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Maintenance of plastid RNA editing activities independently of their target sites

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RNA editing in plant organelles is mediated by site-specific, nuclear-encoded factors. Previous data suggested that the maintenance of these factors depends on the presence of their rapidly evolving cognate sites. The surprising ability of allotetraploid *Nicotiana tabacum* (tobacco) to edit a foreign site in the chloroplast *ndhA* messenger RNA was thought to be inherited from its diploid male ancestor, *Nicotiana tomentosiformis*. Here, we show that the same *ndhA* editing activity is also present in *Nicotiana sylvestris*, which is the female diploid progenitor of tobacco and which lacks the *ndhA* site. Hence, heterologous editing is not simply a result of tobacco's allopolyploid genome organization. Analyses of other editing sites after sexual or somatic transfer between land plants showed that heterologous editing occurs at a surprisingly high frequency. This suggests that the corresponding editing activities are conserved despite the absence of their target sites, potentially because they serve other functions in the plant cell.

Keywords: RNA editing; chloroplast; cybrid; plastid transformation; *Nicotiana*

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INTRODUCTION

RNA editing in higher plant plastids and mitochondria changes the genetic information encoded in messenger RNAs by modifying nucleotides at highly specific positions. It is predominantly characterized by C-to-U modifications that restore codons conserved in evolution at a given site (Maier *et al*, 1992). Unlike the cognate editing sites of animal editing factors APOBEC1 or ADAR1, which are conserved at least in vertebrates (Bass, 2001; Levanon *et al*, 2005), editing sites in plant organelles are evolutionarily highly dynamic, with even closely related taxa having different sets of sites (Freyer *et al*, 1995; Schmitz-Linneweber *et al*, 2002; Sasaki *et al*, 2003). Indirect evidence suggested that the cognate editing factors are evolving rapidly: a spinach-specific and a maize-specific site introduced artificially into the tobacco plastid chromosome remained unedited (Bock *et al*, 1994; Reed & Hanson, 1997). In addition, no editing of a tobacco-specific editing site was found in a pea *in vitro* editing system (Miyamoto *et al*, 2002). Taken together, these data suggested that an RNA editing site and its cognate site-specific editing factors form an evolutionary unit that is rapidly evolving and is either present or absent in a given species.

The first case reported of a foreign site edited when transferred into plastids of a different species despite the absence of an endogenous homologue was that of the spinach *ndhA*-189 site by tobacco (Schmitz-Linneweber *et al*, 2001). Tobacco is an allotetraploid species that originated from a natural interspecific fertilization event that occurred between the progenitors of the modern diploid species *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. Site *ndhA*-189 is present and processed in *N. tomentosiformis*, but absent from *N. sylvestris* (Schmitz-Linneweber *et al*, 2001). On the basis of these results, the authors concluded that the most parsimonious explanation for the *ndhA*-189 editing activity found in tobacco was that the *N. tomentosiformis* gene for the factor persisted in the allotetraploid tobacco nuclear genome.

Here we show that the heterologous *ndhA* editing activity previously identified in tobacco is also present in its female progenitor *N. sylvestris*. We describe other heterologous editing events in an alloplasmic line and cybrid plants. The unexpectedly high frequency of heterologous editing suggests that there is a subgroup of editing factors that are conserved between plant taxa independently of their target sites. Possibly, such factors are retained because they edit several sites in plastid transcriptomes (Chateigner-Boutin & Hanson, 2002, 2003; Tillich *et al*, 2005).

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Table 1 | Editing sites in foreign genetic backgrounds

	Nicotiana tabacum	*Nicotiana sylvestris*	*Nicotiana glutinosa*	*Nicotiana tomentosiformis*	*Atropa belladonna*	*Pisum sativum*	*Spinacia oleracea*	*Zea mays*
ndhA	L189[1,7]	L189[2]	L189[3]	S189L[1]	S189L[4]	L189[5]	S191L[1]	S188L[6]
ndhD	L293[7]	L293[7]	S293L[7]	S293L[7]	S293L[4]	S293L[3]	S293L[3]	S293L[6]
psbE	P72S[8]	P72S[9]	P72S[3]	P72S[9]	S72[10]	S72[5]	S72[11]	S72[12]
rps14	P50L[8]	P50L[9]	P50L[3]	P50L[9]	L50[10]	L50[5]	S50L[3]	L53[12]
atpA	P264L[8]	P264L[9]	P264L[3]	P264L[9]	L264[10]	L264[5]	L264[11]	L264[12]
ndhD	S200L[8]	S200L[9]	S200L[3]	S200L[9]	L200[10]	L200[5]	L200[11]	L200[12]
ndhD	S225L[8]	S225L[9]	S225L[3]	S225L[9]	L225[10]	S225L[5]	L225[11]	L225[12]
psbF	F26[13]	F26[14]	F26[3]	F26[15]	F26[16]	S26F[5]	S26F[13]	F26[12]
rpoB	L208[17]	L208[14]	L208[3]	L208[15]	L208[16]	L208[5]	L208[11]	P206L[6]
rpoB	L809[10]	L809[14]	L809[3]	L809[15]	S809L[4]	ND	S809[3]	L814[12]

The chart summarizes information on editing sites that have been transferred between species. The table is divided by the thick line in editing sites processed in a heterologous nuclear background (above) and those not processed (below). Numbers refer to the codons affected, with the amino acids encoded before (left of the number) and after editing (right of the number) given in one-letter code. Shading: dark grey, sites known to be edited with codon transition and number of codon given (in contrast to previous investigations (Inada *et al*, 2004), we found *ndhD*-293 fully edited in pea plants (supplementary Fig 4D online); possibly, differences in growth conditions account for these contradicting observations); white, editing site absent, but capacity to edit the site heterologously was experimentally proven (Schmitz-Linneweber *et al*, 2005; this study); black, editing site absent, and heterologous editing upon experimental introduction of these sites failed (Bock *et al*, 1994; Reed & Hanson, 1997; Schmitz-Linneweber *et al*, 2005); light grey, site absent; hatching, site present but not edited. [1]Schmitz-Linneweber *et al* (2001); [2]Fig 1; [3]supplementary Fig 4 online; [4]Schmitz-Linneweber *et al* (2002); [5]Inada *et al* (2004); [6]Maier *et al* (1995), Tillich *et al* (2001); [7]Fig 2; [8]Hirose *et al* (1999); [9]Sasaki *et al* (2003); [10]Schmitz-Linneweber *et al* (2005); [11]accession no. AJ400848; [12]accession no. X86563; [13]Bock *et al* (1994); [14]accession no. AB237912; [15]accession no. AB240139; [16]accession no. AJ316582; [17]Reed & Hanson (1997); ND, not determined.

RESULTS

N. sylvestris harbours an activity to process *ndhA*-189

Previously, we used transformation vector pCEE to introduce the spinach *ndhA*-189 editing site into tobacco chloroplasts (Schmitz-Linneweber *et al*, 2001). Here, we used the same vector to transform *N. sylvestris* by particle bombardment (Svab & Maliga, 1993) to test whether this species also possesses an activity to process the foreign editing site *ndhA*-189, a site widespread in angiosperms (Table 1; Fig 1A). After bombardment of ten leaves, one spectinomycin-resistant line containing the *aadA* cassette was isolated. From this single transformant, two subclones, designated as SylCEE-2.8 and SylCEE-10.2, were established and maintained by micropropagation *in vitro*. Correct integration of the transgene was verified by PCR and Southern analysis (Fig 1B,C). Southern analysis further showed that the primary isolates were already homoplastomic for the mutation (Fig 1C).

Total leaf RNA from SylCEE-2.8 and SylCEE-10.2 was reverse transcribed and transgene-specific complementary DNAs were amplified by PCR and sequenced to assess RNA editing. Surprisingly, the transcripts containing spinach-derived sequences were processed in *N. sylvestris* plastids (Fig 1D), demonstrating that *N. sylvestris* harbours an activity to process *ndhA*-189. RNA editing of this site is only partial, as there is also a signal corresponding to the unedited transcript visible. It is noteworthy that SylCEE-10.2 showed a higher editing efficiency than SylCEE-2.8, which is paralleled by a lower steady-state level of transgenic *ndhA* transcript in line SylCEE-10.2 (supplementary Fig 1 online), suggesting that the differences in editing of the two transgenic lines may be linked to the differential abundance of target RNAs in these plants, as suggested for similar transformation experiments (Reed & Hanson, 1997; Reed *et al*, 2001a,b; Chateigner-Boutin & Hanson, 2002).

Why is the *ndhA*-189 activity maintained in *N. sylvestris*? Possibly, the underlying editing factors serve a cluster of plastid editing sites. Interestingly, *ndhA*-189 shares sequence homology with site *ndhF*-97 (supplementary Fig 2 online), a potential member of a cluster of sites (Chateigner-Boutin & Hanson, 2002, 2003). Processing of this or other sites could be affected in SylCEE plants, because the corresponding factor might be titrated by the competing transgene transcript (Reed *et al*, 2001a). We therefore tested editing of all described *N. sylvestris* sites (Sasaki *et al*, 2003), but did not find any significant reduction in editing anywhere (supplementary Fig 3 online). This is preliminary evidence that the *ndhA*-189 activity does not serve a further known editing site in the plastid transcriptome.

Heterologous editing in alloplasmic and cybrid plants

To determine whether heterologous editing is a more widespread phenomenon, we analysed plants that harbour foreign plastid genomes. Specifically, we tested the ability of the tobacco nucleus to edit foreign sites introduced into a tobacco nuclear background either by sexual or somatic hybridization.

Nicotiana tabacum Samsoun (NN) is a tobacco variety that carries an introgressed N gene, which confers disease resistance against the tobacco mosaic virus (Holmes, 1938). The N gene was introduced genetically by a backcrossing scheme from the donor species *N. glutinosa* making *N. tabacum* Samsoun (NN) effectively an alloplasmic line containing the *N. glutinosa* plastome (Clausen & Godspeed, 1925; Holmes, 1938; Fig 2A). We confirmed this by sequencing 2,809 bp of the *N. glutinosa* and *N. tabacum* Samsoun (NN) plastid chromosomes. They were identical, but had 12 point mutations relative to *N. tabacum* Petit Havanna. Next, we analysed the *N. glutinosa* plastid chromosome for the presence

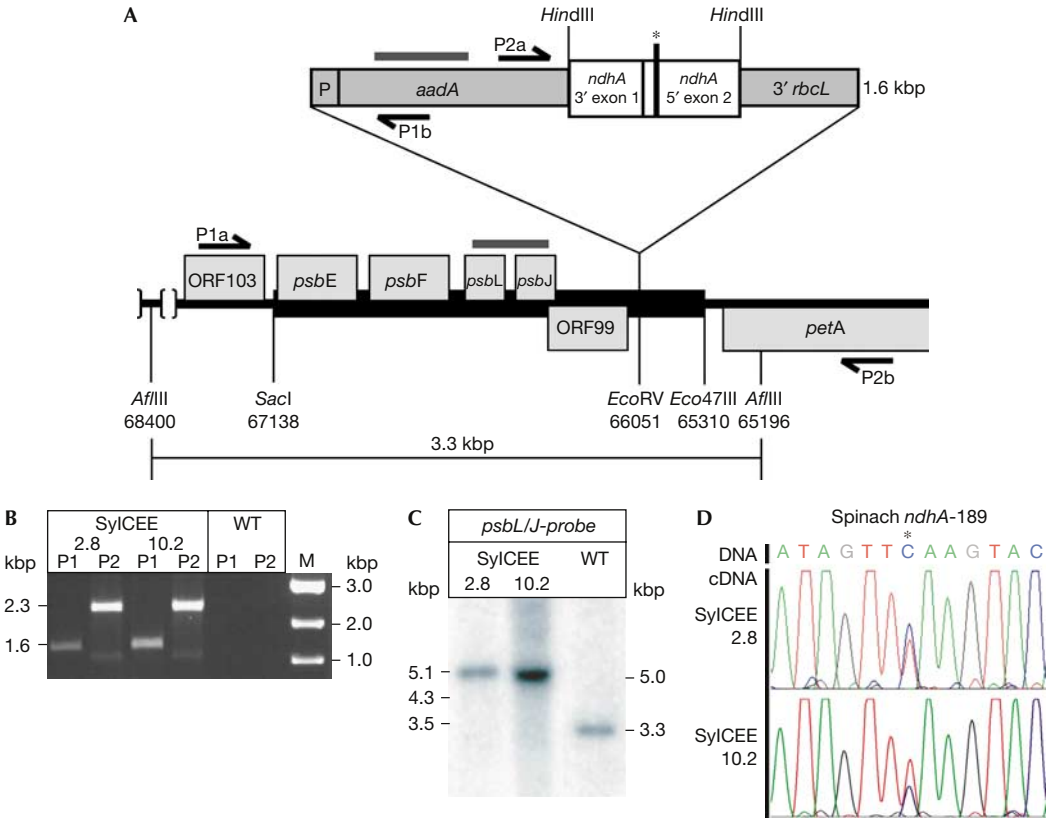

Fig 1 | Introduction of a recombinant spinach *ndhA* construct into the *Nicotiana sylvestris* plastid chromosome. (**A**) Schematic representation of parts of the plasmid vector pCEE, as described by Schmitz-Linneweber *et al* (2001). The thick black bar represents the tobacco plastid DNA fragment used for targeting the recombinant cassette to the *petA–psbJ* intergenic spacer. The grey bars indicate regions corresponding to probes used in Southern and RNA gel blot analysis. P, 16S ribosomal DNA promoter; 3′*rbcL*, 3′ stabilizing element. Arrows indicate primers. The asterisk denotes the position of the editing site *ndhA*-189. Numbers refer to positions in the tobacco plastid chromosome (accession no. Z00044). (**B**) PCR analysis of correct integration of the transgene into the *N. sylvestris* plastid chromosome in the two spectinomycine-resistant subclones SylCEE-2.8 and SylCEE-10.2 recovered after transformation. PCR product P1, 1,624 bp; P2, 2,320 bp (see (**A**) for positions of primers). M, molecular weight marker. (**C**) Southern analysis of *Afl*III-digested total genomic DNA (5 μg) of transplastomic lines SylCEE-2.8 and SylCEE-10.2. The probe covers the *psbL/J* genes (see (**A**)). Signals obtained correspond to calculated fragment length (indicated on the left; also see (**A**)). No signal corresponding to wild-type plastid DNA was detected in transplastomic plants, suggesting that they are homoplastomic for the transgene. (**D**) Complementary DNA sequence analysis of the introduced *ndhA* editing site in transplastomic lines SylCEE-2.8 and SylCEE-10.2. An excerpt of the corresponding chromatograms is shown with the edited nucleotide in the centre marked by an asterisk. A C-peak corresponding to unedited messages and a T-peak corresponding to edited messages were found in the plant lines analysed (top: corresponding DNA sequence).

of editing sites known to be absent from tobacco but present in other dicots, because such sites could be analysed for heterologous editing in *N. tabacum* Samsoun (NN). In total, ten editing sites not present in tobacco have been identified in dicots, namely *accD*-284, *clpP*-187, *matK*-219, *ndhA*-189, *ndhB*-419, *ndhD*-293, *ndhD*-296, *petL*-2, *psbF*-26 and *rpoB*-809 (supplementary Fig 4 online). In addition, we included a maize site, *rpoB*-206 (codon 208 in tobacco; supplementary Fig 4 online), previously tested for heterologous editing (Reed & Hanson, 1997). The corresponding regions in the *N. glutinosa* plastid chromosome were amplified and sequenced. This led to the identification of editing site *ndhD*-293 in *N. glutinosa*, which was also shown to be present in *N. tabacum* Samsoun (NN) and represents a widely distributed editing site in angiosperms (Table 1). cDNA analysis showed that this site was processed in *N. glutinosa* and in *N. tabacum* Samsoun (NN; Fig 2A). Thus, *ndhD*-293 is processed after sexual introduction into an *N. tabacum* nuclear background. Apparently, the tobacco nucleus contains the factor for editing this site despite its absence from the plastid genome, although it cannot be excluded that a gene tightly linked to the *N. glutinosa* N gene is coding the required activity.

Apart from alloplasmic lines, a second source for the study of heterologous editing are somatically generated cytoplasmic hybrids (cybrids) that combine plastid editing sites from one species with the nuclear background of another. Nt(Ab) cybrids have a nuclear genome of tobacco, Nt, and a plastid genome of *Atropa belladonna* (nightshade), (Ab). These cybrids were generated by fusion of somatic cells *in vitro*, and phenotypically resemble normal wild-type tobacco (Babiychuk *et al*, 1995). The nightshade plastid genome contains three editing sites not present in tobacco (Schmitz-Linneweber *et al*, 2002). Two of these heterologous sites, *ndhA*-189 and *ndhD*-293, were fully processed

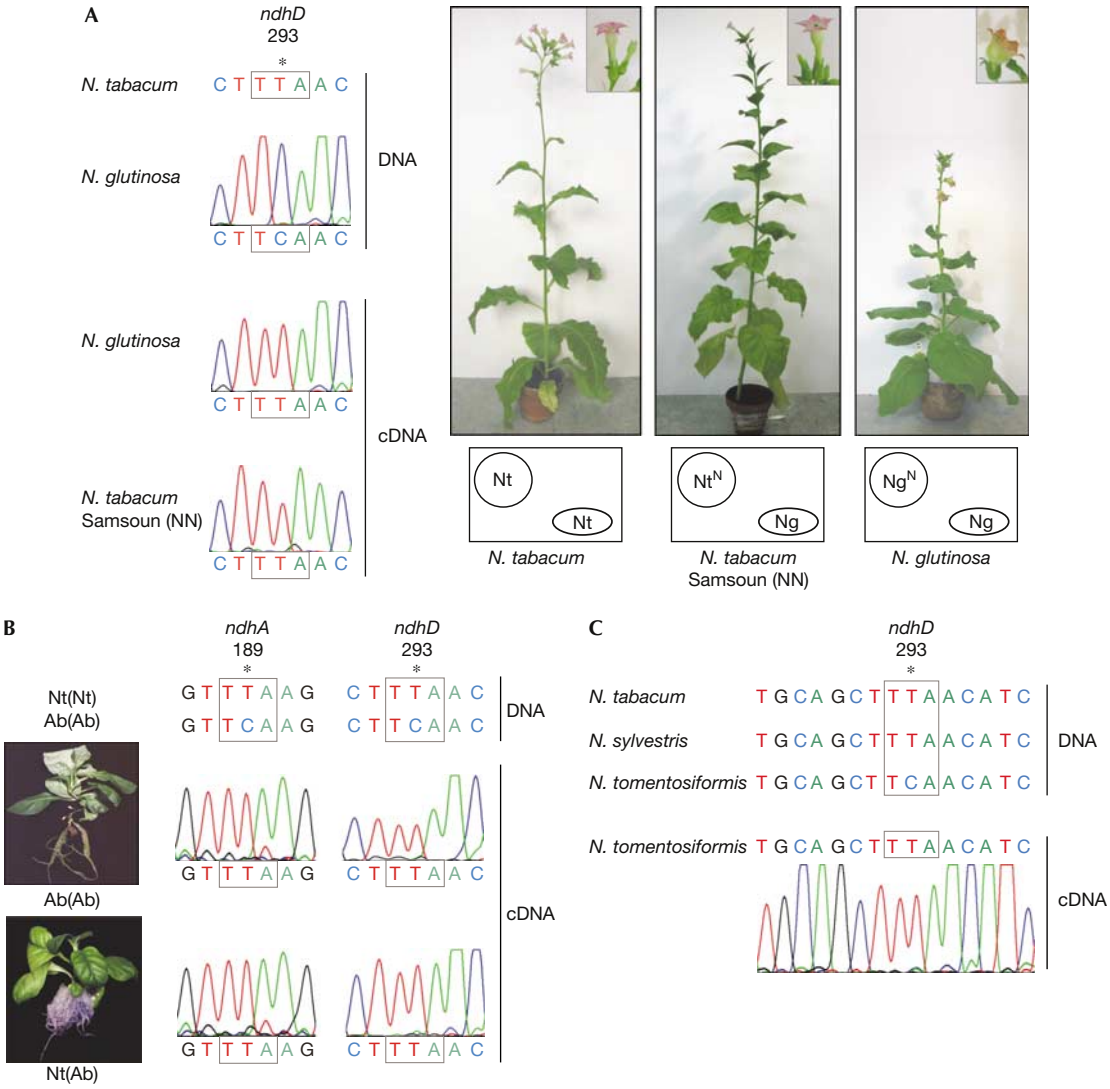

Fig 2 | Heterologous processing of editing sites after somatic or sexual transfer into a foreign genetic background. (**A**) Editing of *ndhD*-293 in *Nicotiana glutinosa*, *Nicotiana tabacum* Petit Havanna and the introgression line *N. tabacum* Samsoun (NN). Excerpts of the sequencing chromatograms are shown, with the edited position marked by an asterisk and the affected codon boxed. Phenotypes of mature plants are shown, with the inset showing the different flower morphologies. Circles indicate the nuclei and ovals the plastids. Nt, *N. tabacum* Petit Havanna; Ng, *N. glutinosa*. Superscript N indicates the presence of the N-gene conferring resistance against tobacco mosaic virus. (**B**) Complementary DNA sequence analysis of editing sites *ndhA*-189 and *ndhD*-293 in nightshade Ab(Ab) and cybrid Nt(Ab) seedlings. Relevant chromatogram excerpts are shown, with the edited position and codons marked as in (**A**). (**C**) *Nicotiana tomentosiformis* possesses and processes editing site *ndhD*-293, whereas this site is absent in *Nicotiana sylvestris* and *N. tabacum*. Partial *ndhD* DNA sequences for the three *Nicotiana* species are aligned. Below is an excerpt of a cDNA sequence from *N. tomentosiformis* together with the corresponding chromatogram. Editing sites and affected codons are marked as in (**A**).

in cybrids (Fig 2B), whereas the third site, *rpoB*-809, remained unedited (Schmitz-Linneweber *et al*, 2005). This confirms previous findings that site *ndhA*-189 is processed when brought into a tobacco nuclear background by plastid transformation (Schmitz-Linneweber *et al*, 2001) and shows that the respective activity present in tobacco chloroplasts is efficiently processing site *ndhA*-189 when expressed from its natural promoter. This finding further supports that the partial editing observed in transplastomic tobacco (Schmitz-Linneweber *et al*, 2001) and *N. sylvestris* lines (Fig 1D) is due to a titration effect of the *ndhA*-189 activity by overexpression of the target site. The presence of an activity responsible for *ndhD*-293 editing in the Nt(Ab) cybrid suggests that this activity was not co-introduced with the N gene into *N. tabacum* Samsoun (NN), but is already encoded in the nuclear genome of tobacco. Interestingly, like site *ndhA*-189, *ndhD*-293 is present in tobacco's diploid male progenitor *N. tomentosiformis*, but absent from its female progenitor and donor of plastids *N. sylvestris* (Fig 2C; Schmitz-Linneweber *et al*, 2001). Whether *N. sylvestris* is able to process this site, similar to the situation for *ndhA*-189, remains to be determined.

DISCUSSION

This study draws attention to the fact that several plant organellar editing sites are processed after transfer into a foreign nuclear background. Independent transfer events of editing sites between species using three alternative models (transplastomic plants, alloplasmic lines and cybrid lines) prove that editing factors can persist in plant genomes despite the absence of their target site (Figs 1,2; Schmitz-Linneweber *et al*, 2001). Four novel heterologous editing events have been found concerning two sites (*ndhA*-189 in SylCEE and Nt(Ab) and *ndhD*-293 in Nt SNN and Nt(Ab)). Thus, out of a total of ten different heterologous editing sites analysed here and previously (Bock *et al*, 1994; Reed & Hanson, 1997; Schmitz-Linneweber *et al*, 2001, 2005), four are edited. Apparently, the nuclear-encoded activities for these four sites have not been lost after loss of their cognate site, but are maintained. Possibly, such maintenance of editing factors is simply due to chance: the factor is not (yet) lost, because loss of the target site occurred rather recently in evolution. Alternatively, some factors might be maintained by selection, because they are required to fulfil another function.

We assumed earlier that the tobacco activity for *ndhA*-189 was inherited during allotetraploidization from tobacco's diploid male parent, *N. tomentosiformis* (Schmitz-Linneweber *et al*, 2001). Similar arguments have been made for the inheritance of other tobacco editing activities (Sasaki *et al*, 2003). Here, we show however by a transgenic approach that even though its cognate site is absent, the editing activity for *ndhA*-189 is present in *N. sylvestris*. Therefore, the existence of at least this hidden editing activity in tobacco cannot be adequately explained by allotetraploidy alone. Instead, the presence of this activity in both ancestors of tobacco and its survival through the—from a genomic point of view—stressful allotetraploidization event towards tobacco argue for long-term maintenance of the factor and tentatively suggest that some other sort of selective pressure for the stable maintenance of editing activities besides editing the *ndhA* site guarantees its maintenance. Such a selective pressure for the stable maintenance of editing activities was predicted earlier (Covello & Gray, 1993) and would also help to understand the unexpected high frequency of heterologous editing described here.

What could be the nature of such putative additional selective pressures/functions for editing factors? Most parsimoniously, they could be required for processing a different editing site. This would agree with recent findings that editing sites tend to form evolutionarily and functionally linked clusters, potentially served by group-specific editing factors (Chateigner-Boutin & Hanson, 2003; Tillich *et al*, 2005) and supports earlier suggestions that editing factors are maintained because they serve not only one target site, but many (Covello & Gray, 1993). Alternatively, the factors could also be involved in a task unrelated to editing. The only specificity factor of plant organelle RNA editing isolated so far belongs to the pentatricopeptide repeat (PPR) protein family (Kotera *et al*, 2005), members of which have diverse roles in organellar RNA metabolism (Lurin *et al*, 2004). Further analyses are needed to show whether more editing factors are PPR proteins and whether such factors are exclusively involved in RNA editing or do have several functions, for instance in other aspects of organellar RNA metabolism.

METHODS

Preparation of nucleic acids and their analysis. Plants were grown on soil in the green house or on agar-solidified MS medium (Murashige & Skoog, 1962) under sterile conditions in a growth chamber. Total cellular DNA was extracted using a standard cetyltrimethylammonium bromide protocol. Total cellular RNA was isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany). First-strand cDNA synthesis was carried out with Superscript II RNase H^- Reverse Transcriptase (Invitrogen). Amplification of DNA fragments by PCR was carried out according to standard protocols (Sambrook *et al*, 1989). PCR products were sequenced as described previously (Schmitz-Linneweber *et al*, 2002). All oligonucleotides were bought from MWG-BIOTECH (Ebersberg, Germany) (supplementary Table 1 online). For gel blot analyses, nucleic acids were separated by gel electrophoresis and transferred onto nitrocellulose membranes (Sambrook *et al*, 1989).

Plastid transformation of *Nicotiana sylvestris*. Young leaves were harvested from sterile grown *N. sylvestris* plants and bombarded with plasmid DNA-coated gold particles by using the biolistic PDS-1000/He unit (Bio-Rad, Munich, Germany; Svab & Maliga, 1993). Spectinomycine-resistant shoots were selected on RMOP regeneration medium containing 500 mg/l spectinomycine dihydrochloride (Svab & Maliga, 1993). Plastid transformants resulting from homologous recombination events were identified by PCR, and homoplastomy was verified by Southern blot analysis.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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