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 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 extend 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.

For plant mitochondria, the in vitro RNA editing system from the pea has shown that for recognition by the RNA editing activity, only ~20 nucleotides are essential upstream, 40 are optimal, and basically none is necessary downstream of the first editing site in the atp9 mRNA. Analysis of the cis-requirements by targeted mutations of the template and competition experiments have narrowed the sequence requirements for the site specificity to the region 5–20 nucleotides upstream of this site in the atp9 mRNA (Takenaka et al. 2004). Transfections of isolated wheat mitochondria with cox2 mRNA (Farré and Araya 2001; Farré et al. 2001; Staudinger and Kempken 2003) and mutational analysis of two sites in this transcript showed that similarly 16–20 nucleotides upstream are required to define these sites (Choury et al. 2004). However, in addition to these upstream elements, one or more nucleotide positions downstream were found to be crucial for efficient editing. These sequence requirements suggest that individual recognition elements vary between different editing sites. Extensive mutational analysis revealed that the important nucleotide identities are different for the two sites, confirming the variation of cis-elements between individual RNA editing sites (Choury et al. 2004).

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

RESULTS

The cauliflower in vitro mitochondrial extract

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

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

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).
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 nucleotides(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-
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.
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 \textit{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 respective coding regions, which is 23 nucleotides upstream of this editing site (Fig. 2).

**FIGURE 5.** The effects of scanning mutations around the first \textit{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.

**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 \textit{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 adaptive 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-
showing that the distance from the wild-type sequence. No activity was observed with either template, element and the edited nucleotide was investigated by deleting or position has occurred.

results in the loss of position through all three alternative nucleotides. Any of these changes the editing site. The influence of the identity of the first nucleotide tested in four separate experiments, and the mean percentages of the in vitro editing activity for distance alterations between the upstream error is indicated for each mutant. The tolerance of the in vitro editing complex in cauliflower mitochondria, only molecules derived from mRNAs fully edited at this site are observed (data not shown).

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 interconnected 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...
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 were introduced by the same procedure using the respectively EcoRI or XbaI, respectively, and self-ligated. The point mutations were constructed in an adapted pBluescript SK+ to allow run-off transcription of the respective complement pentanucleotide in primers M1-M10. PCR was performed on deletion clone atp9–40 with primer inversion1 and primers M1, M2, M3, M4, and M5, respectively, and in the second series with primer inversion2 and primers M6, M7, M8, M9, and M10, respectively. The resulting 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 performed on deletion clone atp9–40 with primer inversion1 and primers M1, M2, M3, M4, and M5, respectively, and in the second series with primer inversion2 and primers M6, M7, M8, M9, and M10, respectively. The resulting fragments were digested with EcoRI 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. 

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 uncleaved DNA fragments. The ratio of cleaved (i.e., edited) fragment to uncleaved 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 inversion1 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 inversion2 and primers +10 and +6, 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 inversion1 and primers M1, M2, M3, M4, and M5, respectively, and in the second series with primer inversion2 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 pBluescriptISK+ 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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