Extraplasmidic site-specific factors mediate RNA editing in chloroplasts

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Single nucleotides in higher plant organellar mRNAs are subject to post-transcriptional alterations by RNA editing, typically resulting in changes of the encoded protein sequence. Although some information has been acquired on the general features of the editing processes in both plastids and plant mitochondria, the mechanisms and factors involved in the selective recognition of the nucleotide to be edited are still unknown. To gain a better understanding of how an editing site is specifically selected by the organellar RNA editing machinery, we have attempted to rescue a previously generated tobacco plastid editing mutant. Using an interspecific protoplast fusion approach, we were able to restore RNA editing activity for a specific site in the *psbF* transcript that otherwise remained unedited. Our results suggest (i) that site-specific *trans*-acting factors mediate chloroplast editing site recognition and (ii) that these factors are of extraplasmidic origin.

Keywords: chloroplast/*Nicotiana tabacum*/*psbF*/RNA editing/RNA processing

Introduction

Gene expression in a variety of genetic systems is affected by a post-transcriptional nucleotide modification mechanism termed RNA editing (Benne, 1996). In plants, RNA editing occurs in two cellular compartments, mitochondria and plastids (Covello and Gray, 1989; Gualberto *et al.*, 1989; Hiesel *et al.*, 1989; Hoch *et al.*, 1991; Kudla *et al.*, 1992). In both organelles, editing involves mainly cytidine→uridine conversions (Bonnard *et al.*, 1992; Kossel *et al.*, 1993; Schuster and Brennicke, 1994; Maier *et al.*, 1996), with few examples of reverse events (Gualberto *et al.*, 1990; Schuster *et al.*, 1990; Yoshinaga *et al.*, 1996). The selective modification of only very few nucleotides within an mRNA poses the intriguing question of how these residues are recognized specifically by the organellar editing machinery. In the absence of efficient *in vitro* editing systems (Araya *et al.*, 1991; Yu and Schuster, 1995), evidence for the site specificity of the editing reaction has come from two sets of plastid transformation experiments. Firstly, a spinach editing site transferred into the tobacco plastid genome remained unmodified in the transgenic tobacco organelles (Bock *et al.*, 1994), suggesting that an essential site-specific (*cis*- or *trans*-acting) component was missing. Secondly,

introduction of additional copies of the tobacco *psbL* editing site revealed that the editing activity for this site is depletable (Chaudhuri *et al.*, 1995; Chaudhuri and Maliga, 1996).

We have used the above-mentioned plastid RNA editing mutant resulting from the interspecific editing site transfer (Bock *et al.*, 1994) to investigate the nature and cellular origin of specificity factors for plastid editing site selection. This mutant carries a modified version of the *psbF* gene containing the spinach *psbF* editing site in an internal segment of 35 bp complete sequence identity (Figure 1). Editing of this site changes a genomically encoded serine codon into a phenylalanine codon (Bock *et al.*, 1993) which is evolutionarily conserved in the *psbF*-encoded proteins (β -subunit of cytochrome *b559*) of all photosynthetically active organisms. The *psbF* editing site is not present in tobacco wild-type plants since here the correct phenylalanine codon is already specified at the DNA level (Figure 1). We have shown previously that replacement of part of the tobacco *psbF* gene with the homologous region from spinach results in a mutant phenotype owing to the absence of any detectable *psbF* editing activity in the transgenic plastids (Bock *et al.*, 1994).

Three possible reasons for the lack of editing of the spinach site in tobacco can be envisaged: (i) the 35 bp region of the spinach *psbF* gene used to replace the homologous tobacco sequence is not sufficient to direct editing, i.e. the missing factor is a *cis*-acting sequence element outside this 35 bp segment; (ii) a plastid-encoded *trans*-acting specificity factor for *psbF* editing is missing in tobacco chloroplasts; and (iii) a nuclear-encoded specificity factor for *psbF* editing is present and imported into the chloroplast compartment in spinach but not in tobacco. To distinguish between these three possibilities, we set out to test whether combination of the mutant chloroplasts with a spinach nucleus would restore *psbF* editing activity.

Results

Generation of interspecific hybrid cells by fusion of spinach and mutant tobacco protoplasts

The transplastomic tobacco mutant Nt-pRB8-S6 carries the spinach *psbF* editing site and the surrounding sequence motif from –16 to +18 (Bock *et al.*, 1994). This segment replaces the homologous tobacco *psbF* sequences which naturally do not contain the RNA editing site (Figure 1). We have shown recently that the tobacco plastid RNA editing machinery is incapable of editing the heterologous spinach site, resulting in a single amino acid substitution within the *psbF* gene product (β -subunit of cytochrome *b559*). In homoplasmic plants, this lack of editing is associated with a mutant (i.e. photosynthetically deficient) phenotype (Bock *et al.*, 1994). The phenotype is stable

Tobacco Wt DNA	..CTAGCTGTACCTACCGTCTTTTGGGATCAATATCAGC..
Tobacco Wt RNA	..CUAGCUGUACCUACCGUCUUUUUUUGGGAUCAAUAUCAGC..
Spinach DNA	..CTGGCTGTACCTACCGTTCTTTTGGGGTCAATATCCGC..
Spinach RNA	..CUGGCUGUACCUACCGUUUUUUUGGGUCAAAUAUCGC..
Tobacco Mutant DNA	..CTAGCTGTACCTACCGTTCTTTTGGGGTCAATATCAGC..
Tobacco Mutant RNA	..CUAGCUGUACCUACCGUUCUUUUUGGGUCAAAUAUCAGC..

Fig. 1. Partial sequence alignment of the region containing the *psbF* RNA editing site in spinach. DNA and mRNA sequences are shown for wild-type tobacco (Shinozaki *et al.*, 1986), spinach (Carrillo *et al.*, 1986; Bock *et al.*, 1993) and the tobacco mutant harboring a 35 bp spinach segment with the editing site (Bock *et al.*, 1994). Note RNA editing in spinach (C in DNA, U in mRNA; position marked by vertical arrow), but absence of the editing site in wild-type tobacco (T already at the DNA level). When introduced into the tobacco plastid genome, the spinach editing site remains unmodified (C in both DNA and mRNA of the tobacco *psbF* editing mutant). + indicates nucleotide positions in the chimeric *psbF* gene of the mutant which are specific for spinach; positions specific for tobacco are marked by asterisks. The codon changed by RNA editing from serine to phenylalanine is underlined.

under all environmental conditions tested, indicating that neither spontaneous restoration of *psbF* editing nor elimination of the editing site by a genomic C→T mutation occurs (R.Bock, unpublished results).

The plastid *psbF* gene is part of the plastid *psbE* operon containing the *psbE*, *psbF*, *psbL* and *psbJ* reading frames. Theoretically, sequences outside the introduced -16 to +18 fragment could be required for editing of the *psbF* site and thus could be the cause of the lack of editing in the transplastomic tobacco line. If this were the case, the missing editing factor in tobacco plastids would be a distant *cis*-acting element. Alternatively, a *trans*-acting specificity factor could participate in the *psbF* editing site recognition. Evolutionary loss of the *psbF* editing site in tobacco would most likely be accompanied by the loss of the site-specific *trans*-factor in the absence of selective pressure. In view of the extremely high degree of sequence similarity of the spinach and tobacco *psbE* operons (Carrillo *et al.*, 1986; Bock *et al.*, 1993), we considered a missing distant *cis*-acting factor a less likely explanation for the lack of *psbF* editing in the Nt-pRB8-S6 tobacco line. Moreover, recent data indicate that in most cases the essential *cis*-elements for plastid RNA editing site recognition reside in close proximity to the editing site (Bock *et al.*, 1996; Chaudhuri and Maliga, 1996) and thus are likely to be contained in the 35 bp segment of complete sequence identity. We speculated, therefore, that a *trans*-acting specificity factor for *psbF* editing is present in spinach but absent from tobacco plastids. We favored the idea that this factor is provided by the nucleus rather than encoded by the plastid genome for two reasons: (i) plastid RNA editing was shown to be entirely independent of chloroplast translation (and thus of plastid-encoded proteins; Zeltz *et al.*, 1993) and (ii) we have been unable to detect guide RNA-like molecules in plastids (Bock and Maliga, 1995; our unpublished results from low stringency hybridization analyses) which, by analogy to the kinetoplast editing system (Simpson *et al.*, 1993; Benne, 1996; Kable *et al.*, 1996), could serve as *trans*-acting specificity factors also for chloroplast editing. If our nuclear-encoded specificity factor hypothesis were correct, combination of the mutant tobacco chloroplasts with a spinach nucleus should restore *psbF* editing activity.

Interspecific combinations of a given cytoplasm with a

different nuclear background can be achieved by using cell fusion technologies. We attempted, therefore, to generate hybrid cells resulting from spinach and Nt-pRB8-S6 tobacco protoplasts. To facilitate microscopic selection of heterologous fusion products and to exclude unfused protoplasts as well as homologous tobacco–tobacco and spinach–spinach fusion products, we used sterile etiolated spinach seedlings and prepared protoplasts from their bright yellow cotyledons (Figure 2A and C). The presence of undifferentiated plastids (etioplasts) in the spinach protoplasts is unlikely to interfere with editing of the *psbF* site. We have shown previously that *psbF* editing is equally efficient in spinach chloroplasts and etioplasts (Bock *et al.*, 1993).

Protoplast fusion was induced by DC pulses following dielectrophoretic protoplast alignment in an AC field. Since the fusion does not lead to immediate mixing of the cytoplasm of the fusion partners, tobacco–spinach hybrid protoplasts can easily be identified by their partially green and partially yellow cytoplasm as well as by their chimeric internal structure (Figure 2E and F). Microscopic selection was carried out using a modified computer-based, stepmotor-driven microdevice (Koop and Schweiger, 1985; Schweiger *et al.*, 1987; Koop and Spangenberg, 1989; Spangenberg and Koop, 1992). Individual heterologous fusion products were identified and taken up by a microcapillary (connected to a nanoliter pump) in a volume of 10 nl. Computer-directed micro-motors then transferred the capillary to a microdroplet in a collection dish (2 µl culture medium overlaid with mineral oil) where the hybrid cell was released. This way, 15–20 fusion products per experiment were selected and subsequently subjected to microdroplet culture in the dark. The hybrid cells did not undergo cell divisions, which is most likely due to incompatibility of the nuclei and/or cytoplasm of the only distantly related species tobacco and spinach. However, when kept in suitable cultivation media, the fusion products maintained physiological activity for a few days.

Analysis of *psbF* editing in spinach–tobacco hybrid cells

To facilitate the analysis of *psbF* editing in as few as 15 fusion products, we have optimized the RNA isolation, cDNA synthesis and PCR procedures resulting in reproducible amplification of *psbF* cDNA from five tobacco protoplasts. Since the hybrid cells contain both spinach and mutant tobacco plastids, the spinach chloroplast *psbF* cDNA molecules needed to be selectively excluded from amplification. This was made feasible by choosing a PCR primer derived from a variable region in the downstream *psbL/J* intergenic spacer (Shinozaki *et al.*, 1986; Bock *et al.*, 1993). *psbF* cDNA can be amplified with primers located outside the coding region since the plastid *psbE*, *psbF*, *psbL* and *psbJ* genes are part of a tetracistronic transcription unit which does not undergo post-transcriptional processing into monocistronic mRNAs (Haley and Bogorad, 1990; Willey and Gray, 1990).

As expected, PCR with the tobacco-specific primer resulted in cDNA amplification products for tobacco and for the hybrid cells, but not for spinach (Figure 3). In order to verify that indeed cDNA and no contaminating DNA was amplified, we first sequenced the downstream

Fig. 2. Experimental strategy to test for restoration of *psbF* editing in mutant plastids by combination with a spinach nucleocytoplasmic background. Etiolated spinach cotyledons (A) and a green leaf from the tobacco mutant (B) were used for protoplast isolation. The spinach (C) and tobacco (D) protoplasts are thus easily distinguishable by their color and internal structure. DC pulse-induced fusion does not result in immediate mixing of the cytoplasm, thereby facilitating recognition of heterologous spinach-tobacco hybrid cells (E and F). The fusion procedure also results in the production of multiple fusion products, such as the spinach-tobacco-spinach hybrid cell shown in (F).

psbL portion of the PCR products. The *psbL* initiation codon has been shown to be created by RNA editing in both tobacco and spinach (Kudla *et al.*, 1991; Bock *et al.*, 1993). The presence of a virtually completely edited AUG initiation codon indicated that exclusively cDNA was amplified (data not shown).

We then tested the editing status of the *psbF* site in fusion products cultured for 2 days. Primer extension analysis revealed, in addition to a signal for the unedited nucleotide, also a significant peak for the edited nucleotide

(Figure 4A), indicating that combination of the mutant tobacco plastids with a spinach nucleus may have indeed resulted in restoration of editing activity. Direct sequencing of the amplified cDNAs confirmed the presence of a mixed cDNA population in the cultured hybrid cells: besides molecules with a C, *psbF* cDNAs with a T at the editing position are also detected (Figure 4B). Quantitation of editing efficiency (Bock *et al.*, 1996) using a Phosphor-Imager yielded an approximate proportion of 25% edited mRNAs.

To provide direct proof for the existence of edited transcript molecules in the hybrid cell plastids, we have analyzed individual cDNA clones. Sequencing of 20 clones resulted in the detection of four clones reflecting edited *psbF* mRNA sequences (Figure 4C). The presence of all diagnostic sequence deviations both upstream and downstream of the editing site (Figures 1 and 4C) confirmed that the edited mRNA molecules originate from the chimeric *psbF* gene of the mutant tobacco plastids and not from co-amplified spinach *psbF* cDNA molecules. This as well as the presence of completely unedited tobacco and fully edited spinach *psbF* cDNA populations in an unfused control sample also excludes template switching by the *Taq* polymerase as a possible cause of the amplification of edited cDNA molecules.

No significant restoration of editing could be observed in the fusion products immediately after fusion, strongly suggesting that a steady flow of a *trans*-acting specificity factor into the mutant plastids is the cause of the observed *psbF* editing in the hybrid cells. To examine the time course of the restoration more precisely, we analyzed

fusion products after 0, 1, 2 and 5 days incubation and measured the *psbF* editing efficiencies. The detected amount of edited mRNA was ~10% after 1 day of microdroplet culture, ~25% after 2 days and ~15% after 5 days [values determined as described previously (Bock *et al.*, 1996) and rounded to full 5 or 10%]. The observed decrease from day 2 to day 5 is likely to reflect the loss

Fig. 3. Amplification of *psbF* DNA and cDNA sequences from protoplast samples. Amplification with a primer pair specific for the tobacco plastid *psbE* operon yields a 299 bp product for both tobacco DNA and cDNA (lanes a and b) but no product for spinach DNA or cDNA (lanes c and d). The 299 bp product is also obtained with a mixture of unfused tobacco and spinach protoplasts (lane e) and with hybrid cell samples not incubated (lane f) or incubated in the dark for 2 days (lane g). M, molecular weight marker.

Fig. 4. Analysis of hybrid cells for restoration of *psbF* editing activity. (A) Primer extension analysis of amplified cDNA samples. The primer chosen extends the non-coding strand. The presence in the reaction of dATP and dCTP only results in an 11 nucleotide extension of the primer on an unedited template and a 16 nucleotide extension on an edited template. The upper (green) curve represents an unfused mixture of spinach and mutant tobacco protoplasts, the red curve hybrid cells immediately after fusion, and the black curve hybrid cells incubated for 2 days. Note the presence of two major peaks in the green and red curves (primer peak and 11 nucleotide extension product) and the appearance of a third peak (16 nucleotide extension product; arrowhead) in the black curve, reflecting the edited mRNA population in the cultured hybrid cells. (B) Direct sequencing of amplified cDNA samples. Owing to the polarity of the sequencing primer, the sequence ladder shown here reflects the strand complementary to the mRNA sequence. Sequences for hybrid cells immediately after fusion (0 d) and for cultured hybrid cells (2 d) are shown. The editing position is marked by arrowheads. Note the absence of editing in the 0 d sample (G signal only) but partial restoration of *psbF* editing in the 2 d sample (G and A signal, reflecting C and U at the mRNA level). (C) Sequence analysis of two cDNA clones derived from the hybrid cell sample incubated for 2 days. The upper panel shows a sequence reflecting an unedited cDNA, the lower panel displays an edited cDNA sequence. Arrowheads point to the editing position. Diagnostic nucleotide positions are marked as in Figure 1.

of viability of the hybrid cells as caused by the nucleocytoplasmic incompatibility.

Discussion

The lack of *psbF* editing in a transplastomic tobacco mutant resulting from the interspecific transfer of the spinach *psbF* editing site into the tobacco plastid genome (Bock *et al.*, 1994) can be explained by three hypotheses: (i) lack of an essential distant *cis*-acting sequence element located outside the transferred *psbF* segment; (ii) absence of a plastid-encoded *trans*-acting specificity factor for *psbF* editing; or (iii) lack of a site-specific *trans*-factor provided by the spinach nucleus. In the course of this work, we have experimentally tested the hypothesis that a nuclear-encoded *trans*-acting factor confers the site specificity of the *psbF* editing reaction. We have shown that *psbF* editing can be partially restored by combining the mutant Nt-pRB8-S6 chloroplasts with a spinach nucleocytoplasmic background. This finding provides direct evidence for the involvement of site-specific *trans*-factors in plastid RNA editing site recognition.

We have considered the possibility that this *trans*-factor is synthesized in the spinach plastids and introduced into Nt-pRB8-S6 plastids by recombination between spinach and tobacco plastids. However, recombination between chloroplasts is known to be an extremely rare event that may occur only accidentally (Medgyesy *et al.*, 1985) and is even undetectable under non-selective conditions (Kutzelnigg and Stubbe, 1974; Chiu and Sears, 1985). This is most probably due to a very low frequency of chloroplast fusion (Birky, 1995) and contrasts with the situation in plant mitochondria where organelle fusion and genome recombination appear to be regular events accompanying cell fusions (Akagi *et al.*, 1995). The possibility of recombination between chloroplasts after cell fusion was studied extensively, and attempts to identify chloroplast recombinants have mostly failed (e.g. Fluhr *et al.*, 1984). Applying strong selection for plastid recombination, Maliga and co-workers were able to isolate a single chloroplast recombinant from a large number of heterologous tobacco protoplast fusions (Medgyesy *et al.*, 1985). This recombinant line was analyzed thoroughly and found to contain multiple sites of recombination between the two parental genomes (Maliga and Fejes, 1985), suggesting that the rate-limiting step of chloroplast recombination is the extremely low probability of chloroplast fusion events (Maliga *et al.*, 1987). The chloroplast recombination frequency was at best 10^{-6} [calculated according to Maliga *et al.* (1987) for the average number of 100 chloroplasts per tobacco cell (Chen *et al.*, 1977)], which by no means can account for any significant restoration of *psbF* editing in our protoplast fusion experiments.

We conclude, therefore, that the spinach nucleus is the source of the *trans*-acting specificity factor that restored *psbF* editing in the Nt-pRB8-S6 plastids. This markedly contrasts the editing site selection mechanism in a different, evolutionarily unrelated editing system, the kinetoplasts of trypanosomes (Benne *et al.*, 1986). In this system, the specificity factors are not imported into the organelle. Instead, they are encoded as small RNA molecules (termed

guide RNAs) by the kinetoplast genome itself (Simpson *et al.*, 1993; Benne, 1996).

It was shown recently that the sequences immediately surrounding the nucleotide to be edited act as important *cis*-guiding determinants in editing site selection (Bock *et al.*, 1996; Chaudhuri and Maliga, 1996). Thus it seems reasonable to assume that the *trans*-acting site-specific factor shown to be involved in *psbF* editing is the molecule specifically binding to these *cis*-acting sequence elements. The molecular interaction of RNA substrate and specificity factor may then facilitate binding of the (hypothetical) general component(s) of the editing machinery which may carry the catalytic cytidine-modifying activity (e.g. a nuclear-encoded cytidine deaminase).

In spite of incubation over several days, only a partially edited *psbF* mRNA pool was detected in the generated spinach–tobacco hybrid cells. The incomplete restoration of *psbF* editing may be due to limiting amounts of the *trans*-factor (i.e. competition between the spinach and tobacco chloroplasts for the factor provided by the spinach nucleus) or, alternatively, could be caused by an inefficient uptake of the spinach nuclear factor by the tobacco chloroplast import machinery. Interestingly, recent studies have suggested that the introduction of supernumerary editing sites (and the concomitant increase in the number of substrate molecules) can exhaust the capacity of the plastid editing apparatus (Chaudhuri *et al.*, 1995; Chaudhuri and Maliga, 1996). Similarly, usage in both spinach and tobacco plastids may lead to a depletion of the *psbF*-specific *trans*-factor synthesized by the spinach nucleus only.

How specific are such *trans*-factors for plastid RNA editing? Besides a strict site specificity, it also seems possible that one *trans*-factor mediates the editing of more than one site. Certain plastid editing sites were found to share common sequence elements (Maier *et al.*, 1992; Freyer *et al.*, 1995), raising the possibility that they are recognized by one and the same *trans*-acting factor(s). Our data suggest that the evolutionary elimination of the *psbF* editing site in tobacco (by a genomic C→T mutation) led to a subsequent loss of the corresponding *trans*-acting specificity factor owing to the lack of selective pressure. Thus it appears unlikely that this factor was required for the editing of other substrates in tobacco plastids. We propose, therefore, that at least this *trans*-factor is strictly specific for the *psbF* site. Given the high number of plastid RNA editing sites (Maier *et al.*, 1995) and the at least two orders of magnitude higher number of plant mitochondrial editing sites, we consider it unlikely that these site-specific *trans*-acting factors have the property of an editing enzyme. We rather view them as small molecules whose interaction with the editing site allows for binding of the catalytic component of the editing machinery. Future investigations will be aimed at testing this model and determining the molecular identity of the factors involved. The knowledge that site-specific *trans*-acting editing factors exist and are encoded by nuclear genes, as well as the availability of mutant phenotypes linked to plastid RNA editing, will now allow us to devise suitable genetic screens and to employ the powerful tools of plant nuclear genetics in order to identify these factors.

Materials and methods

Plant material

Tobacco mutant and wild-type plants (*Nicotiana tabacum* cv Petit Havana) were grown under sterile conditions on agar-solidified MS medium (Murashige and Skoog, 1962) containing 30 g/l sucrose. The tobacco editing mutant was generated by chloroplast transformation (Svab *et al.*, 1990; Svab and Maliga, 1993; Kanevski and Maliga, 1994) as described previously (Bock *et al.*, 1994). Etiolated spinach seedlings were obtained by germinating surface-sterilized seeds in the dark on sucrose-containing MS medium.

Protoplast isolation procedures

Protoplasts were prepared from cotyledons of sterile etiolated spinach plants (20 pairs) and from a single leaf of the homoplasmic transformed tobacco plastid RNA editing mutant (Bock *et al.*, 1994). Protoplast isolation was carried out by incubation of tissue samples overnight in the dark at 25°C in PIN medium (Koop *et al.*, 1996) with 0.5% cellulase RS, 0.5% pectolyase Y23, 0.5% macerozyme R-10, 500 µg/ml spectinomycin and 100 µg/ml carbenicillin. Purified protoplasts were kept in 0.5 M mannitol/1 mM CaCl₂ and transferred to 0.45 M mannitol/1 mM CaCl₂ immediately before fusion (Eigel *et al.*, 1991).

Protoplast fusion and sorting of hybrid cells

For protoplast fusion, a pair of stainless steel electrodes was inserted into the well of an agarose layer (1% agarose, 0.45 M mannitol, 1 mM CaCl₂). The well was produced by insertion of an appropriate mold into a melted agarose layer and was suitable to take up the electrodes (electrode gap 2.5×0.3×0.6 cm) after solidification of the agarose. A 0.4 ml mixture of spinach and tobacco protoplasts (~10⁴ protoplasts each) were aligned dielectrophoretically in an AC field (60 V/cm, 1 MHz) and subsequently fused by a few DC pulses of 1 kV/cm (50 µs pulse duration). A modified computer-based, stepmotor-driven microdevice (Koop and Schweiger, 1985; Schweiger *et al.*, 1987; Koop and Spangenberg, 1989; Spangenberg and Koop, 1992) was used for microscopic selection of hybrid cells. Individual heterologous fusion products were taken up in a volume of 10 nl by a glass microcapillary and subjected to microdroplet culture in the dark [2 µl PCN culture medium (Koop *et al.*, 1996) with 500 µg/ml spectinomycin overlaid with mineral oil].

Isolation of nucleic acids

Fifteen hybrid cells were cultured in a microdroplet in the dark at 25°C for 1–5 days. RNA was isolated using 100 µl of TRIZOL reagent (Gibco BRL) and adding glycogen (Boehringer Mannheim) as carrier prior to precipitation. The following controls were included: (i) 15 tobacco protoplasts, (ii) 15 spinach protoplasts, (iii) 15 tobacco + 15 spinach protoplasts, unfused and (iv) 15 hybrid cells not subjected to microdroplet culture. Contaminating DNA was removed by digestion with RNase-free DNase I (Boehringer Mannheim), followed by phenol/chloroform treatment and ethanol precipitation after glycogen addition.

DNA was isolated from protoplast samples according to a rapid miniprep procedure described by Doyle and Doyle (1990).

cDNA synthesis and PCR

Reverse transcription was primed with random hexanucleotide primers (1 ng per reaction) and elongated with Moloney murine leukemia virus RNase H-free reverse transcriptase (Gibco/BRL) in a total volume of 10 µl for 45 min. Five µl of the cDNA reaction were amplified in a hot start PCR with the primer pair P7355 (5' GACTATAGATCGAACCTA-TCC 3') / P7652 (5' CCGAATGAGCTAAGAGAATCTT 3') according to standard protocols (45 s at 94°C, 1.5 min at 58 °C, 1 min at 72°C; 30 or 40 cycles). This primer pair is specific for tobacco *psbE* operon-derived cDNA and excludes spinach cDNA from amplification (Figure 3).

Primer extension analysis

Primer extension analyses were carried out using 50 ng of gel-purified PCR product in a 10 µl reaction (30 s at 94°C, 30 s at 58°C, 10 s at 72°C; six cycles) with 2 ng of 5' fluorescence-labeled primer P529 (5' GAACTGCATTGCTGATATTG 3'), 10 M dATP and 10 M dCTP. Extension products were separated in denaturing polyacrylamide gels and detected by the ALF system (Pharmacia).

Cloning and sequencing techniques

DNA and cDNA amplification products were purified by electrophoresis on 2% agarose gels and subsequent extraction from gel slices using the

QIAEX II kit (QIAGEN). For analysis of individual cDNA clones, amplified cDNA was digested with *Sau3AI* and cloned into a Bluescript vector (Stratagene) cut with *EcoRV* and *BamHI*.

Plasmid DNA was sequenced by cycle sequencing reactions using the Sequitherm kit (Biozyme). Direct sequencing of purified PCR products was performed by a modified chain termination method described by Bachmann *et al.* (1990). Oligonucleotide P177 (5' CCCAGTAGA-GACTGGTACG 3') served as sequencing primer. Quantitation of RNA editing efficiencies was carried out as described earlier (Bock *et al.*, 1996).

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References

- Akagi,H., Shimada,H. and Fujimura,T. (1995) High-frequency inter-parental recombination between mitochondrial genomes of rice cybrids. *Curr. Genet.*, **29**, 58–65.
- Araya,A., Domec,C., Begu,D. and Litvak,S. (1991) An *in vitro* system for the editing of ATP synthase subunit 9 mRNA using wheat mitochondrial extracts. *Proc. Natl Acad. Sci. USA*, **89**, 1040–1044.
- Bachmann,B., Luke,W. and Hunsmann,G. (1990) Improvement of PCR amplified DNA sequencing with the aid of detergents. *Nucleic Acids Res.*, **18**, 1309.
- Benne,R. (1996) RNA editing: how a message is changed. *Curr. Opin. Genet. Dev.*, **6**, 221–231.
- Benne,R., Van den Burg,J., Brakenhoff,J.P.J., Sloof,P., Van Boom,J.H. and Tromp,M.C. (1986) Major transcript of the frameshifted *coxII* gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell*, **46**, 819–826.
- Birky,C.W., Jr (1995) Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. *Proc. Natl Acad. Sci. USA*, **92**, 11331–11338.
- Bock,R. and Maliga,P. (1995) *In vivo* testing of a tobacco plastid DNA segment for guide RNA function in *psbL* editing. *Mol. Gen. Genet.*, **247**, 439–443.
- Bock,R., Hagemann,R., Kossel,H. and Kudla,J. (1993) Tissue- and stage-specific modulation of RNA editing of the *psbF* and *psbL* transcript from spinach plastids—a new regulatory mechanism? *Mol. Gen. Genet.*, **240**, 238–244.
- Bock,R., Kossel,H. and Maliga,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.
- Bock,R., Hermann,M. and Kossel,H. (1995) Site-specific factor involved in the editing of the *psbL* mRNA in tobacco plastids. *EMBO J.*, **15**, 5052–5059.
- Bonnard,G., Gualberto,J.M., Lamattina,L. and Grienemberger,J.M. (1992) RNA editing in plant mitochondria. *Crit. Rev. Plant Sci.*, **10**, 503–524.
- Carrillo,N., Seyer,P., Tyagi,A.K. and Herrmann,R.G. (1986) Cytochrome *b-559* genes from *Oenothera hookeri* and *Nicotiana tabacum* show a remarkably high degree of conservation as compared to spinach. *Curr. Genet.*, **10**, 619–624.
- Chaudhuri,S. and Maliga,P. (1996) Sequences directing C to U editing of the plastid *psbL* mRNA are located within a 22 nucleotide segment spanning the editing site. *EMBO J.*, **15**, 5958–5964.
- Chaudhuri,S., Carrer,H. and Maliga,P. (1995) Site-specific factor involved in the editing of the *psbL* mRNA in tobacco plastids. *EMBO J.*, **14**, 2951–2957.
- Chen,K., Wildman,S.G. and Smith,H.H. (1977) Chloroplast DNA distribution in parasexual hybrids as shown by polypeptide composition of fraction I protein. *Proc. Natl Acad. Sci. USA*, **74**, 5109–5112.
- Chiu,W.-L. and Sears,B.B. (1985) Recombination between chloroplast DNAs does not occur in sexual crosses of *Oenothera*. *Mol. Gen. Genet.*, **198**, 525–528.
- Covello,P.S. and Gray,M.W. (1989) RNA editing in plant mitochondria. *Nature*, **341**, 662–666.
- Doyle,J.J. and Doyle,J.L. (1990) Isolation of plant DNA from fresh tissue. *Focus*, **12**, 13–15.

- Eigel, L., Oelmüller, R. and Koop, H.-U. (1991) Transfer of defined numbers of chloroplasts into albino protoplasts using an improved subprotoplast/protoplast microfusion procedure: transfer of only two chloroplasts leads to variegated progeny. *Mol. Gen. Genet.*, **227**, 446–451.
- Fluhr, R., Avaiv, D., Galun, E. and Edelman, M. (1984) Generation of heteroplasmidic *Nicotiana* hybrids by protoplast fusion: analysis for plastid recombinant types. *Theor. Appl. Genet.*, **67**, 491–497.
- Freyer, R., Lopez, C., Maier, R.M., Martin, M., Sabater, B. and Kossel, H. (1995) Editing of the chloroplast *ndhB* encoded transcript shows divergence between closely related members of the grass family (Poaceae). *Plant Mol. Biol.*, **29**, 679–684.
- Gualberto, J.M., Lamattina, L., Bonnard, G., Weil, J.-H. and Grienemberger, J.M. (1989) RNA editing in wheat mitochondria results in the conservation of protein sequences. *Nature*, **341**, 660–662.
- Gualberto, J.M., Weil, J.H. and Grienemberger, J.M. (1990) Editing of the wheat *coxIII* transcript: evidence for twelve C to U and one U to C conversions and for sequence similarities around editing sites. *Nucleic Acids Res.*, **18**, 3771–3776.
- Haley, J. and Bogorad, L. (1990) Alternative promoters are used for genes within maize chloroplast polycistronic transcription units. *Plant Cell*, **2**, 323–333.
- Hiesel, R., Wissinger, B., Schuster, W. and Brennicke, A. (1989) RNA editing in plant mitochondria. *Science*, **246**, 1632–1634.
- Hoch, B., Maier, R.M., Appel, K., Igloi, G.L. and Kossel, H. (1991) Editing of a chloroplast mRNA by creation of an initiation codon. *Nature*, **353**, 178–180.
- Kable, M.L., Seiwert, S.D., Heidmann, S. and Stuart, K. (1996) RNA editing: a mechanism for gRNA-specified uridylation insertion into precursor mRNA. *Science*, **273**, 1189–1195.
- Kanevski, I. and Maliga, P. (1994) Relocation of the plastid *rbcL* gene to the nucleus yields functional ribulose-1,5-bisphosphate carboxylase in tobacco chloroplasts. *Proc. Natl Acad. Sci. USA*, **91**, 1969–1973.
- Koop, H.-U. and Schweiger, H.G. (1985) Regeneration of plants after electrofusion of selected pairs of protoplasts. *Eur. J. Cell Biol.*, **39**, 46–49.
- Koop, H.-U. and Spangenberg, G. (1989) Electric field induced fusion and cell reconstitution with preselected single protoplasts and subprotoplasts of higher plants. In Neumann, E., Sowers, A. and Wolford, S. (eds), *Electroporation and Electroporation in Cell Biology*. Plenum Publishers, New York, pp. 355–366.
- Koop, H.-U., Steinmüller, K., Wagner, H., Roßler, C., Eibl, C. and Sacher, L. (1996) Integration of foreign sequences into the tobacco plastome via polyethylene glycol-mediated protoplast transformation. *Planta*, **199**, 193–201.
- Kossel, H., Hoch, B., Maier, R.M., Igloi, G.L., Kudla, J., Zeltz, P., Freyer, R., Neckermann, K. and Ruf, S. (1993) RNA editing in chloroplasts. In Brennicke, A., and Kuck, U. (eds), *Plant Mitochondria*. VCH Publishers, Weinheim, Germany, pp. 93–102.
- Kudla, J., Igloi, G.L., Metzlaß, M., Hagemann, R. and Kossel, H. (1992) RNA editing in tobacco chloroplasts leads to the formation of a translatable *psbL* mRNA by a C to U substitution within the initiation codon. *EMBO J.*, **11**, 1099–1103.
- Kutznigg, M. and Stubbe, W. (1974) Investigations on plastome mutants in *Oenothera*. I. General considerations. *Subcell. Biochem.*, **3**, 73–89.
- Maier, R.M., Neckermann, K., Hoch, B., Akhmedov, N.B. and Kossel, H. (1992) Identification of editing positions in the *ndhB* transcript from maize chloroplasts reveals sequence similarities between editing sites of chloroplasts and plant mitochondria. *Nucleic Acids Res.*, **20**, 6189–6194.
- Maier, R.M., Neckermann, K., Igloi, G.L. and Kossel, H. (1995) Complete sequence of the maize chloroplast genome: gene content, hotspots of divergence and fine tuning of genetic information by transcript editing. *J. Mol. Biol.*, **251**, 614–628.
- Maier, R.M., Zeltz, P., Kossel, H., Bonnard, G., Gualberto, J.M. and Grienemberger, J.M. (1996) RNA editing in plant mitochondria and chloroplasts. *Plant Mol. Biol.*, **32**, 343–365.
- Maliga, P. and Fejes, E. (1985) Physical evidence for recombination of chloroplast DNA in a somatic hybrid of *Nicotiana tabacum* and *Nicotiana plumbaginifolia*. In Freeling, M. (ed.), *Plant Genetics*. Alan R. Liss Inc., pp. 641–650.
- Maliga, P., Fejes, E., Svab, Z. and Engler, D. (1987) Recombination of chloroplasts in cultured plant cells. In Green, E.C., Somers, D.A., Hackett, W.P. and Biesboer, D.D. (eds), *Plant Tissue and Cell Culture*. Alan R. Liss Inc., pp. 265–274.
- Medgyesy, P., Fejes, E. and Maliga, P. (1985) Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. *Proc. Natl Acad. Sci. USA*, **82**, 6960–6964.
- Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.*, **15**, 473–497.
- Schuster, W. and Brennicke, A. (1994) The plant mitochondrial genome: physical structure, information content, RNA editing, and gene migration. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **45**, 61–78.
- Schuster, W., Hiesel, R., Wissinger, B. and Brennicke, A. (1990) RNA editing in the cytochrome *b* locus of the higher plant *Oenothera berteriana* includes a U to C transition. *Mol. Cell. Biol.*, **10**, 2428–2431.
- Schweiger, H.G., Dirk, J., Koop, H.-U., Kranz, E., Neuhaus, G., Spangenberg, G. and Wolff, D. (1987) Individual selection, culture and manipulation of higher plant cells. *Theor. Appl. Genet.*, **73**, 769–783.
- Shinozaki, K. et al. (1986) The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and gene expression. *EMBO J.*, **5**, 2043–2049.
- Simpson, L., Maslov, D.A. and Blum, B. (1993) RNA editing in *Leishmania* mitochondria. In Benne, R. (ed.), *RNA Editing. The Alteration of Protein Coding Sequences of RNA*. Ellis Horwood, New York, pp. 53–85.
- Spangenberg, G. and Koop, H.-U. (1992) Low density cultures: microdroplets and single cell nurse culture. In Linsey, K. (ed.), *Plant Tissue Culture Manual*. Suppl. 1, 10A, Kluwer Academic Publishers, pp. 1–28.
- Svab, Z. and Maliga, P. (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc. Natl Acad. Sci. USA*, **90**, 913–917.
- Svab, Z., Hajdukiewicz, P. and Maliga, P. (1990) Stable transformation of plastids in higher plants. *Proc. Natl Acad. Sci. USA*, **87**, 8526–8530.
- Wiley, D.L. and Gray, J.C. (1990) An open reading frame encoding a putative haem-binding polypeptide is cotranscribed with the pea chloroplast gene for apocytochrome *f*. *Plant Mol. Biol.*, **15**, 347–356.
- Yoshinaga, K., Iinuma, H., Masuzawa, T. and Ueda, K. (1996) Extensive RNA editing of U to C in addition to C to U substitutions in the *rbcL* transcript of hornwort chloroplasts and the origin of RNA editing in green plants. *Nucleic Acids Res.*, **24**, 1008–1014.
- Yu, W. and Schuster, W. (1995) Evidence for a site-specific cytidine deamination reaction involved in C to U RNA editing in plant mitochondria. *J. Biol. Chem.*, **31**, 18227–18233.
- Zeltz, P., Hess, W.R., Neckermann, K., Börner, T. and Kossel, H. (1993) Editing of the chloroplast *rpoB* transcript is independent of chloroplast translation and shows different patterns in barley and maize. *EMBO J.*, **12**, 4291–4296.

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