Amino acid sequence variations in Nicotiana CRR4 orthologs determine the species-specific efficiency of RNA editing in plastids

Kenji Okuda¹, Yuya Habata², Yoshichika Kobayashi² and Toshiharu Shikanai¹,*

¹Department of Botany, Graduate School of Science, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan and ²Graduate School of Agriculture, Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

Received August 1, 2008; Revised September 10, 2008; Accepted September 12, 2008

ABSTRACT

In flowering plants, RNA editing is a posttranscriptional process that converts specific C to U in organelle mRNAs. Nicotiana tabacum is an allotetraploid species derived from the progenitors of Nicotiana sylvestris and Nicotiana tomentosiformis. These Nicotiana species have been used as a model for understanding the mechanism and evolution of RNA editing in plastids. In Nicotiana species, the ndhD-1 site is edited to create the translational initiation codon of ndhD that encodes a subunit of the NAD(P)H dehydrogenase (NDH) complex. An analysis of this RNA editing revealed that editing efficiency in N. tomentosiformis is lower (15%) than that in N. tabacum (42%) and N. sylvestris (37%). However, this level of editing is sufficient for accumulating the NDH complex and its activity. The heterogous complementation of Arabidopsis crr4-3 mutant, in which RNA editing of ndhD-1 is completely impaired, with CRR4 orthologous genes derived from Nicotiana species suggested that the reduction in editing efficiency in N. tomentosiformis is caused by amino acid variations accumulating in CRR4.

INTRODUCTION

RNA editing is a posttranscriptional process in which a specific C nucleotide is altered to U in an RNA molecule in the mitochondria and plastids of flowering plants (1–3). In contrast, U to C conversions occur frequently in both ferns and hornworts (4,5). In flowering plants, about 30 editing sites have been detected in plastid genomes and more than 400 editing sites in mitochondrial genomes (6–9). In contrast to other RNA maturation steps in plant organelles including RNA splicing, intergenic RNA cleavage and RNA stabilization, RNA editing sites are highly divergent among species. Unlike introns, which are phylogenetically conserved in their positions, and structures in plastids, even closely related species exhibit distinct editing site patterns (10–13), suggesting the dynamic evolution of editing sites even in current establishments of species. An analysis of transplastomic lines suggested that the cognate editing factors corresponding to specific editing sites are co-evolving rapidly: spinach- and maize-specific sites introduced into the tobacco plastid genome remained unedited (14,15). In addition, a tobacco-specific editing site was not edited in a pea in vitro editing system (16). Thus, the RNA editing machinery in plastids appears to be phylogenetically dynamic.

Recent work employing plastid transformation and in vitro RNA editing system has shed some light on the molecular mechanisms of plastid RNA editing. For site-specific RNA editing in plastids, a cis-element is essential and consists of fewer than 30 nt surrounding the editing site and also a further upstream sequence in some cases (17–20). The case is similar for mitochondria (21). In addition, a genetic study of photosynthetic electron transport led to the discovery of nucleus-encoded factors responsible for specific RNA editing events. The Arabidopsis crr4 (chlororespiratory reduction) and crr21 mutants are defective in RNA editing for sites 1 (ndhD-1) and 2 (ndhD-2), respectively, in ndhD mRNA (22,23). The ndhD gene encodes a subunit of the chloroplast NAD(P)H dehydrogenase (NDH) complex, which is involved in the cyclic electron flow around photosystem I (24). CRR4 and CRR21 genes both encode members of the pentatricopeptide repeat (PPR) protein family (22,23). More recently, it was found that Arabidopsis PPR protein, CLB19, is involved in RNA editing of rpoA and...
**Materials and Methods**

**Plant materials**

*Nicotiana tabacum* (L. var. Xanthi), *N. sylvestris* and *N. tomentosiformis* leaves were harvested from 6-week-old plants grown in a growth chamber at 28°C under 16 h light/8 h dark conditions.

**Chlorophyll fluorescence analysis**

Chlorophyll fluorescence was measured by using a MINI-PAM (pulse amplitude modulation) portable chlorophyll fluorometer (Walz, Effeltrich, Germany). The transient increase in chlorophyll fluorescence after turning off the actinic light (AL) was monitored as previously described (45).

**Analysis of RNA editing**

Total RNA was isolated from green leaves of *Nicotiana* species by using an RNAeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and treated with DNase I (Invitrogen, Carlsbad, CA, USA). DNA-free RNA (2.5 µg) was reverse-transcribed with random hexamers. The sequence including the ndhD-1 editing site was amplified by PCR with primers ndhD-1-FW and ndhD-1-RV (Supplementary Data 1). The RT–PCR products were sequenced directly. To analyze the editing efficiency of ndhD-1, the sequence including the ndhD-1 editing site was amplified by PCR using the primers ndhD-1-FW2 and ndhD-1-RV (Supplementary Data 1). The RT–PCR products were cloned into the pTAC-1 vector (BioDynamics Laboratory, Tokyo, Japan) and transformed in *Escherichia coli*. PCR products were amplified by using primers BD-FW and BD-RV (Supplementary Data 1) from 100 independent clones, digested with *Nla*III and analyzed with 8% polyacrylamide gel.

For analysis of the RNA editing efficiency of ndhD-1 in *Arabidopsis*, cDNAs were isolated as previously described (22). The sequence including the ndhD-1 editing site was amplified by PCR using the primers AtndhD-1-FW and AtndhD-1-RV (Supplementary Data 1). The RT–PCR products were cloned into the pTAC-1 vector (BioDynamics Laboratory) and transformed in *E. coli*. PCR products were amplified by using primers BD-FW and BD-RV (Supplementary Data 1) from 100 independent clones, digested with *Nla*III and analyzed with 8% polyacrylamide gel. For further high-throughput quantitative analysis of editing efficiency, the RT–PCR products, which were digested with *Nla*III, were analyzed with QIAxcel System (Qiagen). The efficiency of RNA editing was quantified by comparing the signal intensity of the sensitive and resistant DNA fragments to *Nla*III using the software of Biocalculator (Qiagen).
Protein blot analysis

Chloroplasts were isolated from the leaves of 4-week-old plants as previously described (22). The protein samples were separated by 12.5% SDS–PAGE and used for immunodetection.

Isolation and sequence analysis of cDNAs

ESTs encoding for protein that shows a high sequence identity with Arabidopsis thaliana CRR4 (AtCRR4) was sought by the RIKEN Transcriptome Analysis of BY-2, EST search database (http://mrg.psc.riken.go.jp/struc/blast.html). Total RNA was isolated from green leaves of Nicotiana species by using a RNAeasy Plant Mini Kit (Qiagen) and treated with DNase I (Invitrogen). DNA-free RNA (2.5 μg) was reverse-transcribed with oligo(dT)20 primer. cDNA was amplified by PCR using appropriate primers designed from the EST sequence and the resulting DNAs were cloned into the pGEM-T vector (Promega, Madison, WI, USA), and its sequence was determined.

The 5′-terminal portion of the cDNA was isolated by 5′-rapid amplification of cDNA ends (5′-RACE) using primers designed from the sequences determined in this study. The procedure is as described in the instruction manual for the 5′-RACE System for Rapid Amplification of cDNA Ends, version 2.0 Kit (Invitrogen) and the resulting cDNAs were cloned into the pGEM-T vector (Promega), and their sequences were determined. The 3′-terminal portion of the cDNA was isolated by 3′-rapid amplification of cDNA ends (3′-RACE) using primers designed from the sequences determined in this study. The procedure is as described in the instruction manual for 3′-Full RACE Core Set (Takara, Kyoto, Japan), and the resulting cDNAs were cloned into the pGEM-T vector (Promega), and their sequences were determined.

Plant transformation

For heterogous complementation of crr4-3, the nucleotide sequences encoding the putative CRR4 orthologous genes were amplified from the genomic DNA of N. sylvestris (NsyICRR4) and N. tomentosiformis (NtomCRR4) by PCR using primers NsyICRR4-FW and -RV and NtomCRR4-FW and -RV, respectively (Supplementary Data 1). The amplified DNA fragments were translationally fused with the promoter and 5′-UTR of AtCRR4 PCR-amplified using primers ProCRR4-FW and ProCRR4-RV (Supplementary Data 1). The resultant DNA fragments were subcloned into the pBIN19 vector and introduced into crr4-3 via Agrobacterium tumefaciense MP90.

Quantitative real-time RT–PCR

Total RNA was isolated from green leaves of Arabidopsis by using a RNAeasy Plant Mini Kit (Qiagen) and treated with RNase-free DNase Set (Qiagen). DNA-free RNA (1.0 μg) was reverse transcribed with oligo(dT)20 primer. For quantitative RT–PCR, gene-specific primers were designed for NsyICRR4, NtomCRR4, and a potential control gene, AtACT8 (Supplementary Data 1). Primers were validated using serial dilutions of purified plasmids containing the corresponding genes. Standard curves were plotted and primers with amplification efficiencies of 100 ± 10% were selected for quantitative RT–PCR analysis. Dissociation curves were performed after the PCR reaction to confirm that single, specific products were produced in each reaction. The PCR amplification reaction was carried out with the MX3000P QPCR system (Stratagene, LA Jolla, CA, USA). PCR reaction mixture (20 μl) contained 10 μl of 2× Brilliant II SYBR Green QPCR Master Mix, 0.5 μM of each primers and 1 μl of a 20th diluted cDNA template. The PCR reaction was 95°C for 10 min, 40 cycles of 95°C for 30s and 60°C for 1 min and then the fluorescence was measured at each cycle at 60°C. Threshold cycle (Ct) values from triplicate samples were averaged and the SD value was calculated. The logarithmic average Ct value for each gene and the control gene was converted to a linear value using the 2−ΔCt term (46). Converted values were normalized to the AtACT8 gene by dividing average value for each gene by the average value of the control gene AtACT8.

RESULTS

NDH complex is active in N. tomentosiformis

The ndhD-1 site is edited to create the translational initiation codon of ndhD in N. tabacum and N. sylvestris, but the site was reported to remain unedited in N. tomentosiformis (12). To investigate whether the NDH complex is functional in N. tomentosiformis, we analyzed NDH activity by using PAM fluorometry in Nicotiana species. The chloroplast NDH complex catalyzes electron donation to plastoquinone from the stromal electron pool and its activity can be monitored as a transient increase in chlorophyll fluorescence after turning off AL (45). The increase in fluorescence is due to the reduction of plastoquinone by the stromal electron pool, which accumulates during AL illumination. Figure 1 shows a typical trace of the chlorophyll fluorescence level in N. tabacum, in which a transient increase in chlorophyll fluorescence after AL illumination was detected. In tobacco ΔndhB mutant, in which the ndhB gene was disrupted by the insertion of an aadA cassette (45), this transient increase in fluorescence level was suppressed (Figure 1), indicating that NDH activity is impaired. In N. tomentosiformis, the transient increase in fluorescence level was detected as well as in N. tabacum and N. sylvestris (Figure 1), indicating that NDH complex is active in N. tomentosiformis.

RNA editing activity of ndhD-1 site is reduced in N. tomentosiformis

The detection of NDH activity in N. tomentosiformis suggests that the ndhD mRNA would be translated. The crr4 phenotype indicates that the translation is severely suppressed in the absence of this RNA editing (22). To investigate whether the site is also edited in N. tomentosiformis, RNA editing of the ndhD-1 site was analyzed by direct sequencing RT–PCR products (Figure 2A).
Results are expressed as the total averages of clone analyzes using three independent plants. Approximately 100 clones were analyzed in each experiment. Consistent with a previously reported result (12), the ndhD-1 site was partially edited in leaves of both N. tabacum and N. sylvestris (Figure 2A). However, in contrast with the previous results, the ndhD-1 site was also partially edited in leaves of N. tomentosiformis (Figure 2A). The editing efficiency of ndhD-1 was analyzed as described in Materials and methods section. The primer ndhD-1-FW2 was used to create the restriction enzyme site of ndhD-1. The editing efficiency of ndhD-1 was analyzed as described (50). In N. tomentosiformis, the ndhD-1 site was partially edited in leaves of both N. tabacum and N. sylvestris (Figure 2B). These results indicate that N. tomentosiformis (Figure 2B). These results indicate that N. tomentosiformis (Figure 2B). These results indicate that N. tomentosiformis (Figure 2B). These results indicate that N. tomentosiformis (Figure 2B). These results indicate that N. tomentosiformis (Figure 2B). These results indicate that N. tomentosiformis (Figure 2B). These results indicate that N. tomentosiformis (Figure 2B). These results indicate that N. tomentosiformis (Figure 2B). These results indicate that N. tomentosiformis (Figure 2B). These results indicate that N. tomentosiformis (Figure 2B). These results indicate that N. tomentosiformis (Figure 2B). These results indicate that N. tomentosiformis (Figure 2B).
RNA editing. In ndhD-1 RNA editing in *Arabidopsis*, a PPR protein, CRR4, specifically recognizes the *cis*-element of the ndhD-1 site, which consists of at least the 25-nt upstream and 10-nt downstream sequences surrounding the editing site (37). It is possible that *N. tomentosiformis* does not have either an appropriate *cis*-element or *trans*-factor for the efficient editing of ndhD-1. We first compared the 35 nt surrounding the ndhD-1 site among *Nicotiana* species to show that the sequence is completely conserved (Figure 4A). Furthermore, the *cis*-element is also similar to that of *Arabidopsis thaliana*, suggesting that the element is recognized by a *trans*-factor similar to AtCRR4 in *Nicotiana* species.

We next investigated the possibility that the activity or the expression level of the *trans*-factor for the ndhD-1 RNA editing is reduced in *N. tomentosiformis*. We surveyed the RIKEN Transcriptome Analysis of BY-2 EST search database and found the EST that exhibits a high nucleotide sequence identity for AtCRR4 in *N. tabacum*. Based on this EST, full-length cDNAs were isolated from both *N. sylvestris* (NsylCRR4) and *N. tomentosiformis* (NtomCRR4). They contain an open reading frame (ORF) of 1878 nt that encode a putative CRR4 protein.
orthologous protein of 626 amino acids (Figure 4B). PCR amplification of the genomic genes indicated that these genes are not disrupted by any introns in either Nicotiana species, which is not the case with Arabidopsis (data not shown). The program ChloroP 1.1 predicted that the first 79 amino acids were the target signal to the plastids (Figure 4B). NsylCRR4 and NtomCRR4 show 60% and 57% identity to AtCRR4, respectively (Figure 4B). In contrast, a BLAST search revealed that NsylCRR4 and NtomCRR4 did not exhibit significant sequence identity to any other PPR proteins in Arabidopsis (about 25% identity or less).

On the basis of bioinformatic analysis, the PPR protein family is subdivided into the P and PLS subfamilies (27). The PLS subfamily exhibits a variable tandem repeat of a standard pattern of three PPR motifs (27). Based on the association or not of this repeat with three non-PPR motifs at their C-terminus, the PLS subfamily is subdivided into a further four subgroups: PLS, E, E + and DYW (27). Both NsylCRR4 and NtomCRR4 contain 11 characteristic PPR motifs and the E and E + motifs following a tandem array of PPR motifs, whose motif structures are very similar to AtCRR4 except that Nicotiana CRR4 has a longer E + motif than AtCRR4 (Figure 4B). The amino acid sequences of NsylCRR4 and NtomCRR4 were 93% identical (97% similar), indicating that 37 amino acids were divergent in the mature region between NsylCRR4 and NtomCRR4 (Figure 4B). Thirty-two amino acid alterations were detected in the N-terminal PPR motifs and the C-terminal E/E + motifs were highly conserved for NsylCRR4 and NtomCRR4 (Figure 4B).

Low editing efficiency at ndhD-1 site in N. tomentosiformis caused by low CRR4 activity

Given the high sequence similarity, it is likely that these Nicotiana proteins are Arabidopsis CRR4 orthologs. To confirm this possibility, NsylCRR4 and NtomCRR4 genes were expressed in Arabidopsis crr4-3, in which the RNA editing of the ndhD-1 site is completely impaired (22). NDH activity was detected in both transgenic lines (Figure 5A) and the NhD level was also restored close to the wild-type level (Figure 5B). We also confirmed that the introduction of these genes restored the RNA editing of ndhD-1 in crr4-3 (Figure 5C). We conclude that NsylCRR4 and NtomCRR4 are orthologs of AtCRR4 and function as trans-factors of ndhD-1 RNA editing in Nicotiana species. Consistent with the conservation of cis-elements among Arabidopsis and Nicotiana species (Figure 4A), the mechanism of the RNA editing of ndhD-1 is conserved.

We then investigated the possibility that the lower level of the editing efficiency of ndhD-1 in N. tomentosiformis is due to the low level of NtomCRR4 transcripts. To verify this possibility, the transcript level of endogenous CRR4 orthologs in two Nicotiana species was analyzed by quantitative RT–PCR. The transcript level in NtomCRR4 was about a half of that in NsylCRR4 (Supplementary Data 2).

Although the introduction of NtomCRR4 restored the accumulation of NDH complex in crr4-3, a quantitative analysis of the RNA editing efficiency using enzymatic digestion of PCR products from 100 independent clones clarified that the editing level was lower than those in the wild-type Arabidopsis and crr4-3 complemented by the introduction of NsylCRR4 (Figure 5C). This result appears to reflect the difference between the editing efficiencies of N. sylvestris and N. tomentosiformis (Figure 2B). However, it is possible that the difference may simply reflect the expression of transgenes. To eliminate this possibility, we analyzed the transcript level of transgenes in 11 independent lines transformed with each gene using quantitative RT–PCR analysis (Figure 5D). The transcript levels of transgenes were similar between lines expressing the different Nicotiana CRR4 orthologs, although there are minor fluctuations in the transcript level between lines (Figure 5D). We also determined the efficiency of the RNA editing using the QIAxcel system, in which PCR products are quantitatively analyzed in the automated capillary-type electrophoresis. The average of editing efficiency in crr4-3 complemented by NsylCRR4 and by NtomCRR4 were 39% and 19%, respectively (Figure 5D), consistent with the editing efficiency determined by cloning of PCR products (Figure 5C). We conclude that NtomCRR4 has slightly lower activity in editing of the ndhD-1 site in Arabidopsis, and the same story is probably true in N. tomentosiformis. We cannot eliminate the possibility that NtomCRR4 is slightly less stable than NsylCRR4 both in N. tomentosiformis and Arabidopsis.

DISCUSSION

In this study, we found that the ndhD-1 site is edited in N. tomentosiformis, although its efficiency is lower than those in N. tabacum and N. sylvestris (Figure 2). This observation is inconsistent with a previous report in which direct cDNA sequencing of this site could not detect any editing in N. tomentosiformis (12). A similar discrepancy was also reported in Arabidopsis. Although Lutz and Maliga (47) reported that the ndhD-1 site was not edited in Arabidopsis leaves, it was partially edited as detected in other plants (13,22). This disagreement would be explained in terms of the influences imposed by plant development, tissue type, age and environmental factors (48,49). Furthermore, the site is partially edited even in wild-type leaves, which makes it difficult to detect low levels of RNA editing under certain conditions using a low resolution method such as the direct sequencing of RT–PCR products.

Heterogenous complementation by NtomCRR4 restored the crr4-3 defect with lower efficiency (21%) compared with that realized by NsylCRR4 (40%) (Figure 5C and D). We believe that the difference reflects the variations in the CRR4 sequence. Since the efficiency was evaluated with an identical genetic background and both transgenes were driven by the AtCRR4 promoter, 5'- and 3'-UTRs and NOS terminator, we also believe that the difference in CRR4 activity reflected the RNA editing efficiency.
In fact, results from the quantitative RT–PCR analysis did not show the significant differences in transcript level between *NsylCRR4* and *NtomCRR4* lines (Figure 5D). We did not detect any tight correlations between the expression level of the transgene and the editing efficiency, except for two lines, #6 and #11, transformed with *NtomCRR4*, in which both transcript level and RNA editing efficiency were decreased (Figure 5D). We do not eliminate the possibility that the minor reduction in *NtomCRR4* transcript level in *N. tomentosiformis* somewhat influences the RNA editing efficiency. However, the RNA editing efficiency is significantly higher in *NsylCRR4* lines compared to that in *NtomCRR4* lines accumulating a similar level of transcripts (Figure 5D). Furthermore, the overexpression of *AtCRR4* under the control of the cauliflower mosaic virus 35S promoter did not increase the efficiency of RNA editing, indicating that the efficiency of the RNA editing is not restricted by the CRR4 transcript level (22,23). Thus, it is likely that difference in CRR4 function determine the RNA editing efficiency between *N. sylvestris* and *N. tomentosiformis*, rather than the difference in gene expression. We do not eliminate the possibility that the amino acid variations of CRR4 between *Nicotiana* species influence the protein stability and consequently the RNA editing efficiency. Due to the low accumulation level of CRR4 *in vivo*, we cannot evaluate the stability of *Nicotiana* CRR4 (22,23). We conclude that the different efficiency of the RNA editing between lines complemented by *NsylCRR4* and *NtomCRR4* was reflected by different activity, and/or
stability possibly, of CRR4 in the heterogous system, and this idea provides a likely explanation of the lower RNA editing efficiency in *N. tomentosiformis*.

In flowering plants, molecules involved in plastid RNA editing have been identified only in *Arabidopsis*. This study first facilitated a direct comparison of the RNA editing machinery of different species, although indirect evidence also suggested the involvement of PPR protein in plastid RNA editing in tobacco (50). The discovery of AtCRR4 orthologs in *Nicotiana* species indicates that the RNA editing mechanism of the ndhD-1 site is common at least in *Arabidopsis* and *Nicotiana* species. The translational initiation codon of the *ndhD* gene is generated by RNA editing in dicot plants (6). The cis-element of the ndhD-1 site is highly conserved among dicot plants such as *Arabidopsis*, tobacco and tomato (Supplementary Data 3), implying that AtCRR4 orthologs also recognize the ndhD-1 cis-element in dicot plants. In contrast, the initiation codon of the *ndhD* gene is already encoded by ATG in the genome in monocots and angiosperm (6) and their sequences corresponding to the dicot cis-elements are not conserved (Supplementary Data 3). Bioinformatic analysis of the rice genome suggested that there are no CRR4 orthologous proteins in rice (private communication with Small I). These observations may suggest that the RNA editing system of the ndhD-1 site was acquired during the divergence to monocots and dicot. This is the first molecular information on the coevolution of the RNA editing site and its cognate trans-factor.

Why does the editing efficiency of the ndhD-1 site remain low in *N. tomentosiformis*? The ndhD-1 site has a characteristic that distinguishes it from other editing sites. This RNA editing creates a translational initiation codon rather than altering the coding amino acid. This site is partially edited even in leaves where the *ndhD* gene is mainly expressed and its editing extent appears developmentally regulated (48), suggesting that the RNA editing may play a role in the regulation of *ndhD* translation. However, NDH activity and protein blot analysis suggested that 15% of the editing is sufficient for accumulating a level of NDH complex comparable to that observed in other species (Figure 3) and for activity detected in our chlorophyll fluorescence analysis (Figure 1). Thus, the low level of editing in the ndhD-1 site may not be a result of regulation, although we cannot rule out the possibility that the RNA editing level limits translation under certain conditions. The population of *ndhD* mRNA with the translational initiation codon depends on the species, and the developmental and environmental conditions. This initiation codon is encoded by ATG in the genomes of monocots (6), suggesting that the regulation of RNA editing efficiency is not physiologically essential. The editing extent in tobacco, *Arabidopsis* and spinach are 45%, 61% and 41%, respectively (51). Taking account of our observation that 15% editing is sufficient for the function of the NDH complex in *N. tomentosiformis*, it may be more likely that the sites do not need to be edited completely. The RNA editing of ndhD-1 in an etiolated seedling (25%) is lower than that in a green leaf (40%) (51), also suggesting that the translation of *ndhD* is regulated by RNA editing via light. However, the NdH level of *N. tomentosiformis* (15% editing) was comparable to those of *N. tabacum* (45% editing) and *N. sylvestris* (42% editing) (Figure 3). It is unclear whether the level of RNA editing (25%) limits the translation in etioplasts. Actually the NDH complex is already present in etioplasts and de-etiolation does not upregulate the accumulation (52). When we take account of all the information, the lower editing efficiency of ndhD-1 in *N. tomentosiformis* might be a result of evolution permitting a reduction in editing efficiency simply because high editing efficiency was not required. PPR proteins appear to be evolutionarily highly flexible (53), and this fact might be essential to respond to newly occurring RNA editing sites in plant organelles.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

**ACKNOWLEDGEMENTS**

We thank Asako Tahara for skilled technical support. We are grateful to Tsuyoshi Endo (Kyoto University, Kyoto, Japan) for the gift of antibody. We also thank Masahiro Sugiyama (Nagoya City University, Nagoya, Japan) and Mamoru Sugita (Nagoya University, Nagoya, Japan) for giving us *N. sylvestris* and *N. tomentosiformis* seeds.

**FUNDING**

Ministry of Education, Culture, Sports, Science, and Technology of Japan (Grant-in-Aid 16085206 for Scientific Research on Priority Areas; Grant 17GS0316 for Creative Science Research; Ministry of Agriculture, Forestry and Fisheries of Japan (Genomics for Agricultural Innovation, GPN0008). Funding for open access charge: Grant-in-Aid 16085206.

Conflict of interest statement. None declared.

**REFERENCES**

1. Brennicke,A., Marchfelder,A. and Binder,S. (1999) RNA editing. *FEMS Microbiol. Rev.*, 23, 297–316.
2. Bock,R. (2000) Sense from nonsense: how the genetic information of chloroplasts is altered by RNA editing. *Biochimie*, 82, 549–557.
3. Shikanai,T. (2006) RNA editing in plant organelles: machinery, physiological function and evolution. *Cell Mol. Life Sci.*, 63, 698–708.
4. Kugita,M., Yamamoto,Y., Fujikawa,T., Matsumoto,T. and Yoshinaga,K. (2003) RNA editing in hornwort chloroplasts makes more than half the genes functional. *Nucleic Acids Res.*, 31, 2417–2423.
5. Wolf,R.G., Rowe,C.A. and Hasebe,M. (2004) High levels of RNA editing in a vascular plant chloroplast genome: analysis of transcripts from the fern *Adiantum capillus-veneris*. *Gene*, 339, 89–97.
6. Tsuzuki,T., Nakasugi,T. and Sugiuira,M. (2001) Comparative analysis of RNA editing sites in higher plant chloroplasts. *J. Mol. Evol.*, 53, 327–332.
7. Handa,H. (2003) The complete nucleotide sequence and RNA editing content of the mitochondrial genome of rapeseed (*Brassica napus* L.); comparative analysis of the mitochondrial genomes of rapeseed and *Arabidopsis thaliana*. *Nucleic Acids Res.*, 31, 5907–5916.
8. Giege,P. and Brennicke,A. (1999) RNA editing in Arabidopsis mitochondria effects 441 C to U changes in ORFs. Proc. Natl Acad. Sci. USA, 96, 15324–15329.

9. Notsu,Y., Masood,S., Nishikawa,T., Kubo,N., Akiduki,G., Nakazono,M., Hirai,A. and Kadowaki,K. (2002) The complete sequence of the rice (Oryza sativa L.) mitochondrial genome: frequent DNA sequence acquisition and loss during the evolution of flowering plants. Mol. Genet. Genomics, 268, 434–445.

10. Ftazer,R., Lopez,M., Maler,R.M., Martin,M., Sabate,B. and Kossel,H. (1995) Editing of the chloroplast ndtB encoded transcript shows divergence between closely related members of the grass family (Poaceae). Plant Mol. Biol., 29, 679–684.

11. Schmitz-Linneweber,C., Regel,R., Du,T.G., Hupfer,H., Herrmann,R.G. and Maier,R.M. (2002) The plastid chromosome of Arrope belladonna and its comparison with that of Nicotiana tabacum: the role of RNA editing in generating divergence in the process of plant speciation. Mol. Biol. Evol., 19, 1602–1612.

12. Sasaki,T., Yukawa,Y., Miyamoto,T., Obokata,J. and Sugiiura,M. (2003) Identification of RNA editing sites in chloroplast transcripts from the maternal and paternal progenitors of tobacco (Nicotiana tabacum): comparative analysis shows the involvement of distinct trans-factors for ndtB editing. Mol. Biol. Evol., 20, 1028–1035.

13. Tille, M., Funahashi,T., Schmitz-Linneweber,C., Pabate,B., Martin,M. and Maier,R.M. (2005) Editing of plastid RNA in Arabidopsis thaliana ecotypes. Plant J., 43, 708–715.

14. Bock,R., Kossel,H. and Maligna,P. (1994) Introduction of a heterologous editing site into the tobacco plastid genome: the lack of RNA editing leads to a mutant phenotype. EMBO J., 13, 4623–4628.

15. Read,M.L. and Hanson,M.R. (1997) A heterologous maize rpoB editing site is recognized by transgenic tobacco chloroplasts. Mol. Cell. Biol., 17, 6948–6952.

16. Nakanuma,T., Obokata,J. and Sugiiura,M. (2002) Recognition of RNA editing sites is directed by unique proteins in chloroplasts: biochemical identification of cis-acting elements and trans-acting factors involved in RNA editing in tobacco and pea chloroplasts. Mol. Cell. Biol., 22, 6726–6734.

17. Chaudhuri,S. and Maliga,P. (1996) Sequences directing C to U editing in trans tabacum. Sabater,B., Martin,M. and Maier,R.M. (2005) Editing of plastid psbL RNAs. Plant Cell., 17, 1480–1485.

18. Hashimoto,M., Endo,T., Peltier,G., Tasaka,M. and Shikanai,T. (2003) A nucleus-encoded factor, CR2, is essential for the expression of chloroplast ndtB in Arabidopsis. Plant J., 36, 541–549.

19. Hao,T., Miyake,H. and Sugita,M. (2007) A pentatricopeptide repeat protein is required for RNA processing of cdpf pre-mRNA in moss chloroplasts. J. Biol. Chem., 282, 10773–10782.

20. Fisk,D.G., Walker,M.B. and Barkan,A. (1999) Molecular cloning of the gene for the maternal genome donor of Nicotiana sylvestris plastid. Proc. Natl Acad. Sci. USA, 96, 913–917.

21. Okuda,K., Nakamura,T., Sugita,M., Shimizu,T. and Shikanai,T. (2006) A pentatricopeptide repeat protein is a site recognition factor in chloroplast RNA editing. Mol. Genet. Genomics, 275, 367–373.

22. Hirose,T. and Sugita,M. (2001) Involvement of site-specific trans-acting factor and a common RNA-binding protein in the editing of chloroplast mRNAs: development of a chloroplast in vitro RNA editing system. EMBO J., 20, 1144–1152.

23. Reed, M.L., Peeters,N.M. and Hanson,M.R. (2001) A single alteration 20 nt 5' to an editing target inhibits chloroplast RNA editing in vivo. Nucleic Acids Res., 29, 1507–1513.

24. Sasaki,T., Yukawa,Y., Wakisugi,T., Yamada,K. and Sugiiura,M. (2006) A simple in vitro RNA editing assay for chloroplast transcripts using fluorescent dideoxynucleotides: distinct types of sequence elements required for editing of ndtB transcripts. Plant J., 47, 802–810.

25. Takenaka,M., Neuwirt,J. and Brennicke,A. (2004) Complex cis-elements determine an RNA editing site in pea mitochondria. Nucleic Acids, Res., 32, 4137–4144.

26. Kotera,E., Tasaka,M. and Shikanai,T. (2005) A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature, 433, 326–330.

27. Okuda,K., Myouga,F., Motohashi,R. Shinozaki,K. and Shikanai,T. (2007) Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. Proc. Natl Acad. Sci. USA, 104, 8178–8183.

28. Munekage,Y., Hashimoto,M., Miyake,C., Tomizawa,K. Endo,T. Tasaka,M. and Shikanai,T. (2004) Cyclic electron flow around photosystem 1 is essential for photosynthesis. Nature, 429, 579–582.

29. Chatagnier-Boutin,A.L., Ramos-Vega,M., Guerra-Garcia,A., Andres, C., de la Luz Gutierrez-Nava,M., Cantero,A., Delannoy,E., Jimenez,L.F., Lucir,H., Small,J. et al. (2008) CLB19, a pentatricopeptide repeat protein required for editing of rpoA and cdpf chloroplast transcripts. Plant J., doi:10.1111/j.1365-313x.2008.03634.x.

30. O’Toole,N., Hattori,M., Andres,C., Iida,K., Lurin,C. Schmitz-Linneweber,C., Sugita,M. and Small,I. (2008) On the expansion of the pentatricopeptide repeat gene family in plants. Mol. Biol. Evol., 25, 1120–1128.

31. Kurin,C., Andres,C., Aubourg,S., Bellouard,M., Bitton,F., Brure,C., Cabocho,M., Debast,C., Gaulberto,J., Hoffmann,B. et al. (2004) Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organellar biogenesis. Plant Cell, 16, 2089–2103.

32. Pfalz,J., Lierk,K., Kandlbinder,A., Dietz,K.J. and Oelmüller,R. (2006) petTAC2, -6, and -12 are components of the transcriptionally active plastid chromosome that are required for plastid gene expression. Plant Cell, 18, 176–197.

33. Schmitz-Linneweber,C., Williams-Carreir,E.R., Williams-Voelker,P.M., Kroeger,T.S., Vichas,A. and Barkan,A. (2006) A pentatricopeptide repeat protein facilitates the trans-splicing of the maize chloroplast rps12 pre-mRNA. Plant Cell, 18, 2621–2630.

34. de Longevialle,A.F., Meyer,E.H., Andrés,C., Taylor,N.L., Lurin,C., Millar,A.H. and Small,I.D. (2007) The pentatricopeptide repeat gene OTP43 is required for trans-splicing of the mitochondrial nadl intron 1 in Arabidopsis thaliana. Plant Cell, 19, 3256–3265.

35. Meierhoff,K., Felder,S., Nakamura,T., Bechtold,N. and Schuster,G. (2003) HCF152, an Arabidopsis RNA binding pentatricopeptide repeat protein involved in the processing of chloroplast psbB-psbT pre-mRNAs. Plant Cell., 15, 1480–1485.

36. Yamazaki,H., Tasaka,M. and Shikanai,T. (2004) PPR motifs of the nucleus-encoded factor, PGR3, function in the selective and distinct steps of chloroplast gene expression in Arabidopsis. Plant J., 38, 152–163.

37. Okuda,K., Nakamura,T., Sugita,M., Shimizu,T. and Shikanai,T. (2006) A pentatricopeptide repeat protein is a site recognition factor in chloroplast RNA editing. Mol. Gen. Genomics, 275, 367–373.

38. Hirosue,T., Kusumegi,T. and Sugita,M. (2006) The chloroplast genome of Nicotiana sylvestris and Nicotiana tomentosiformis: complete sequencing confirms that the Nicotiana sylvestris progenitor is the maternal genome donor of Nicotiana tabacum. Mol. Genet. Genomics, 275, 367–373.

39. Hirosue,T., Kusumegi,T. and Sugita,M. (2006) The chloroplast genome of Nicotiana sylvestris and Nicotiana tomentosiformis: complete sequencing confirms that the Nicotiana sylvestris progenitor is the maternal genome donor of Nicotiana tabacum. Mol. Genet. Genomics, 275, 367–373.

40. Hirosue,T., Kusumegi,T. and Sugita,M. (2006) The chloroplast genome of Nicotiana sylvestris and Nicotiana tomentosiformis: complete sequencing confirms that the Nicotiana sylvestris progenitor is the maternal genome donor of Nicotiana tabacum. Mol. Genet. Genomics, 275, 367–373.

41. Hirosue,T., Kusumegi,T. and Sugita,M. (2006) The chloroplast genome of Nicotiana sylvestris and Nicotiana tomentosiformis: complete sequencing confirms that the Nicotiana sylvestris progenitor is the maternal genome donor of Nicotiana tabacum. Mol. Genet. Genomics, 275, 367–373.
46. Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods, 25, 402–408.

47. Lutz, K.A. and Maliga, P. (2001) Lack of conservation of editing sites in mRNAs that encode subunits of the NAD(P)H dehydrogenase complex in plastids and mitochondria of Arabidopsis thaliana. Curr. Genet., 40, 214–219.

48. Hirose, T. and Sugiura, M. (1997) Both RNA editing and RNA cleavage are required for translation of tobacco chloroplast ndhD mRNA: a possible regulatory mechanism for the expression of a chloroplast operon consisting of functionally unrelated genes. EMBO J., 17, 6804–6811.

49. Peeters, N.M. and Hanson, M.R. (2002) Transcript abundance supercedes editing efficiency as a factor in developmental variation of chloroplast gene expression. RNA, 8, 497–511.

50. Kobayashi, Y., Matsuoi, M., Sakamoto, A., Wakasugi, T., Yamada, K. and Obokata, J. (2007) Two RNA editing sites with cis-elements of moderate sequence identity are recognized by an identical site-recognition protein in tobacco chloroplasts. Nucleic Acids Res., 36, 311–318.

51. Chateigner-Boutin, A.L. and Hanson, M.R. (2003) Developmental co-variation of RNA editing extent of plastid editing sites exhibiting similar cis-elements. Nucleic Acids Res., 31, 2586–2594.

52. Kanervo, E., Singh, M., Suorsa, M., Paakkarinen, V., Aro, E., Battchikova, N. and Aro, E.M. (2008) Expression of protein complexes and individual proteins upon transition of etioplasts to chloroplast in pea (Pisum sativum). Plant Cell Physiol., 49, 396–410.

53. Rivals, E., Bruyère, C., Toffano-Nioche, C. and Lecharny, A. (2006) Formation of the Arabidopsis pentatricopeptide repeat family. Plant Physiol., 141, 825–839.