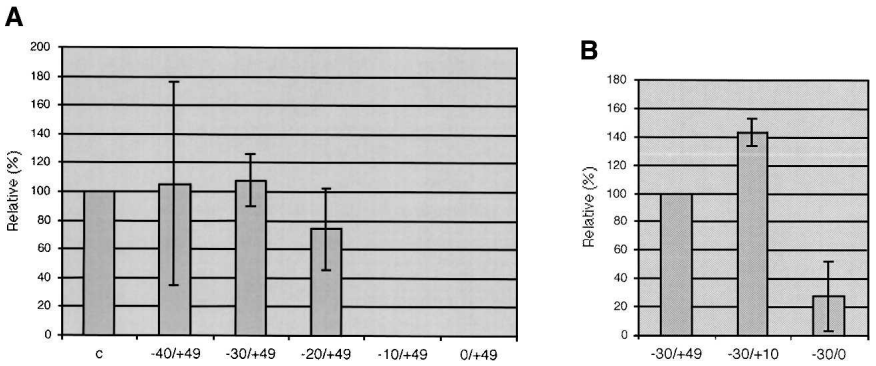

FIGURE 4. Delineation of the *cis*-recognition region by deletion clones in the cauliflower mitochondrial lysate. The sequences upstream and downstream of the monitored editing site were deleted in steps of 10 nucleotides; that is, they were in effect substituted by bacterial vector sequences. (*A*) 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

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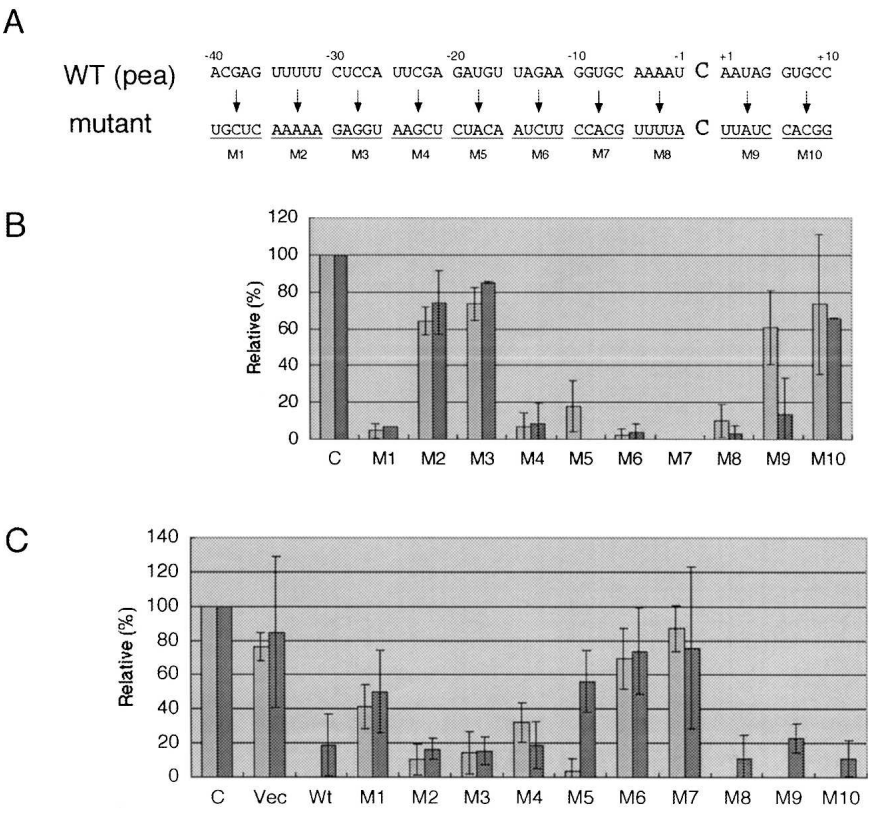

FIGURE 5. The effects of scanning mutations around the first *atp9* editing site as substrates and as competitors on in vitro RNA editing in pea and cauliflower mitochondrial lysates are compared. (*A*) The respective nucleotide quintet altered to its complementary sequence in each set of experiments 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-

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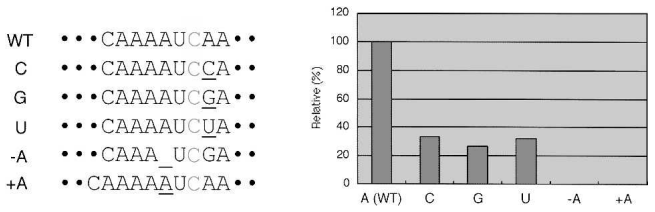

FIGURE 6. Importance of individual nucleotide alterations around the editing site. The influence of the identity of the first nucleotide downstream of the edited nucleotide was determined by mutating this position through all three alternative nucleotides. Any of these changes results in the loss of ~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

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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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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.
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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

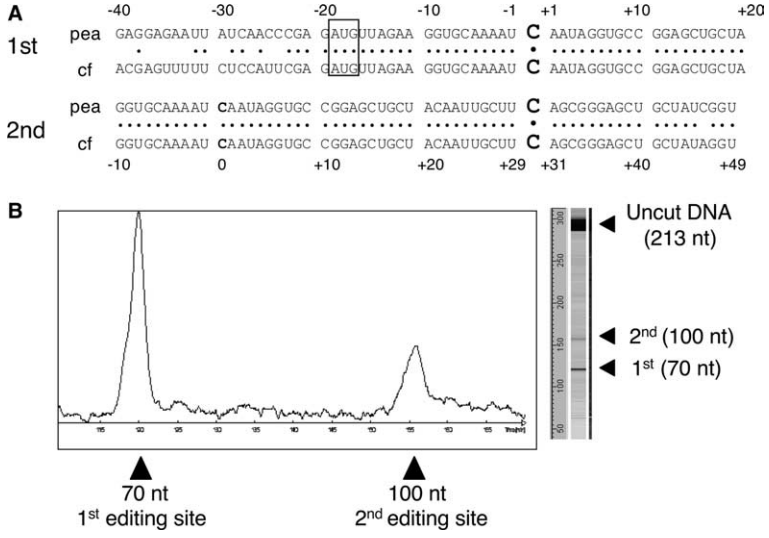

Fig. 1. Two neighbouring RNA editing sites are processed in an in vitro lysate from cauliflower mitochondria (*Brassica oleracea*). (A) Nucleotide sequence alignment of the first two editing sites (1st and 2nd) in the *atp9* mRNA reveals no sequence similarity around the sites. The edited C residues 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.

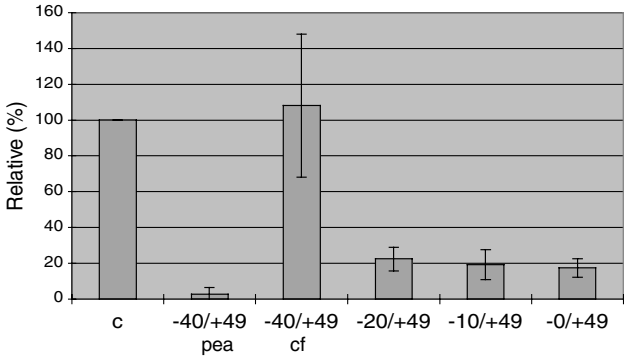

Fig. 2. Analysis of the *cis*-reqirements for in vitro RNA editing at the second site. The *atp9* sequences retained in the respective template RNAs are indicated underneath each column. All templates contain the second site at nucleotide +30 from the first site. In the three series of experiments summarized 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

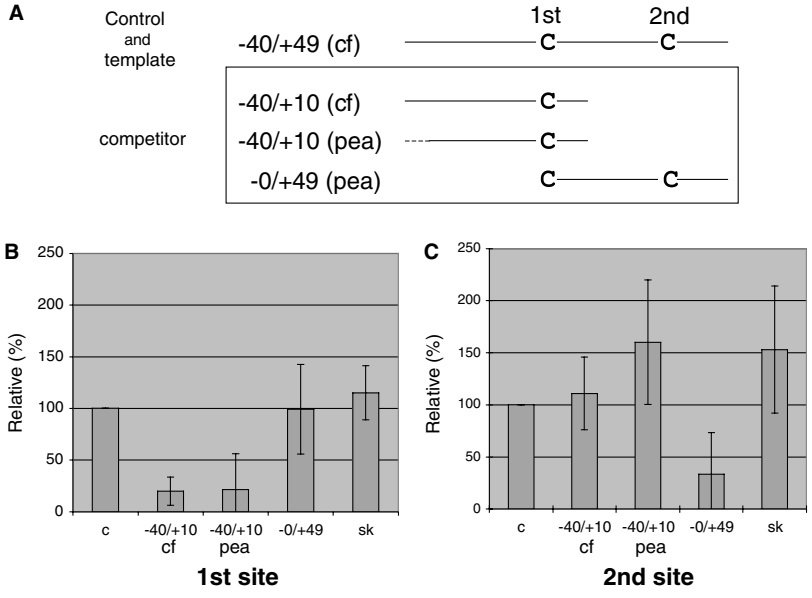

Fig. 3. Determination of competition for *trans*-factors between the respective core recognition sequences of the two neighbouring sites. (A) Scheme of the template *atp9* RNA from cauliflower (cf; top line), the competitors for the upstream site from cauliflower (second line) and from pea (third line), 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 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

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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.

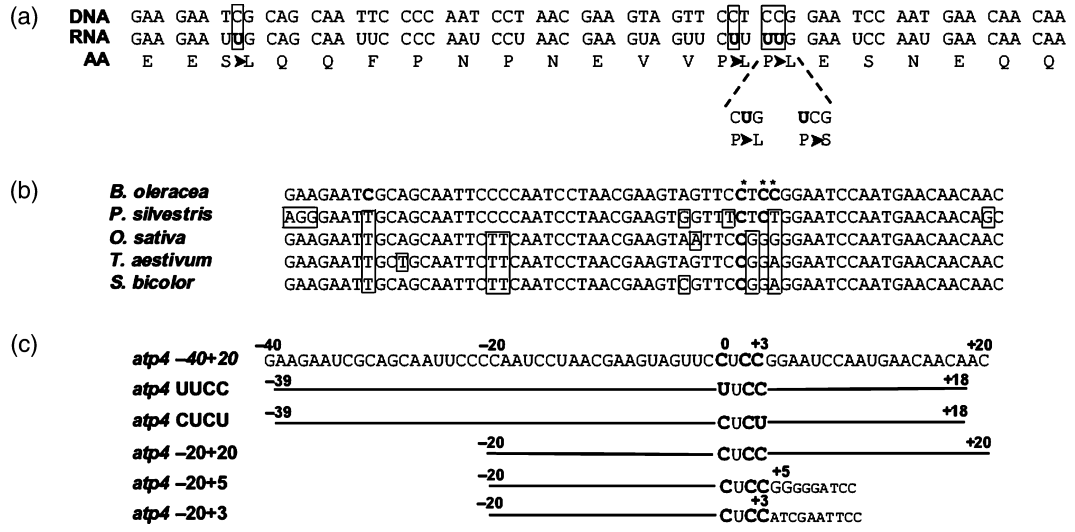

Figure 1. Three RNA editing sites are clustered within four nucleotides in the *atp4* coding sequence.
(a) Effect of RNA editing at the clustered sites on the encoded amino acid sequence. After editing the third site, the second site becomes a silent editing event, which does not change the amino acid 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.

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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

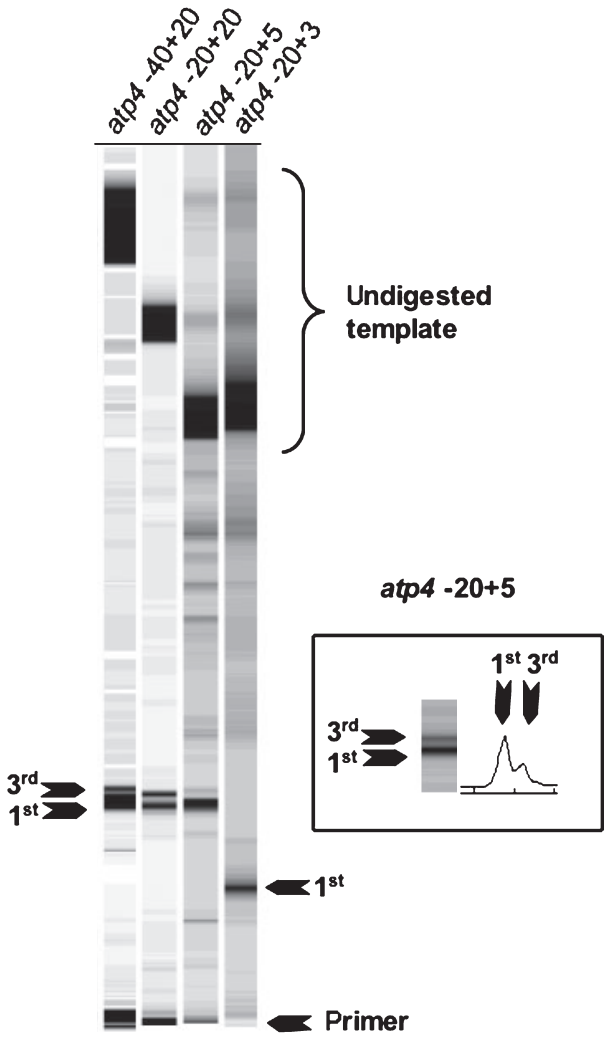

Figure 2. Determination of the minimal recognition site for the *atp4* editing site cluster. The longest substrate shown here (left lane, −40 + 20), covering 40 nucleotides upstream and 20 nucleotides downstream of the first editing site, is edited rather efficiently at both the first and third sites. 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).

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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

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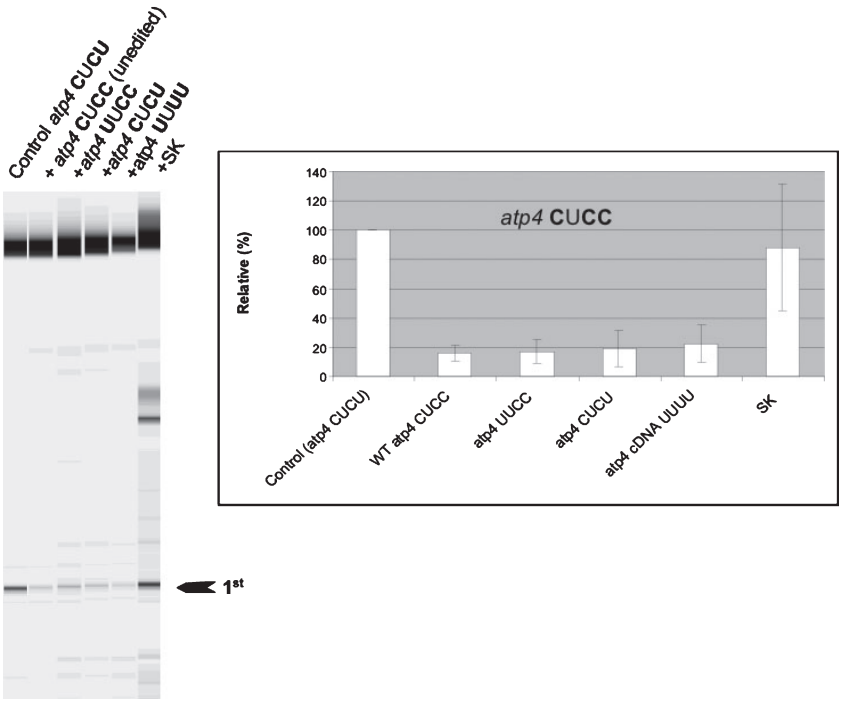

Figure 4. Competitor experiments of the first editing site with the *atp4* substrate region in various stages of RNA editing. The left-hand panel shows a representative gel image, the bar graph on the right summarizes the results of three independent experiments. To monitor editing only at the first 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.

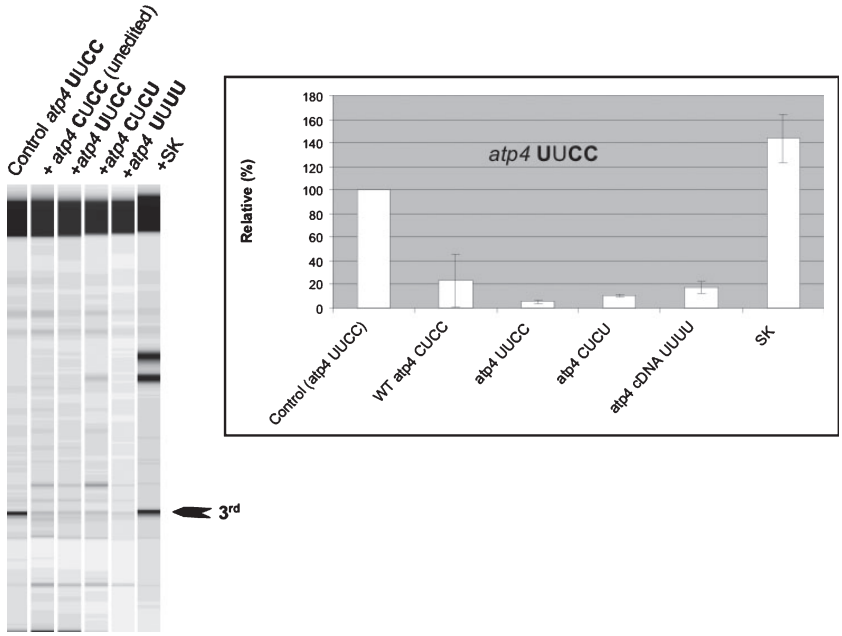

Figure 5. Competitor experiments for editing site three with the *atp4* substrate region in various stages of RNA editing. The left-hand panel shows a gel image, the bar graph on the right summarizes the results of three independent experiments. To monitor editing only at the third site, the editing 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

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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.

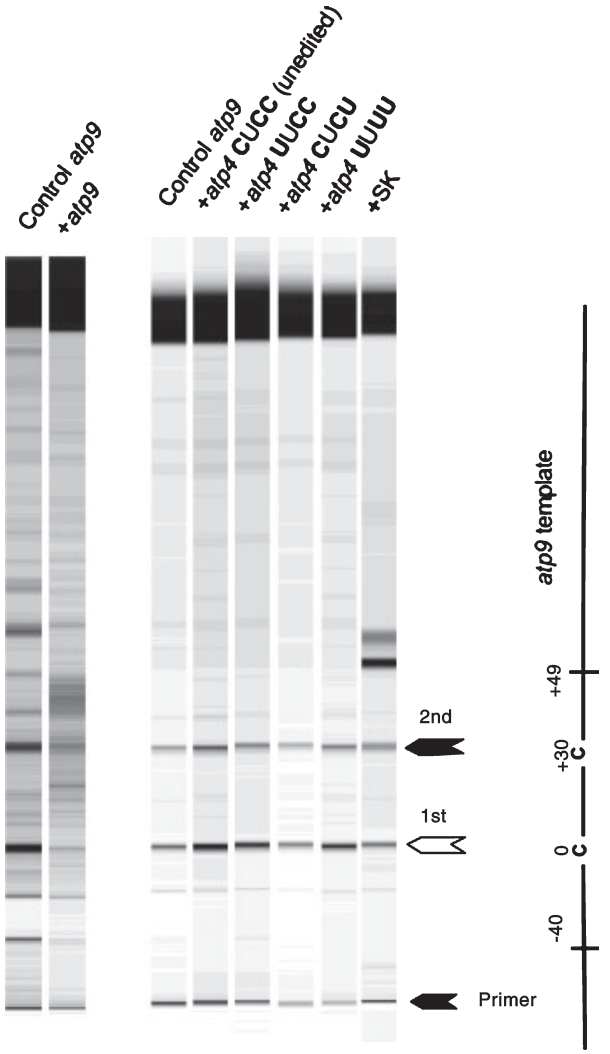

Figure 7. Heterologous competitor experiments to test the specificity of *trans*-factor binding. Two *atp9* editing sites located 30 nucleotides apart were monitored for the effect of 1000-fold competitors of the *atp4* substrate region in various stages of RNA editing. The locations of the two *atp9* 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

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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

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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.

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In vitro RNA editing in plant mitochondria does not require added energy

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Abstract RNA editing in flowering plant mitochondria is investigated by in vitro assays. These cauliflower mitochondrial lysates require added NTP or dNTP. We have now resolved the reason for this requirement to be the inhibition of the RNA binding activity of the glutamate dehydrogenases (GDH). Both GDH1 and GDH2 were identified in RNA–protein cross-links. The inhibition of in vitro RNA editing by GDH is confirmed by the ability of the GDH-specific herbicide phosphinothricin to substitute for NTP. NADH and NADPH, but not NAD or NADP, can also replace NTP, suggesting that the NAD(P)H-binding-pocket configuration of the GDH contacts the RNA. RNA editing in plant mitochondria is thus intrinsically independent of added energy in the form of NTP.
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Keywords: RNA editing; Plant mitochondria; Glutamate dehydrogenase; RNA-binding; In vitro RNA editing; Cauliflower

1. Introduction

In plants RNA molecules are in both organelles altered by RNA editing. In plastids of flowering plants about 35 and in mitochondria about 400 selected cytosines are changed to uridines. The direct biochemical effect of RNA editing in these plants is thus a site-specific deamination. Despite years of investigation neither the reaction mechanism nor the enzymes involved have been identified. Several clues on – or rather conditions of – the biochemistry of the reaction have emerged mostly from in vitro analyses of plastid and mitochondrial lysates, respectively. The first in vitro assays of plant mitochondrial lysates suggested that the sugar-phosphate bonds of the affected RNA molecule are not disrupted in the polynucleotide chain [1]. This observation excludes insertional editing which would excise and exchange either the nucleotide or the base. Thus either direct deamination or transamination are the most likely mechanisms. The first reaction would per se require no added energy, while for the latter, the transamination reaction, additional molecules of higher molecular energy would most likely be involved.

For mitochondrial lysates a strict requirement for added ATP has been observed [2]. Similarly in plastids additional ATP has been reported to be required in most lysates [3–5], while in some residual activity can be seen without any added ATP [6,7]. Surprisingly the added ATP can be substituted to full effect by some or all of the other NTPs and even dNTPs [2,6,7]. While all of these molecules are virtually interchangable in mitochondrial lysates, they vary in their effect in plastid extracts [6,7]. In both organelles at least one of the dNTPs is as effective as ATP, suggesting that one of the few enzymes accepting either triphosphate is involved. One group of such enzymes is a class of RNA helicases and their participation has consequently been proposed [2].

We have now investigated the requirement for nucleotide triphosphates in detail and come to the surprising conclusion that this dependence is at least in mitochondria almost entirely explained by the behaviour of the enzyme glutamate dehydrogenase (GDH).

2. Materials and methods

2.1. Preparation of mitochondrial extracts

Cauliflower mitochondria were purified by differential centrifugation steps and a Percoll gradient as described [2,8]. Isolated mitochondria were lysed, the lysate was cleared and the supernatant was recovered and dialyzed as detailed previously [2,8].

2.2. RNA substrates

DNA clones were constructed in an adapted pBluescript SK^+ to allow run-off transcription of the editing substrate RNA as described [2,8]. Substrate RNAs containing vector sequences at the 5′- and 3′-ends of the mitochondrial insert sequences were synthesized from the T7 RNA polymerase promoter in the linearized template DNA. The bordering bacterial sequences were used for specific amplification of the substrate RNAs by RT-PCR after the in vitro assay [9,10]. The ^{32}P-labelled RNA templates for the gel shift assays were obtained by direct incorporation of labelled ATP into the RNA during run-off transcription from the DNA template.

2.3. In vitro RNA editing reactions

The in vitro RNA editing reactions were performed as described [8,11]. 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). The TDG treated fragments were separated and the Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (GE Healthcare).

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2.4. RNA binding proteins

Proteins in the mitochondrial lysate binding to RNA were analysed by incubating 1 pmol RNA template for 30 min in the reaction mixture. In the template RNA every uridine was labeled with ^{32}P by in vitro transcription without adding cold UTP. The proteins in contact with the RNA were irradiated with UV light for 20 min (Stratalinker 1800, stratagene). This step was followed by digestion of the RNA with RNase A at 37 °C for 30 min. Protein samples were dissolved in buffer and separated by SDS–PAGE on a 10% gel. Labelled proteins were visualized with a Bioimaging Analyzer BAS-3000 (Fuji Photo Film Co).

2.5. Protein identification

RNA editing templates were labelled with biotin (biomers GmbH) and 500 pmol were incubated with 100 µl of mitochondrial lysate in a total volume of 200 µl under standard in vitro RNA editing conditions in the presence of 1 mg total yeast RNA [2]. The proteins in contact with the RNA were cross-linked by UV irradiation for 20′ (UV Stratalinker 1800, Stratagene). The biotin-labelled RNA–protein complexes were bound to streptavidine sepharose™ High performance (GE healthcare). Unbound proteins were washed off by six rinses in spin columns with 400 µl wash buffer each (30 mM HEPES-KOH pH 7.7, 3 mM magnesium acetate, 45 mM potassium acetate, 30 mM ammonium acetate, 10% glycerol). The sepharose beads were treated with RNase A to release the RNA-bound proteins, which were collected with wash buffer. Proteins with their bound residual RNA fragments were analysed by PAGE and/or were (directly) identified by liquid chromatography coupled (LC) electrospray ionization (ESI) tandem mass spectrometry (MSMS). Proteins cross-linked to RNA were digested within the gel using trypsine according to Shevchenko et al. [12]. For digestion of proteins in solution after extraction from the beads, the samples are denatured in 4 M urea and 2 M thiourea, diluted to a final concentration of $\leqslant$1 M urea and incubated with 2 µg trypsine overnight at room temperature. Peptides are desalted and separated by nanoLC equipped with a pre-column working in backflush and directly analyzed by ESI MSMS in a Q-ToF (Q-ToF ultima, Waters) or a hybrid triple quadrupole/linear iontrap mass spectrometer (4000 QTrap, ABI) under standard conditions. Fragment spectra of peptides are searched against the NCBInr database using Mascot as search engine. Matches with resulting protein identification were usually obtained against the *Arabidopsis thaliana* sequences, which is phylogenetically closely related to the here analysed cauliflower (*Brassica oleracea*). Since different species are compared, similarities/differences in the respective orthologous protein sequences additionally influence the generated scores.

3. Results and discussion

3.1. NTP as well as dNTP support the in vitro RNA editing reaction

The strict requirement for added NTP in the in vitro RNA editing reaction is documented in Fig. 1. No RNA editing product with uridine in the relevant position of RNA editing is detectable if no NTP is added. Addition of ATP at the optimal concentration of 15 mM yields about 2–5% C to U converted nucleotide, the amount depending upon the lysate, the source of the mitochondria and the template used. The positive effect of ATP can be fully recovered by substitution with CTP, shown as example here (Fig. 1). Any of the NTPs or dNTPs can replace ATP and will yield comparable editing rates [2]. Since in plastid as in mitochondrial in vitro systems NTPs and dNTPs can substitute for ATP, analogous factors may be responsible for the in vitro ATP requirement in both organelles.

3.2. Influence of the added NTP on template RNA–protein interactions

To investigate the protein moieties affected by the added NTP/dNTP in their ability to contact and bind to the RNA substrate molecules, we performed gel shift experiments with radioactively labelled RNA editing substrates (data not shown). Comparisons of the protein patterns cross-linked with the template RNA in the presence or absence of ATP on PAGE-gels and by more sensitive mass spectroscopy (MS)-analyses did not reveal any qualitative differences. The overall quantity of the proteins labelled with the cross-linked RNA, however, changes dramatically with the presence or absence of NTP: Without added ATP the same proteins are labelled much stronger (Fig. 2). This suggests that addition of ATP disturbs all protein–RNA interactions non-specifically.

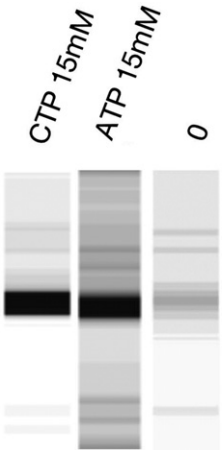

Fig. 1. In vitro RNA editing of an *atp4* mRNA template requires the addition of ATP or CTP. Similar to the in vitro editing of an *atp9* RNA [2], dNTP-nucleotides can substitute for the NTP in this reaction (data not shown).

3.3. Identification of proteins bound by the RNA editing template

Since the gel shift and UV cross-linking experiments (Fig. 2) did not yield detectable proteins differentially affected by NTP in their affinity to the RNA editing template, we employed a more sensitive affinity purification scheme to identify respective polypeptides. This procedure achieved an enrichment of the proteins bound to the editing template RNA (Fig. 3). The Coomasie-stained SDS–PAGE patterns of proteins retained by the RNA template in the presence or absence of ATP, however, again showed only little qualitative difference and no clear candidate protein could be assigned by its ATP-dependent affinity to the template in this gel analysis (data not shown).

Because the ATP-dependence of the in vitro reaction may not manifest in abundant specific RNA–protein interactions and the PAGE analysis may not be sensitive enough, we proceeded to analyse the set of proteins interacting with the RNA directly by the much more sensitive MS without prior gel separation. The proteins bound by the RNA editing template were hydrolyzed and peptides were identified by liquid chromatography (LC) coupled tandem MS (MSMS) analysis. To identify the proteins, the obtained fragment spectra were searched against all entries in the NCBInr database. Since cauliflower and *A. thaliana*, which latter is completely sequenced, are closely related and the protein sequences of many household enzymes are to a large extent identical, we should be able to identify cauliflower proteins through their

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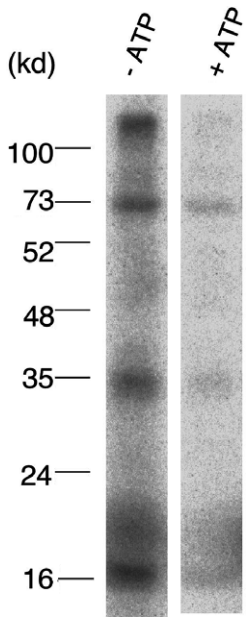

Fig. 2. Similar proteins are cross-linked to radiolabelled RNA in the presence or absence of ATP, but ATP reduces the overall amount of the unspecifically bound proteins. Proteins contained in the mitochondrial lysate from cauliflower inflorescences used for in vitro RNA editing assays were UV-cross-linked to ^{32}P labelled *atp4* RNA in the absence (−ATP) or presence (+ATP) of 15 mM added ATP. The RNA template was digested with RNase A and proteins were separated by SDS–PAGE in a 10% gel. Sizes of marker proteins are indicated in kDa on the side of the autoradiogramme of the dried gel.

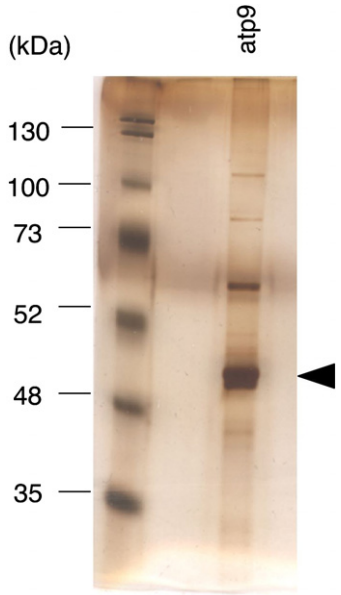

Fig. 3. Cauliflower proteins binding to the atp9 RNA editing template were purified by cross-linking to the RNA. The *atp9* RNA editing template contains 40 nucleotides upstream and 10 nucleotides downstream of the first editing site. Proteins were spread on an analytical PAGE-gel and silver-stained. The arrow indicates the position of the GDH as determined from the molecular weight of the *A. thaliana* protein(s). It is assumed that the cauliflower protein analysed here has a comparable molecular weight.

orthologues in *A. thaliana*. The proteins contained in the total complement enriched by the affinity purification procedure and identified by conserved peptide sequences indeed yielded a list of mitochondrial proteins from *A. thaliana* (Table 1).

Within this list we focussed on proteins which might be involved in a deaminase or transaminase reaction. Among the general mitochondrial proteins such as heat shock proteins, malate dehydrogenase and such, some of the more prominent proteins were representatives of the glutamate dehydrogenase group of proteins, i.e. GDH1 and GDH2 (Table 1) [13]. GDH can catalyze amination as well as deamination reactions depending on the presence and concentration of various allosteric and isosteric regulators (Fig. 4). There is thus a (albeit remote) possibility that one or more of these enzymes have been recruited into the RNA editing reaction to catalyse the deamination step from C to U.

Furthermore GDH-proteins have been previously identified as RNA binding proteins [14] and have been investigated particularly with respect to their participation in RNA editing in kinetoplasts of trypanosomes. Simpson and coworkers showed that the preferential binding of GDH to guide RNA is fortuitous and not related to RNA editing [15]. However, in kinetoplasts RNA editing involves the insertion and deletion of specific uridines rather than the deamination events found in mitochondria and plastids of flowering plants.

3.4. Is the GDH involved in the RNA editing reaction?

To investigate a potential participation of the GDH in the C to U deamination reaction, we tested the influence of various cofactors of this enzyme on the in vitro editing assay. The most prominent and essential cofactors of the normal GDH catalyzed reactions are NADH and NADP for the deamination and amination reactions, respectively. NAD, NADP and their respective reduced forms NADH and NADPH were individually added to the in vitro RNA editing reaction. The assay also contained the suboptimal concentration of 10 mM ATP to be able to observe either enhancing or detrimental effects. NADH as well as NADPH stimulated the reaction while the oxidized dinucleotides NAD and NADP did not (data not shown). Since only enhancing effects were detected, the next series of assays investigated the effect of NADH and the other dinucleotides in the absence of ATP to see whether they can substitute at least partially for the mononucleotide. Both NADH and NADPH were able to replace the NTP requirement, while NAD and NADP did not support the in vitro RNA editing reaction (Fig. 5).

3.5. Investigation of the role of the GDH in the in vitro RNA editing assay

This result of NADH and NADPH being able to substitute for NTP suggests that NTP may not be required for the RNA editing reaction per se, but may be required to alleviate the inhibitory effect of the GDH by releasing this protein from the template RNA or by keeping it away a priori. To test this hypothesis that the GDH might be a major inhibitor of the in vitro RNA editing activity, we analysed the effect of a direct blocker of the GDH proteins.

The compound phosphinothricin is such a specific inhibitor of the GDH in plants and binds irreversibly to the active site of the enzyme. Addition of phosphinothricin to the in vitro RNA editing reaction indeed recovered the editing activity in the absence of any added NTP (Fig. 6). This result shows that NTP solely serves to keep the GDH protein(s) away from the tem-

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Table 1
Typical protein identification results of an MS analysis after enrichment and UV-cross-linking of cauliflower mitochondrial proteins binding to the *atp9* RNA editing template

Gene identifier	Mass	Score	Queries matched	Name
gi|4210330	116581	839	19	2-oxoglutarate dehydrogenase, E1 subunit
gi|7076784	114147	575	12	2-oxoglutarate dehydrogenase, E1 subunit-like protein
gi|2654210	72330	489	9	Heat shock 70 protein
gi|17939849	63332	435	7	Mitochondrial F1 ATP synthase beta subunit
gi|15238762	44496	324	7	GDH1 (GLUTAMATE DEHYDROGENASE 1); oxidoreductase
gi|520478	39163	258	4	Pyruvate dehydrogenase E1 beta subunit
gi|4210332	49908	226	7	2-oxoglutarate dehydrogenase E2 subunit
gi|415733	62316	210	4	Mitochondrial chaperonin
gi|15240793	44671	191	4	GDH2 (GLUTAMATE DEHYDROGENASE 2); oxidoreductase
gi|710400	43003	134	5	Pyruvate dehydrogenase E1 alpha subunit
gi|6435320	25248	123	3	Nucleoside diphosphate kinase

The orthologous proteins from *A. thaliana* with relevant scores listed here are those expected in plant mitochondria and previously identified in proteome analyses [16].

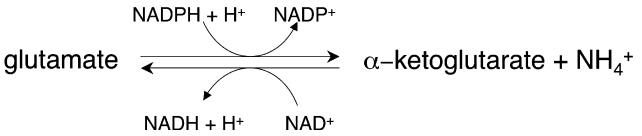

Fig. 4. The reactions catalyzed by the glutamate dehydrogenase include deamination and amination steps. The GDH enzymes bind NAD(P) or NAD(P)H for the activities of amination and deamination, respectively. The direction of the dominant reaction is influenced by the levels of ATP. It is possible that one of these or a similar enzyme has mutated to access also RNA molecules and deaminate specific cytidines.

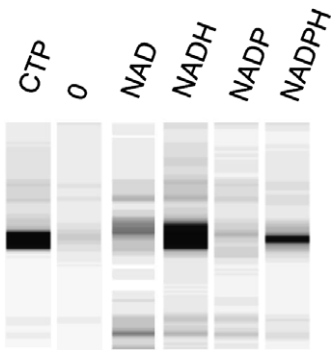

Fig. 5. NADH and NADPH can substitute the requirement for NTP in the in vitro RNA editing reaction. These assays monitor the first editing site in an *atp4* template RNA in which no other site can be edited since the adjacent downstream nucleotides have been altered [9]. The lane marked 0 contains no added (di)nucleotide in the control in vitro incubation. The lanes marked CTP, NADH, NAD, NADP, NADPH contain 15 mM of the respective compounds in the in vitro reaction mix. The gel image shown is the relevant portion of the fluorescent detection of the Cy5 labelled RT-PCR products after TDG treatment, which recognizes the mismatches at the T moieties introduced by in vitro editing.

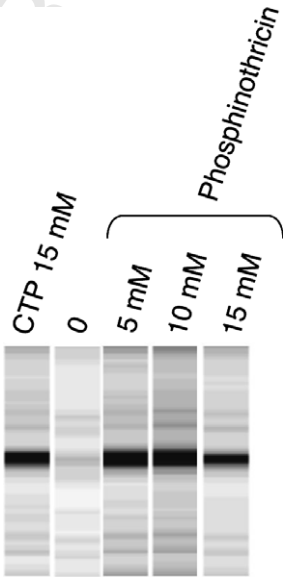

Fig. 6. The GDH-specific inhibitor phosphinothricin can substitute the requirement for NTP in the in vitro RNA editing reaction. All assays monitor the first editing site in an *atp4* template RNA. In the control in vitro incubation no nucleotide was added (lane marked 0). The lanes marked "phosphinothricin" contain this compound but no added NTP or dNTP in the in vitro reaction mix. Only the relevant portion of the gel image is shown.

plate and that no added energy is required for the RNA editing reaction per se.

The equivalent capability of NADH and the other reduced dinucleotide NADPH suggests that the site of action of these compounds is the GDH itself. Whether the added NTPs and dNTPs also bind to the GDH remains open.

If the NTPs and dNTPs do not directly interact with the GDH, the NTP/dNTP and the NADH/NADPH/phosphinothricin effects could be achieved by different modes of action: While the first may activate an RNA helicase [2] which removes the inhibiting bound GDH, the latter compounds may have the same final effect by directly blocking the GDH. The ultimate result observed with both sets of added chemicals, namely active in vitro RNA editing, could be the outcome from either mechanism. To decide this question, the effect of added (d)NTPs and NAD(P)H on the RNA binding of the GDH will have to be investigated with the purified GDH enzyme. The observation that all unspecific RNA-binding proteins in a mitochondrial extract are diminished upon addition of ATP to the gel-shift or cross-linking reaction (Fig. 2) suggests that the NTP/dNTP effect is indeed not a direct action on the GDH, but could be rather a separate general RNA-wiping effect, possibly mediated by an RNA helicase.

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Nevertheless, the substitution of NTP by phosphinothricin shows that if another, general RNA-clearing enzyme such as a helicase is stimulated by the added NTP/dNTP, the activity of this enzyme is not essential for the actual RNA editing reaction, but at least in vitro mainly serves to remove the inhibitory GDH.

3.6. RNA editing does not require added NTP in vitro

The observed in vitro RNA editing in the absence of added NTP suggests that the actual biochemical reaction does not consume energy. The in vitro observed requirement of added NTPs, dNTPs, NADH or phosphinothricin may not be relevant in the in vivo situation: in the intact mitochondrion, RNA editing as well as splicing and 5′- and 3′-processing most likely occur compartimentalized and in safe distance from the location of the GDH.

In chloroplast extracts in vitro RNA editing is similarly stimulated by or depends on the addition of NTP or dNTP moieties. Here the plastid located NADPH-dependent GDH may play an analogous inhibitory role and corresponding specific inhibitors such as phosphinothricin should be tested to resolve this question. It has been reported however that neither NADH nor NADPH have any effect on the in vitro reaction, which may argue against an analogous role [6]. On the other hand the effects of individual NTP and dNTP identities vary between chloroplast extracts from different plant species [3,6,7].

Of course one cannot formally exclude the presence of low amounts of ATP in the mitochondrial or chloroplast lysates which escape the dialysis step by being bound to larger molecules during the extract preparation. However, direct determination of the ATP content in a plastid lysate showed less than 10 nM ATP to be present, suggesting that the dialysis indeed efficiently removes these small molecules [6].

The correct in vitro RNA editing in a plant mitochondrial lysate in the presence of GDH-inhibiting phosphiniothricin shows that the GDH is not involved in the deamination of C to U although this enzyme regularly catalyzes an analogous reaction and can bind RNA molecules. These conclusions make a direct deamination process by a modified cytidine deaminase or an analogous enzyme more probable than a transaminase reaction, for which a requirement of some additional source of activation energy would be more likely – although energetically not strictly necessary.

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1992 Finished high school in Johannesburg with University exemption

1995 Enrolled at the Rand Afrikaans University (RAU) studying mechanical engineering

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2002 Received M.Sc. Degree titled: Cloning and Characterisation of the *orfx* gene in *Nicotiana Tabacum.*

2003 Enrolled in a PhD. Programme under the guidance of Prof. Brennicke in Germany.

Work Experience: Worked in a pathological laboratory between 1998 and 2002 performing routine biochemical and haematological blood tests.

7.3 List of Publications

Posters:

2003 Poster at the 7th International Congress of Plant Molecular Biology in Barcelona: Identification and characterisation of an orfx/mttB/tatC orthologue in the *Nicotiana tabacum* mitochondrial genome. van der Merwe J.A. and Dubery I.

2004 Poster at the Botanikertakung in Braunschweig: Characterisation of *cis*-and-*trans* elements involved in RNA editing of *atp*9. Neuwirt J., van der Merwe J.A., 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 RNAs in RNA editing. van der Merwe J.A., 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 J.A., Verbitskiy D. and Brennicke A.

2007 Poster at the RNA Editing Gordon research conference in Ventura: Glutamate dehydrogenases (GDH) inhibit *in vitro* RNA editing in cauliflower mitochondria. Takenaka M., Neuwirt J., van der Merwe J.A., Verbitskiy D. and Brennicke A.

Publications

2005 Neuwirt J., Takenaka M., van der Merwe J.A. and Brennicke A. An in vitro editing system from cauliflower mitochondria: Editing site parameters can vary in different plant species. RNA 11: 1563-1570

2006 van der Merwe J.A., Takenaka M., Verbitskiy D., Neuwirt J. and Brennicke A. RNA editing sites in plant mitochondria can share *cis*-elements. FEBS Letters 580: 268-272

2006 van der Merwe J.A. and Dubery I.A. Benzothiadiazole inhibits mitochondrial NADH:ubiquinone oxidoreductase in tobacco. Journal of plant physiology 168 8: 877-882

2006 Verbitskiy D., Takenaka M., Neuwirt J., van der Merwe J.A. and Brennicke Partially edited RNAs are intermediates of RNA editing in plant mitochondria. Plant Journal 47: 408-416

2007 Takenaka M., Verbitskiy D., van der Merwe J.A., Zehrmann A., Plessmann U., Urlaub H. and Brennicke A. *In vitro* RNA editing in plant mitochondria does not require energy. FEBS Letters (under review)

Reviews

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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 May 2007

Johannes Andreas van der Merwe

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