Two RNA editing sites with cis-acting elements of moderate sequence identity are recognized by an identical site-recognition protein in tobacco chloroplasts

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ABSTRACT

The chloroplast genome of higher plants contains 20–40 C-to-U RNA editing sites, whose number and locations are diversified among plant species. Biochemical analyses using in vitro RNA editing systems with chloroplast extracts have suggested that there is one-to-one recognition between proteinous site recognition factors and their respective RNA editing sites, but their rigidness and generality are still unsettled. In this study, we addressed this question with the aid of an in vitro RNA editing system from tobacco chloroplast extracts and with UV-crosslinking experiments. We found that the ndhB-9 and ndhF-1 editing sites of tobacco chloroplast transcripts are both bound by the site-specific trans-acting factors of 95 kDa. Cross-competition experiments between ndhB-9 and ndhF-1 RNAs demonstrated that the 95 kDa proteins specifically binding to the ndhB-9 and ndhF-1 sites are the identical protein. The binding regions of the 95 kDa protein on the ndhB-9 and ndhF-1 transcripts showed 60% identity in nucleotide sequence. This is the first biochemical demonstration that a site recognition factor of chloroplast RNA editing recognizes plural sites. On the basis of this finding, we discuss how plant organellar RNA editing sites have diverged during evolution.

INTRODUCTION

RNA editing is a process in which the nucleotide sequences of transcripts are changed by insertion/deletion or conversion of nucleotides, and various types of RNA editing have been found in diverse organisms (1,2). The organelles of vascular plants, specific C residues on the transcripts are converted to U, and U-to-C editing rarely occurs (1,3,4). Chloroplast and mitochondrial genomes of higher plants have 20–40 and 400–500 RNA editing sites, respectively, and in most cases, RNA editing restores phylogenetically conserved codons (1,3,4), including those of functional importance (5–8). Therefore, RNA editing is an indispensable process for plant organellar genomes to produce functional proteins.

An intriguing issue of plant organellar RNA editing is the mechanism by which specific C residues are recognized for editing substrates, since no consensus motif or secondary structure is found in the vicinity of the editing sites. With the aid of transplastomic plants and of in vitro RNA editing systems from chloroplast lysates, cis-elements required for RNA editing were analyzed for several tobacco chloroplast editing sites and revealed that cis-elements are generally located within 20 nucleotides upstream of the editing sites (9–17). Proteinous trans-acting factors that specifically bind to the cis-elements were evidenced by UV-crosslinking experiments with in vitro RNA editing systems; cis-elements of tobacco psbL, psbE and petB editing sites are bound by the proteins of 25, 57 and 70 kDa, respectively (12,14,15).

From the study of an Arabidopsis mutant deficient in the editing activity of the ndhD-1 site, a site-specific RNA-binding protein, CRR4, was identified (8). CRR4 is a member of the pentatricopeptide repeat (PPR) family (18) and specifically binds to the immediate upstream region of the ndhD-1 editing site (19). PPR proteins constitute an extraordinarily large family in higher plants, and many are involved in the maturation processes of organellar transcripts (20). As CRR4 does not have a catalytic domain, it is likely to recruit a catalytic subunit of unknown identity to the editing site (8,19).

The above findings suggest that sequence-specific binding of the trans-acting factors to the upstream...
cis-elements is a crucial process of the accurate site recognition of chloroplast RNA editing. If so, how specifically do the trans-acting factors recognize their respective cis-elements? Site recognition mechanisms of RNA editing seem analogous between chloroplasts and mitochondria (21–26). If so, how many trans-acting factors are necessary to recognize whole editing sites of plant organelles? Overexpression of the psbL editing site in tobacco chloroplasts reduced the editing efficiency of endogenous psbL mRNA, but not of the other editing sites, implying that the psbL-specific trans-acting factor is exclusively recruited to the psbL editing site (27). As mentioned above, trans-acting factors specifically binding to tobacco psbL, psbE, petB, and Arabidopsis ndhD-1 editing sites have distinct molecular masses (8,12,14,15). These genetic and biochemical observations might support a ‘one factor to one site’ hypothesis for plant organellar RNA editing. However, this hypothesis is now challenged from other viewpoints. The Arabidopsis genome encodes ca. 450 PPR proteins (20), while the total number of chloroplast and mitochondrial editing sites of this plant amounts to more than 480 (28–30). If trans-acting factors responsible for site recognition are exclusively PPR proteins, ‘one factor to plural sites’ could also be the case. Chateigner-Boutin and Hanson proposed the ‘one factor to plural sites hypothesis’ on the basis of their observations with plastomastic plants; overexpression of the rpoB-2 or ndhF-2 editing sites reduced the editing efficiencies of several sites (31). However, the ‘one factor to plural sites hypothesis’ has not been proven by biochemical investigations.

This study attempted to verify the ‘one factor to plural sites’ hypothesis on the basis of biochemical analysis. We first compared the molecular masses of trans-acting factors specifically binding to several tobacco chloroplast editing sites by UV-crosslinking and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and found that those of the ndhB-9 and ndhF-1 editing sites seem to be very similar. Next, we examined the identities of ndhB-9 and ndhF-1-specific trans-acting factors by cross-competition experiments in the in vitro RNA editing system of tobacco chloroplast extracts. The results unequivocally showed that the ndhB-9 and ndhF-1 editing sites are recognized by the same trans-acting factor. This is the first clear demonstration that ‘one factor to plural sites’ recognition operates in plant organellar RNA editing. Sequence identity in the trans-factor-binding regions of ndhB-9 and ndhF-1 (−15 to −1 relative to the editing site as +1) is only 60%. On the basis of this finding, we discuss how plant organellar RNA editing sites have propagated and diverged during evolution.

MATERIALS AND METHODS

Preparation of RNA substrates

The region from −120 to +21 (relative to the editing site as +1) from the gene of interest, with a 5′ extension of a 20 nt sequence complementary to the T3 primer and a 3′ extension of a 17 nt sequence complementary to the KS primer, was amplified by PCR on plasmids from a tobacco chloroplast DNA clone bank (32) using gene-specific primer pairs (Table 1, PCR forward and PCR reverse). The amplified fragments were cloned into a pGEM-T vector using the pGEM-T Vector System (Promega). From these cloned plasmids, RNA substrates for in vitro editing and UV-crosslinking were prepared as previously described (14) with slight modifications. The upstream region of the respective genes was amplified from the plasmids by PCR using gene-specific primer pairs (Table 1, PCR forward and PCR upstream reverse), and subjected to RNA synthesis with the MEGAScript T3 Kit (Ambion) with purification according to the manufacturer’s instruction. The 5′ terminal two nucleotides of the PCR upstream reverse primers were ribose 2′-methoxy analogs, which hamper nontemplated nucleotide addition by T3 RNA polymerase (33). [5′,32P]-labeled downstream RNAs (20 pmol) (Table 1) were ligated to 60 pmol of the corresponding upstream RNAs (113–123 nt) with the aid of 40 pmol of a bridging DNA oligonucleotide (Table 1) and T4 DNA ligase in 30 μl reaction mixtures at 30°C overnight. The ligated mRNAs were purified by 5% PAGE containing 7 M urea. When mutations were introduced to the RNA substrates, plasmid clones containing respective chloroplast genes were mutagenized using pairs of mutagenesis primers (Table 1) and the QuickChange Site-Directed Mutagenesis Kit (Stratagene), followed by the preparation of RNA substrates as described above.

Preparation of chloroplast extracts

Chloroplast extracts were prepared from tobacco leaves as previously described (34), and utilized for RNA editing reactions and UV-crosslinking.

In vitro RNA editing and UV-crosslinking

RNA editing and UV-crosslinking assays were carried out essentially as previously described (15), with slight modifications. Both reaction mixtures contained 4 μl of chloroplast extract (~50 μg protein) and 10 fmol of mRNA substrate. For RNA editing assays, an mRNA substrate was incubated at 28°C for 1 h. RNA was isolated and digested into 5′ mononucleotides with 1 U of nuclease P1 (Wako) and 120 U of S1 nuclease (TaKaRa) in the presence of 50 mM ammonium acetate (pH 4.8) at 37°C for 3 h. Mononucleotides were separated on cellulose TLC plates (FC-2020, Funakoshi) using isopropanol:HCI:water (70:15:15). For UV-crosslinking assays, an mRNA substrate was incubated at 28°C for 1 h in the editing mixture. Reaction mixtures were irradiated with UV light (254 nm, 1.0 J/cm²) at approximately 10 cm distance using a Funacrosslinker (Funakoshi), then subjected to RNA digestion by 100 ng of RNase A at 37°C for 1 h. Protein samples were separated by 7.5% PAGE containing 0.1% SDS. 32P-labeled mononucleotides on TLC and 32P-crosslinked proteins on PAGE were visualized by STORM (GE Healthcare).

Nomenclature for RNA editing sites

The chloroplast genome of tobacco (Nicotiana tabacum) is known to have 38 RNA editing sites (34–37). In this case, Chateigner-Boutin and Hanson proposed the 'one factor to plural sites' hypothesis on the basis of their observations with plastomastic plants; overexpression of the rpoB-2 or ndhF-2 editing sites reduced the editing efficiencies of several sites (31). However, the 'one factor to plural sites hypothesis' has not been proven by biochemical investigations.
study, the editing sites of tobacco are simply denoted as, for example, ndhB-9 that means the ninth editing site of tobacco mRNA counted from the 5' end. However, ndhF-1 site in this nomenclature corresponds to ndhF2 of the previous reports (31,35) that name was given based on the comparison between tobacco and maize. For circumventing the confusion, the editing sites examined in this study are more precisely defined in Table 2, following the universal nomenclature used by Heyes et al. (16); NTndhB C141 means the editing site at the 141st C of the ndhB mRNA of Nicotiana tabacum.

### RESULTS

Both ndhB-9 and ndhF-1 editing sites are crosslinked with 95 kDa protein

The tobacco chloroplast genome is known to have 38 RNA editing sites (34–37). We previously showed for tobacco psbE-1 and petB-1 RNA editing sites that the proteinous site-specific factors are UV-crosslinked with their respective editing sites (C at +1), as well as the cognate upstream cis-elements (15). If this is also the case for the rest of the RNA editing sites, we could detect and compare the molecular sizes of the respective site-specific

| Name          | Sequence (5’-3’)                      | Purpose             |
|---------------|---------------------------------------|---------------------|
| T3 + ndhB-2For| AATTAACCCCTACCTAAGGGTACTAATGGAATGTA   | PCR forward         |
| T3 + ndhB-9For| AATTAACCCCTCAATAGGGTACTAATGGAATGTA   | PCR forward         |
| T3 + ndhF-1For| AATTAACCCCTCAATAGGGTACTAATGGAATGTA   | PCR forward         |
| T3 + rpoA-1For| AATTAACCCCTCAATAGGGTACTAATGGAATGTA   | PCR forward         |
| T3 + rpoB-1For| AATTAACCCCTCAATAGGGTACTAATGGAATGTA   | PCR forward         |
| T3 + vectorFor| AATTAACCCCTCAATAGGGTACTAATGGAATGTA   | PCR forward         |
| KS + ndhB-2Rev| TCGAGGTGACGGTATGAGACGTATGAGGTATGAGGT | PCR reverse         |
| KS + ndhB-9Rev| TCGAGGTGACGGTATGAGACGTATGAGGTATGAGGT | PCR reverse         |
| KS + ndhF-1Rev| TCGAGGTGACGGTATGAGACGTATGAGGTATGAGGT | PCR reverse         |
| KS + rpoA-1Rev| TCGAGGTGACGGTATGAGACGTATGAGGTATGAGGT | PCR reverse         |
| KS + rpoB-1Rev| TCGAGGTGACGGTATGAGACGTATGAGGTATGAGGT | PCR reverse         |

**Table 1. Oligonucleotide primers used in this study**

| Name          | Sequence (5’-3’)                      | Purpose             |
|---------------|---------------------------------------|---------------------|
| T3 + ndhB-2For| AATTAACCCCTACCTAAGGGTACTAATGGAATGTA   | PCR forward         |
| T3 + ndhB-2For| AATTAACCCCTACCTAAGGGTACTAATGGAATGTA   | PCR forward         |
| T3 + ndhF-1For| AATTAACCCCTACCTAAGGGTACTAATGGAATGTA   | PCR forward         |
| T3 + rpoA-1For| AATTAACCCCTACCTAAGGGTACTAATGGAATGTA   | PCR forward         |
| T3 + rpoB-1For| AATTAACCCCTACCTAAGGGTACTAATGGAATGTA   | PCR forward         |

**Gene targets**

| Gene        | Target Sequence (5’-3’)                      | Purpose             |
|-------------|-----------------------------------------------|---------------------|
| T3 + ndhB-2For| AATTAACCCCTACCTAAGGGTACTAATGGAATGTA   | PCR forward         |
| T3 + ndhB-9For| AATTAACCCCTACCTAAGGGTACTAATGGAATGTA   | PCR forward         |

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Underlines indicate ribose 2'-methoxy analogs.
Figure 1. *Trans*-acting factors specifically binding to the editing sites in the extracts of tobacco chloroplasts. (A) UV-crosslinking was performed with a respective RNA probe that was labeled with $^{32}$P at +1 (C to be edited). Lanes 1, without competitor RNA; lanes 2, a 100-fold molar excess of unlabeled probe RNA was added as a competitor; lanes 3, a 100-fold molar excess of control RNA that was a 161 nt transcript of a pGEM-T vector was added as a competitor. Free indicates the bands of a free probe that migrated in front of the protein bands on SDS–PAGE. (B) Comparison of the electrophoretic mobilities of p95s binding to ndhB-9 (lane 1) and ndhF-1 (lane 2).

| Editing site | Molecular mass | Detection | References |
|--------------|----------------|-----------|------------|
| NTndhB C1481 | 95 kDa         | UV crosslink | This study (ndhB-9) |
| NTndhF C290  | 95 kDa         | UV crosslink | This study (ndhF-1) |
| NTRpoA C680  | 93 kDa         | UV crosslink | This study (pOA-1) |
| NTRpOB C238  | 91 kDa         | UV crosslink | This study (pOB-1) |
| NTRpOB C2000 | 76 kDa         | UV crosslink | This study (pOB-4) |
| NTRpetB C611 | 70 kDa         | UV crosslink | (14,15) |
| PStpetB C611 | 70 kDa         | UV crosslink | (14) |
| ATndhD C2    | 68 kDa         | CRR4 gene   | (8)        |
| NTndhB C467  | 59 kDa         | UV crosslink | This study (ndhB-2) |
| NTpsBE C214  | 56 kDa         | UV crosslink | (14,15) |
| NTpBSD C2    | 25 kDa         | UV crosslink | (31)       |

**Table 2.** *Trans*-acting factors for chloroplast RNA editing, listed in the decreasing order of apparent molecular mass

NT, *Nicotiana tabacum*; PS, *Pisum sativum*; AT, *Arabidopsis thaliana*. Names of the editing sites are according to Heyes et al. (16).

Factors by specific labeling of the C at +1 and subsequent UV-crosslinking. Therefore, we first examined the RNA editing efficiencies of 36 tobacco editing sites in our *in vitro* RNA editing system (Kobayashi et al. will be submitted elsewhere), and picked up six editing sites that exhibited relatively high editing efficiency. Next, we carried out UV-crosslinking experiments for these six editing sites using the RNA substrates (from −120 to +21) labeled at +1 with $^{32}$P, and with tobacco chloroplast extracts that favor *in vitro* editing reactions. After crosslinking, RNA molecules that were not crosslinked with the proteins were digested by RNase, followed by SDS–PAGE. Figure 1 shows autoradiograms of the proteins that were crosslinked with the editing sites (+1). To discriminate specific binding proteins from nonspecific binding proteins, we added either a 100-fold molar excess of the same RNA (lanes 2 in each panel in Figure 1A) or the exogenous control RNA (lanes 3 in Figure 1A) as competitors. This treatment revealed the proteins that specifically bind to the editing sites, and Figure 1A denotes their apparent molecular masses (e.g. the 59 kDa protein is denoted as p59); these factors are summarized in Table 2 in comparison with those of previously reported factors. Among them, proteins specifically binding to the ndhB-9 and ndhF-1 editing sites especially attracted our attention, because their molecular masses appeared to be both 95 kDa. As such, we compared their electrophoretic mobilities on SDS–PAGE once again in adjoining lanes and after a long distance run. However, we could not find any difference between them (Figure 1B). This hints at the possibility that p95s of ndhB-9 and ndhF-1 may be either the same or structurally similar proteins. In the following study, we investigate this possibility by examining the biochemical properties of these proteins.

The 95 kDa proteins are *trans*-acting factors for RNA editing

To ensure that the p95s are involved in RNA editing, we examined the correlation between the RNA editing activity and the binding of p95s to their respective RNA editing sites. Here, we introduced 5 nt scanning mutations to the −15 to −1 regions of the ndhB-9 and ndhF-1 RNA substrates (−120 to +21) as shown in Figure 2A and D, respectively, and then supplemented them to an *in vitro* RNA editing system as competitors.

When wild-type substrates (BW and FW in Figure 2) were added as competitors in a 100-fold molar excess, they trapped the respective *trans*-acting factors, causing the disappearance of C-to-U RNA editing (lanes 3 in Figure 2B and E) as well as the UV-crosslinking signals (lanes 3 in Figure 2C and F). Under the same conditions, mutations spanning −15 to −11 (BM1 and FM1) and −10 to −6 (BM2 and FM2) canceled competition, resulting in the appearance of radiolabeled signals for C-to-U RNA editing (lanes 4 and 5 in Figure 2B and E) and UV-crosslinking (lanes 4 and 5 in Figure 2C and F). These indicate that the upstream sequences from −15 to −6 are essential for recruiting *trans*-acting factors that ensure RNA editing reactions, as well as the contact of p95s to the C at +1. Interestingly, mutations introduced into the region from −5 to −1 (BM3 and FM3 in Figure 2) did not cancel the competition. Rather, they weakened it, resulting in weak detections of both RNA editing and p95’s binding signals (lanes 6 in Figure 2). Therefore, RNA editing activity and the binding of p95s to the editing sites were well correlated over the mutations...
scanning from −15 to −1 for both the ndhB-9 and ndhF-1 editing sites. These correlations strongly suggest that p95s are trans-acting factors indispensable for RNA editing.

We previously reported for tobacco psbE-1 and petB-1 RNA editing sites that the site-specific trans-acting factors are recruited to the upstream cis-elements and then interact with the C residue (+1) to be edited (15). In order to test whether p95s are trans-acting factors with similar properties, we examined if p95s bind to the upstream cis-elements. As described above, cis-elements of the ndhB-9 and ndhF-1 editing sites that recruit trans-acting factors are located in the region from −15 to −6 (Figure 2). Thus, we introduced radiolabels at −10, in the midst of the respective cis-elements (Figure 3A and C, asterisk), and the resultant RNA probes were subjected to UV-crosslinking experiments. Figure 3B represents SDS–PAGE profiles of the proteins bound to −10 and +1 of the ndhB-9 editing site, and Figure 3D represents those of the ndhF-1 site. For both editing sites, p95s appeared to be specifically bound with upstream cis-elements (−10) as well as to the editing site (+1). As for −10 of the ndhF-1 site, another crosslinked signal was detected on SDS–PAGE with an apparent molecular mass slightly larger than that of p95 (Figure 3D). However, this binding signal appeared to be nonspecific, because it did not disappear when a homologous competitor was added in excess (Figure 3D, lane 2).

Taken together with these results, we conclude that the p95s are site-specific trans-acting factors for the ndhB-9 and ndhF-1 RNA editing sites, and that they are recruited by the upstream cis-elements (from −15 to −6) and then interact with the editing site (+1) similarly to p56, the trans-acting factor of the psbE-1 editing site (15).

However, the relationship between these two p95s that specifically bind to ndhB-9 or ndhF-1 is still unknown.

**P95 recognizes both ndhB-9 and ndhF-1 editing sites**

To clarify whether a given p95 can specifically bind to either or both of the ndhB-9 and ndhF-1 editing sites, we tested the binding specificity of the p95s to these sites by a cross-competition experiment (Figure 4).
First, we tested whether the p95 that specifically binds to the ndhB-9 site could also recognize the ndhF-1 site with the aid of ndhB-9 RNA (−120 to +21) radiolabeled at +1 as a probe. As was expected from Figure 2, RNA editing activity (Figure 4B) and the binding of p95 (Figure 4C) to the ndhB-9 site were both inhibited by the addition of the same RNA (BW) as a competitor (lanes 2), but not by BM2, which was mutated on the p95 binding site (lanes 3). Surprisingly, similar results were obtained when FW (−120 to +21 of the ndhF-1 site) and FM2 (the same as FW, but the cis-element was mutated) were added as competitors: FW inhibited both RNA editing and the binding of p95 to the ndhB-9 site (lanes 4), but FM2 did not (lanes 5). This result indicates that the p95 that specifically binds to the ndhB-9 site can also bind to ndhF-1 in a sequence-specific manner.

Next, we examined the reverse case, using the ndhF-1 RNA (−120 to +21) as a probe. The obtained results are shown in Figure 4D and E, indicating that the p95 that specifically binds to the ndhF-1 site can also recognize the ndhB-9 site.

These complementary results let us conclude that the ndhB-9 and ndhF-1 RNA editing sites are corecognized by the identical trans-acting factor, p95.

**DISCUSSION**

This study demonstrated that the C residues at six RNA editing sites of tobacco chloroplast RNAs are bound by their respective site-specific proteins (Figure 1). Table 2 summarizes their apparent molecular masses in comparison with those previously reported for other editing sites (8,12,14,15). The molecular masses distribute from 25 to 95 kDa, implying that the site-specific factors have molecular diversity. CRR4, which specifically binds to the immediate upstream region of the ndhD-1 site in Arabidopsis thaliana, is a member of the PPR protein family (18,20), and CRR4 contains 10 PPR motifs (8,19). If site-specific factors detected by UV-crosslinking are all acting factors are recruited by their respective upstream motifs in conjunction with their specificity to the binding sequences (38).

Competition experiments for ndhB-9 and ndhF-1 RNA editing factors (Figure 2) showed that site-specific trans-acting factors are recruited by their respective upstream cis-elements located from −15 to −6 in a sequence-specific manner, and that a close proximity (−5 to −1) has a weak effect on these interactions. These results are in accordance with previously proposed models for the site recognition of chloroplast RNA editing (9,10,12,14,15,39).

The most notable finding in this study is that the ndhB-9 and ndhF-1 RNA editing sites are recognized by the same trans-acting factor. This conclusion was obtained from cross-competition experiments between ndhB-9 and ndhF-1 RNAs in the in vitro RNA editing and p95-binding reactions (Figure 4). As shown in Figure 5A, the upstream regions (−15 to −1) of ndhB-9 and ndhF-1 represent a 60% identity in nucleotide sequence. This implies that a given trans-acting factor of chloroplast RNA editing could recognize groups of cis-elements that share moderate sequence identity. From this view, we reexamined the 38 RNA editing sites of the tobacco chloroplast genome to search for possible candidates recognized by common trans-acting factors. As shown in Figure 5B, six pairs of editing sites were found to have 60% or higher identity in their upstream sequences (−15 to −1) in addition to ndhB-9 and ndhF-1. If these pairs are really recognized by respective common factors, only 31 site-specific factors could be enough to account for 38 editing sites.

In transplastomic tobacco plants, overexpression of the ndhF-1 editing site caused a decrease in editing efficiency in endogenous ndhF-1, ndhB-3 and ndhD-1 sites, but not in the other sites (31; ndhF-1 site was mentioned as ndhF-2 in this reference; see Materials and Methods). This appears to imply that ndhF-1 editing site shares trans-acting factor(s) with ndhB-3 and ndhD-1 sites but not with ndhB-9 site. However, the present study demonstrated that ndhF-1 and ndhB-9 share the site-recognition factor of 95 kDa. Why was cosuppression not observed for ndhF-1 and ndhB-9 in the transplastomic plants? The putative cis-elements of ndhF-1, ndhB-3 and ndhD-1 do not share sequence identity until gaps are introduced. In plant organellar RNA editing, spacing between upstream cis-elements and editing sites was shown to be critical, with only one base insertion/deletion in such a region causing the complete loss of editing activity (17,22,39). Therefore, one possibility might be that the above cosuppression phenotype in transplastomic plants was caused by competition for some unknown factors other than site recognition protein. In accordance with this speculation, an Arabidopsis mutant deficient in CRR4, the site recognition protein of ndhD-1, cannot edit the ndhD-1 site but still possesses normal editing activity for the ndhF-1 and ndhB-3 sites (8). We should also point out that the cosuppression phenotype of RNA editing by overexpression of given editing sites could vary according to many parameters, including the relative abundance of
We know that such examples exist. The spinach ndhA site I (ndhA-189) was edited when introduced into the chloroplasts of Nicotiana tabacum or Nicotiana sylvestris, although these plants do not have ndhA-189 editing sites (42,43). As the immediate upstream regions (from −15 to −1) of spinach ndhA-189 and tobacco ndhF-1 share 60% sequence identity, ndhA-189 may also be recognized by p95, similarly to ndhB-9 and ndhF-1. This possibility remains to be examined.

In conclusion, this study clearly demonstrated that two RNA editing sites with cis-acting elements of moderate sequence identity are recognized by the same trans-acting factor in tobacco chloroplasts. This finding extends our knowledge that distinct proteins recognize each editing site (8,12,14,15), and suggests that more complex cis-trans recognition networks might be operating in plant organelles.

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Conflict of interest statement. None declared.

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