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Transcript abundance supercedes editing efficiency as a factor in developmental variation of chloroplast gene expression

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ABSTRACT

In maize plastids, transcripts are known to be modified at 27 C-to-U RNA editing sites, affecting the expression of 15 different genes. The relative contribution of editing efficiency versus transcript abundance in regulation of chloroplast gene expression has previously been analyzed for only a few genes. We undertook a comprehensive analysis of the editing efficiency of each of the 27 maize editing sites in 10 different maize tissues, which contain a range of plastid types including chloroplasts, etioplasts, and amyloplasts. Using a reproducible poisoned primer extension assay, we detected variation between RNA editing extent of different sites in the same transcript in the same tissue, and between the same site in different tissues. The most striking editing deficiency is in an editing site in *ndhB* that is edited at only 8% and 1% in roots and callus plastids respectively, whereas green leaf chloroplasts edit this site at 100%. Editing efficiencies of some sites are not affected by the developmental stages we examined and are always edited close to 80–100%. The relative amounts of transcripts of each of the 10 genes that exhibited variable editing extents were determined by real-time PCR. Seven genes exhibited over 100 times lower transcript abundance in either roots or tissue-cultured cells relative to green leaf tissue. The quantitative analysis indicates that a particular editing site can be efficiently edited over a large range of transcript abundance, resulting in no general correlation of transcript abundance and editing extent. The independent variation of editing efficiency of different sites within the same transcript fits with a model that postulates individual *trans*-acting factors specific to each editing site. Because tissues where editing efficiency at certain sites is low invariably also exhibited greatly decreased abundance of the transcripts carrying those sites, decrease in the amounts of particular RNAs rather than a lack of editing is predicted to have the most significant impact on gene expression under steady-state conditions. Our data is consistent with the hypothesis that the role of editing in angiosperm plastids is to correct otherwise detrimental mutations rather than to generate significant protein diversity.

Keywords: maize; poisoned primer extension; real time RT-PCR; RNA editing

INTRODUCTION

RNA processing in the form of C-to-U modifications, known as RNA editing, was discovered in in plant organelle transcripts in the early 1990s by several groups (Covello & Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989; Hoch et al., 1991). Vascular plant chloroplast transcripts exhibit far fewer editing events than mitochondrial transcripts. For example, in *Arabidopsis*, 20 putative chloroplast RNA editing sites are known, whereas 441 C-to-U editing events have been detected in mitochondria (Giege & Brennicke, 1999; Tsudzuki et al., 2001). In both organelles, editing events have been observed to restore a codon to the transcript that will encode a more conserved protein sequence than the one specified by the unedited transcript (Maier et al., 1996). In chloroplasts, the C-to-U editing event usually occurs in the second codon position, thus usually resulting in a change in the encoded amino acid. Only few editing sites are in noncoding regions (e.g., 5′ UTR *ndhG*, in the maize chloroplast; Maier et al., 1995), or do not change the identity of the codon (Hirose et al., 1996). In only a few cases has the function of the protein encoded by an unedited chloroplast transcript been compared to that of the edited transcript. Active acetyl-CoA carboxylase protein is encoded only by edited, not unedited, transcripts (Sasaki et al., 2001), and lack of editing of *psbF* and *petB* mRNAs leads to an aberrant phenotype (Bock et al., 1994; Zito et al., 1997). RNA editing evidently is critical

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for the production of proper protein products of certain genes. Editing is also essential to create the AUG initiation codon of transcripts of certain chloroplast genes (Tsudzuki et al., 2001), so that unedited transcripts have impaired translation.

There have been very few reports concerning chloroplast editing efficiencies of particular editing sites in different tissue types. Editing of the *psbL* initiation codon occurred at high efficiency in both chloroplast and nonphotosynthetic chromoplasts (Kuntz et al., 1992). Editing efficiency of the tobacco *petB* editing site was reported at 100% in both leaves and cultured cells (Hirose et al., 1994). In tobacco *atpA* transcripts, one out of two editing sites is less edited in nonphotosynthetic tissues whereas the second one is not affected by the development stage of the plastid (Hirose et al., 1996). Editing efficiencies of some tobacco sites, particularly those in *ndh* genes, were observed to decrease after tobacco cells were subjected to heat shock or spectinomycin treatment (Karcher & Bock, 1998). Shifting maize plantlets from 20 °C to 37 °C resulted in a decrease in editing of the mRNAs of *rps8*, *rps14*, and *rpl20* (Nakajima & Mulligan, 2001). The editing efficiency of a site in tobacco *rpoA* transcripts was found to be only 70% in green leaves and 50% in nongreen cultured cells (Hirose et al., 1999). From these limited studies, there is as yet no evidence that chloroplast editing is a mechanism for generation of useful, developmentally controlled heterogeneity in proteins, unlike the situation in other systems such as mammalian apolipoprotein B, where the encoded gene product varies between tissues because of tissue-specific differences in editing extent (Powell et al., 1987; Hodges & Scott, 1992; Smith et al., 1997).

To obtain a comprehensive picture of the extent of developmental variation in editing extent, and thus the degree of possible protein heterogeneity in chloroplasts, we have monitored the editing efficiencies of each of the 27 known editing sites on the maize chloroplast genome. We show that our poisoned primer extension technique is both sensitive and robust for assaying editing efficiencies in total RNA preparations. Our results indicate that particular editing sites are more susceptible to variation than others in nonphotosynthetic and reproductive tissues. Though editing efficiency of certain sites varies between tissues, editing extent of some sites is quite high and consistent despite large variations in relative transcript abundance in different tissues. Other sites exhibit large reductions in editing efficiency in particular tissues, especially in nonphotosynthetic tissues. Our genome-wide survey of editing efficiency has detected much more developmental variation in editing efficiency of plastid transcripts than was expected from the previously reported analyses of individual sites, which examined only a few tissues and utilized less precise editing extent assays. Nevertheless, the large changes in transcript abundance we observed between different tissue types are likely to have far more influence on plastid gene expression. The chief function of plastid RNA editing appears to be transcript correction to allow synthesis of functional proteins.

RESULTS

Chloroplast RNA editing sites in maize

Initially 25 editing sites were detected on the maize chloroplast genome (Maier et al., 1995); later an additional editing site was identified (Corneille et al., 2000). In this work, we extend the number of editing sites to 27 by showing that the gene *matK* contains an editing site as suggested by Tsudzuki et al. (2001) and Vogel et al. (1997). Table 1 lists the 27 editing sites. We adopted the nomenclature defined by Tsudzuki et al. (2001), which allows comparison of editing sites in different plants.

TABLE 1. RNA editing sites in maize chloroplasts.

Editing site	Position	Genomic	Edited
atpA-3	384	(uCa) S	(uUa) L
ndhA-1	18	(uCg) S	(uUg) L
ndhA-3	159	(uCa) S	(uUa) L
ndhA-4	189	(uCa) S	(uUa) L
ndhA-5	358	(uCc) S	(uUc) F
ndhB-2	157	(cCa) P	(cUa) L
ndhB-3	197	(Cau) H	(Uau) Y
ndhB-4	205	(uCa) S	(uUa) L
ndhB-6	247	(cCa) P	(cUa) L
ndhB-8	278	(uCa) S	(uUa) L
ndhB-10	495	(cCa) P	(cUa) L
ndhD-3	294	(uCa) S	(uUa) L
ndhF-1	22	(uCa) S	(uUa) L
ndhG-1	−10	(C)	(U)
petB-1	224	(cCa) P	(cUa) L
rpl2-1	2	(aCg) T	(aUg) M
rpl20-1	104	(uCa) S	(uUa) L
rpoB-2	157	(uCg) S	(uUg) L
rpoB-3	183	(uCa) S	(uUa) L
rpoB-4	188	(uCg) S	(uUg) L
rpoB-5	207	(cCg) P	(cUg) L
rpoC2-1	926	(uCg) S	(uUg) L
rps8-1	62	(uCa) S	(uUa) L
rps14-1	28	(uCa) S	(uUa) L
ycf3-1	16	(uCc) S	(uUc) F
ycf3-2	63	(aCg) T	(aUg) M
matK-1	1353	(Cau) H	(Uau) Y

Position in base pair is from the A of the initiator codon. The amino acid change induced by editing is illustrated by the single letter code. ndhG-1 is the only editing site in a noncoding region (Maier et al., 1995; Corneille et al., 2000; Tsudzuki et al., 2001).

Evaluation of the reproducibility of the poisoned primer extension method

We decided to use a poisoned primer extension technique to monitor the 27 editing sites. Though poisoned primer extension has long been a valuable method for editing assays in the mammalian apoB and gluR editing systems (Driscoll et al., 1989; Smith et al., 1991; Schiffer & Heinemann, 1999; Schiffer et al., 2000), most prior reports that assayed editing in plant organelles have instead used a bulk cDNA sequencing method, which is more prone to variation than poisoned primer extension (Reed et al., 2001b). To examine the sensitivity of the technique, a test experiment was carried out on various mixes of cloned edited or nonedited PCR products surrounding the rpoB-2 editing site. Figure 1 shows that the poisoned primer extension (PPE) technique is sensitive and robust. We can indeed detect low percentages of editing such as 10% editing (see Fig. 1B, lane 3), and even 1% editing could be detected (result not shown). In this work, the PPE protocol was carried out on approximately 100 ng of RT-PCR product, but we show (Fig. 1B, lane 13) that as low as 4 ng of RT-PCR product is sufficient.

Sequencing of individual cDNA clones, in sufficient quantity, is considered the most accurate method to measure editing extent, but it is not cost effective for large-scale studies. We compared some of the PPE results by sequencing individual cDNA clones derived from root and callus tissue RNAs for the four first editing sites of *ndhB* and all four editing sites of *rpoB* (Table 2). Remarkably, most editing efficiencies as analyzed by cDNA sequencing are well estimated by the PPE assay. Thus the PPE method provides information comparable to sequencing of cloned cDNAs, but is simpler and more cost effective.

We also considered methods for quantifying the bands on the PPE acrylamide gel corresponding to the edited or nonedit extension product. Quantification of gel slices containing radiolabeled amplification products gives results comparable to the direct quantification of the extension products using the ImageQuant software. Figure 1C illustrates the good correlation of the quantified output of the PPE reaction with the known input. There seems to be a small overestimation of the edited band of the gel that is probably due to a small underestimation of the original concentration of the cloned, edited RT-PCR product. Lanes 7, 17, 19, 20, 21, 22, 23, and 24 of Figure 1C represent eight repetitions of the same input conditions of 50% editing, 100 ng total RT-PCR product. By calculating a mean and standard deviation to compare the two quantifying methods, we observe that the ImageQuant approach is not only easier but more reliable than the radiolabel assay. For the band representing the edited transcript, the mean and standard deviation are 55.1 $\pm$ 2.6 and 55.5 $\pm$ 1.6 for the scintillation counting and the ImageQuant technique, respectively. In this study, we normally used ddGTP in the PPE reaction, which will generate an extension product that is longer when the RT-PCR product is derived from an edited transcript than a nonedited transcript (see Fig. 1). In some cases, dictated by the sequence surrounding the editing position (if many Cs are in the close vicinity of the edited C), in order to generate extension products that have a more significant molecular weight difference, ddATP is used instead of ddGTP. This will generate a longer extension product from nonedited transcript RT-PCR products. For convenience of reading of the PPE gel, we include cloned and sequenced genomic and edited controls in each gel.

Splicing and editing at site ndhA-4

To monitor the editing efficiency using the PPE technique, each of the 27 editing sites on the 15 different maize chloroplast genes had to be amplified by RT-PCR. To avoid introducing an extra level of complexity, splicing status of the edited mRNA was not evaluated. Among the 15 genes, those containing introns (*ndhA*, *ndhB*, *petB*, *rpl2*, and *ycf3*; Maier et al., 1995) were amplified with pairs of oligonucleotides located on the same exons as the editing sites, so that both spliced and unspliced RNAs were amplified. This results in a pair of primers for each exon containing an editing site (Table 3 lists all the primers used).

Because the editing site ndhA-4 is close to the 5′ part of the second exon of the *ndhA* gene, we designed oligonucleotides that would either amplify only the spliced mRNA (primers oNP188, oNP113), or only the unspliced mRNA (oNP190, oNP113). The specificity of these PCR primers were verified on cloned spliced mRNA and genomic DNA (results not shown). As previously shown in barley (del Campo et al., 2000) and recently in tobacco (Schmitz-Linneweber et al., 2001), we observed in maize that the unspliced *ndhA* mRNA is never edited at site ndhA-4 (result not shown). Therefore, the editing data concerning ndhA-4 site is from a PPE assay performed on RT-PCR products corresponding to spliced mRNA. For all other sites, editing was studied regardless of the splicing status of the corresponding mRNA. The lack of editing of unspliced RNA we observed at ndhA-4 site is not a general rule for chloroplast transcripts. Unspliced *petB* tobacco transcripts were found to be completely edited (Hirose et al., 1994).

Site-to-site editing efficiency varies in young green leaf tissue

In most studies involving chloroplast RNA editing, transcripts in green leaves were subjected to analysis. Figure 2 represents the editing efficiency as estimated by PPE of all 27 sites for three young leaf tissue replicates. Editing is generally close to the maximum of

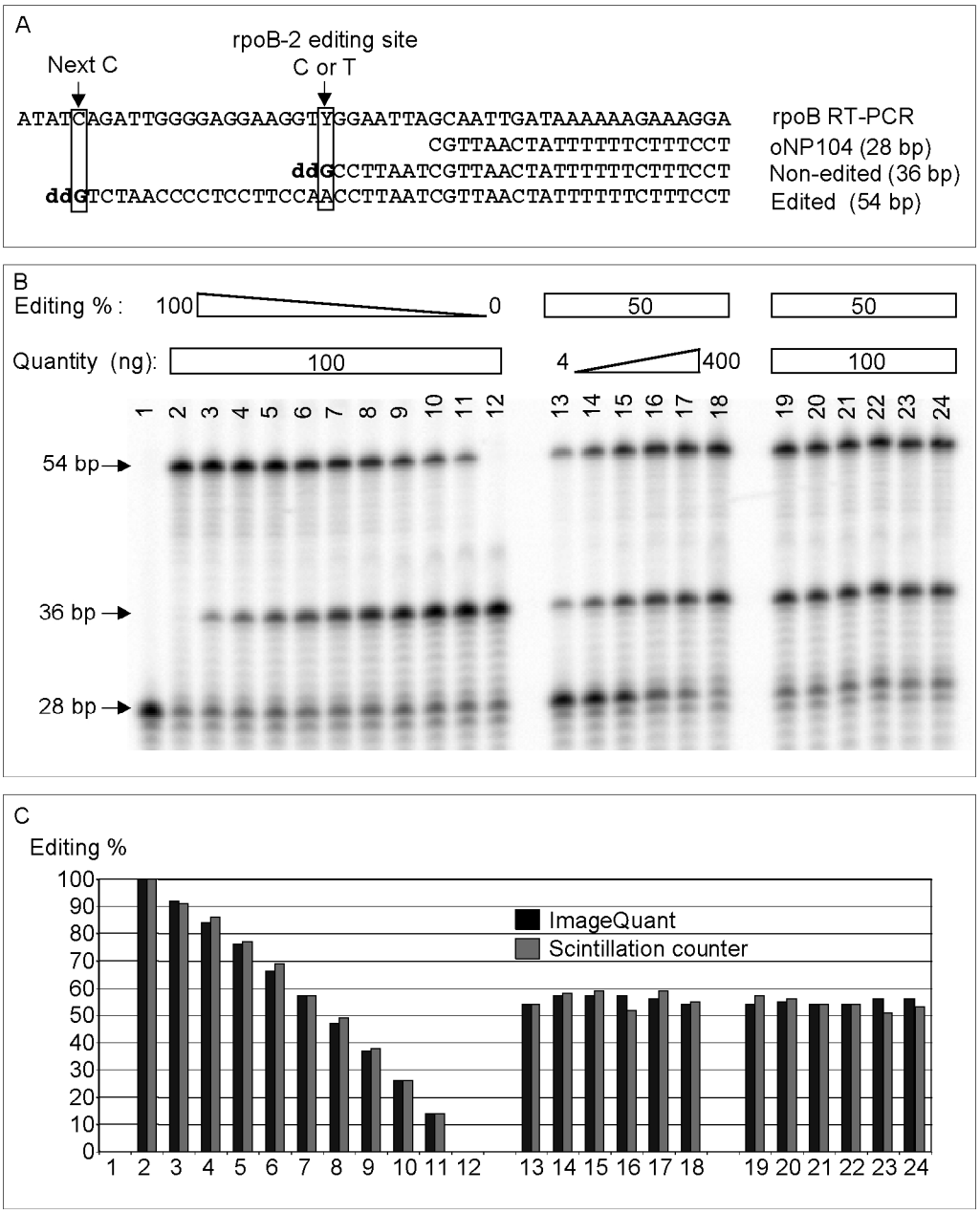

FIGURE 1. Calibration of the poisoned primer extension technique. **A**: Sequence surrounding editing site rpoB-2 and 3′ end of oNP104, the oligonucleotide used in this poisoned primer extension. The editing site and the next C in the sequence are the two possible stops of extension by incorporation of a ddGTP. The corresponding elongated products are 36 bp and 54 bp. **B**: Separation on sequencing gel and phosphorimager exposure of the primer extension products. The band at 28 bp corresponds to the labeled oligonucleotide, 36 bp to the unedited form, and 54 bp to the edited form. The PPE reaction was performed on PCR products of cloned, edited, or nonedited RT-PCR products. After quantification, several mixtures of the two PCR products were made, totaling 100 ng of PCR product. A 10% step decrease in final editing from 100% is in lane 2, 90% in lane 3 to 10% in lane 11, and 0% in lane 12. In lanes 13 to 18, the editing was set up to be 50%, but the final amount of PCR product was increased: 4 ng in lane 13, 10 ng in lane 14, 20 ng in lane 15, 50 ng in lane 16, 100 ng in lane 17, and 400 ng in lane 18. Lanes 19 to 24 are six repetitions of a 50% editing, 100 ng total experiment. **C**: Calculation of the editing percentage by the cutting out each band and quantifying it in a scintillation counter, or by using the ImageQuant software. The X-axis represents the 24 different lanes and the Y-axis is the editing percentage.

efficiency although some editing sites peak in the range of 80–90% efficiency (ndhA-5, petB-1, rpl20-1, rpoB-2, -3, -4, -5, ycf3-2, matK-1). Thus 10–20% of the transcripts of these six genes in some tissues could be potentially translated into a protein carrying a less conserved amino acid than the polypeptide encoded by edited transcripts. Because independently performed editing extent assays of the same RNA vary 5% or

TABLE 2. Comparison of editing efficiency by direct sequencing of individual clones and PPE reaction.

Editing site	Root (sample 2)		Callus (sample 10)	
	Sequencing	PPE	Sequencing	PPE
rpoB-2	33/41 = 80%	78%	31/41 = 76%	69%
rpoB-3	34/41 = 83%	83%	33/41 = 80%	85%
rpoB-4	23/41 = 56%	52%	10/41 = 24%	23%
rpoB-5	31/41 = 76%	73%	20/41 = 49%	52%
ndhB-2	28/42 = 67%	72%	38/44 = 86%	90%
ndhB-3	18/42 = 43%	49%	7/44 = 16%	24%
ndhB-4	2/42 = 5%	8%	0/44 = 0%	1%
ndhB-6	32/42 = 76%	71%	12/44 = 27%	40%

less, and we can easily distinguish 10% differences in editing extent in artificial mixing experiments (Fig. 1), we believe it worthwhile to focus attention on any difference of 20% or more in editing extent between two tissues.

Editing efficiency of transcripts carrying multiple editing sites

Transcripts of four genes contain more than one editing site. All six PPE gels corresponding to the six sites in the *ndhB* transcript are shown in Figure 3A. The corresponding quantification is shown in function of the 10 different plant tissues in Figure 3B. In root (sample 2) and callus (sample 10), five of the six editing sites are poorly edited. Only one site, ndhB-8, is edited at a normal level in roots, and only ndhB-2 exhibits normal levels of editing in callus. Furthermore, the site ndhB-4 is the most affected editing site; it is edited at only 8% in roots, 13% in ovules, and 1% in callus. NdhB-4 editing is also impaired in nongreen vascular tissue (sample 7) and silks (sample 8), but not in etiolated leaves (sample 3) and anthers (sample 6).

Figure 4 represents the editing efficiency of the four editing sites of *ndhA* (A) and *rpoB* (B) and the two editing sites of *ycf3* (C). The PPE gels for editing sites that present little or no variation throughout the various plant tissues are not shown. In *ndhA* (Fig. 4A), one site (ndhA-4) is less edited only in callus tissue, and another site (ndhA-5) is primarily affected in roots and callus tissues and slightly in silks (sample 8) and ovules (sample 9); again this is not a general feature for all editing sites, but only for two out of the four of the editing sites on *ndhA* transcripts. For *rpoB*, three editing sites (rpoB-2, rpoB-4, and rpoB-5) exhibit editing deficiencies in root and callus samples like *ndhB* and *ndhA*, but also in the anther sample (see Fig. 4B, sample 6). Finally, of the two editing sites in the *ycf3* gene, only editing at site ycf3-2 is significantly affected, and only in root tissue (see Fig. 4C).

For the six *ndhB* editing sites, there is always at least one editing site which is edited at levels comparable to green leaf tissue (80 to 100%), even when the editing extent of other *ndhB* editing sites are reduced. For example, this is the case for the site ndhB-8 in root, and the site ndhB-2 in callus (Fig. 3). The site rpoB-3, whose editing efficiency is little affected throughout the different tissues (Fig. 4B), is only 15 bases upstream from site rpoB-4, which is poorly edited in roots and callus. The independent variation of editing efficiency in the same tissue implies that it is not a feature of the entire transcript that affects editing efficiency, but a feature of the particular site.

Editing efficiency is variable in six other sites

Some genes that have single editing sites also exhibit variability in editing throughout the 10 different plant tissues. The ndhD-3, ndhF-1, and ndhG-1 sites are greatly reduced in editing in the root samples (Fig. 5A, around 50% efficiency, compared to the 100% in leaf tissue). Only ndhF-1 and ndhG-1 are affected also in callus (only 20% editing). PetB-1 is edited only around 50–60% in various tissues (Fig. 5B). The rpl20-1 and matK-1 sites are edited at a lower extent only in callus tissue (40% and 70%, respectively).

Editing sites with very little variability

There are five editing sites that did not show any significant reduction in editing in any of the 10 tissues examined. These sites are atpA-3, rpl2-1, rpoC2-1, rps8-1, and rps14-1. Their editing efficiency is always greater than 80% in the tissues we are assaying (results not shown).

Relative abundance of the mRNAs containing editing sites

To determine whether the variation in editing efficiency is correlated with altered transcript abundance, we measured the abundance of mRNA containing editing sites relative to transcripts of a nuclear reference gene, α-actin, and relative to the abundance in the young green leaf tissue (sample 1, called the calibrator). This gene has already been used as a reference gene in other studies (Raizada & Walbot, 2000; Rudenko & Walbot, 2001), but not in as diverse a set of plant tissues as we analyzed. We were able to show that α-actin could be used as a reference gene in this study because its expression, as monitored by real-time RT-PCR, varied only a few-fold in samples containing approximately the same amount of RNA, as measured by absorbance and gel staining. If sample 1 is considered the calibrator sample of value 1, then the relative value of α-actin mRNA abundance in the other sam-

TABLE 3. Oligonucleotides used in this work.

Name	Sequence (5′–3′)	Purpose
oNP91	GAAAGGGATGCTGTGTATGAATCACTCAC	PCR rpoB 5′
oNP92	CCACCTACACAAGCAAATTGTTGATAAA	PCR rpoB 3′
oNP104	CCCATATCCTTTCTTTTTTATCAATTGC	PPE rpoB-2
oNP105	TTTCTCTTAGATTCRAACCCATAGCTGA	PPE rpoB-3
oNP106	GGGTAGGAAACATTATCTAAAATTTCTCTTAGA	PPE rpoB-4
oNP107	ACTCAATCCTCTTCTTCTCCTTAGCAT	PPE rpoB-5
oNP108	GTTCCTGAATAGCTTCYTTCAAAAGGG	PCR atpA 5′
oNP109	CGAGARTTAATTATTGGGGACAGACAGA	PCR atpA 3′
oNP112	ATGATAATAGATAGGGTAGAGGTAGAAACTATCAA	PCR ndhA 5′
oNP113	TCTCTTGTTTGAGAGAACTTTTGTGTTG	PCR ndhA 3′
oNP116	TTATTCGTATTAACAGCTACTCTAGGGGGA	PCR ndhB 5′
oNP117	GGGATCTTGAACTAAGAAATAGACCTAGCA	PCR ndhB 3′
oNP118	GGCTTCCTTATTGCTTATGCTGTCA	PCR ndhD 5′
oNP119	TAGATATTCCCCCCAGTTCTTCGAG	PCR ndhD 3′
oNP120	TGGAACATACATATCAATATGCCTGGG	PCR ndhF 5′
oNP121	CCTATCAATAAATAGGAACACATTCCCACA	PCR ndhF 3′
oNP122	AGTCAGTTCATGAAAAATTTTATACTAGAAATTTC	PCR ndhG 5′
oNP123	AAAGAATTCCATACCACGACGTATC	PCR ndhG 3′
oNP124	TGATCCTGCAYGTATTTCGTGTGTATCT	PCR petB 5′
oNP125	GACCCGAAATACCTTGCTTACGTATCAT	PCR petB 3′, PPE petB-1
oNP126	TATTCCACTTCTAGATAGAGAAACGAACTAAAGGA	PCR rpl25′
oNP127	CCATTGATATAGGAACTTTTGTACCAGAAAC	PCR rpl23′
oNP128	ATGACCAGAGTTCCGCGAGGATATA	PCR rpl205′
oNP129	TATTTTATTGGAAATCGTGTAAAGATTATTTGGA	PCR rpl20, 3′ PPE rpl20-1
oNP132	GATTTACTAACCTCTATAAGAAACGCAGACATG	PCR rps85′
oNP133	CTTCTCCCCCAATTCTGTTTAGTCG	PCR rps83′
oNP134	CAARRAAAAGTTTGATTCAGAGGGAGAAGAAG	PCR rps145′
oNP135	CGGATAGYCCAAAGTCTCGATAGTTAGCTCT	PCR rps143′
oNP136	GCCTAGATCCCGTATAAATGGAAATTTCAT	PCR ycf35′
oNP138	GGCTTGTAATTCTGCGAATTGAGCTAGT	PPE atpA-3
oNP139	GGGTAGAATCCATATAAGTCCGTAGATTTCTTTAA	PPE ndhA-1
oNP140	GCAGCCGCTCGTAGACCGC	PPE ndhA-3
oNP141	ATATTTGGACTGTGCTTCAACTATATCAACTGT	PPE ndhA-4
oNP142	TACTGTTATTGTATTCTTATTTATAGTGAAACTAG	PPE ndhA-5
oNP143	CAGACAATAGGTAGGAACATAAACTGAAACAT	PPE ndhB-2
oNP144	CCCAGATAAACCATATAGCCAAGAGAAAC	PPE ndhB-3
oNP145	AAGCTCGATCTCCCCCCCAGA	PPE ndhB-4
oNP146	CGTCAGGAGTCCATTGATGAAAAGG	PPE ndhB-6
oNP147	CGAGAATTCGCGTGGCTAAAGC	PPE ndhB-8
oNP148	AATTGCAAGAATGGGGTTCATTGATAT	PPE ndhB-10
oNP149	TTTTTGAAATTTCGTTGACCAAGAGAAGT	PPE ndhD-3
oNP150	GTTGCTGTAGGAATAAGAAAAAGTCCAAATC	PPE ndhF-1
oNP151	GTATTGGTCCAGGTAAATCCATTATGGA	PPE ndhG-1
oNP152	GGGATAGGTGTTTTGTATAAATGTTTCGC	PPE rpl2-1
oNP153	ATTTCATATGCGACTGCAGGTCGA	PPE rpoC2-1
oNP154	TAGTCTTTCTCCTTTGATGTCGCAAAGT	PPE rps8-1
oNP155	ACTCAAAGGGGAAACTTTGCTTCTTATC	PPE rps14-1
oNP156	ATTTGCAATAAAATATTGGCTACAATCGA	PPE ycf3-1
oNP157	AGTATATAACTTCGATCATAGGGATCAATTTCTAG	PPE ycf3-2
oNP158	GCGCTATAGCTTGTTTCCAATACTCAG	PCR ycf35′
oNP159	CCAAGCTTCCGCAATTTCCGA	PCR ycf35′
oNP160	GAAGGAATATGGGACTTTAGAAGAAGACTC	PCR rpoC2 5′
oNP161	TTCCCAATTCACTACTAAGCAAGTTCGA	PCR rpoC2 3′
oNP187	GGTATTTCATAACTAATAGATTGAGCAGCC	PCR ndhA 3′
oNP188	TTTTGTGTACTAGCAATATCTCTACTATCTAATAG	PCR ndhA 5′
oNP189	CCAGTCGTTGCTTTTCTTTCTGTTAC	PCR ndhB 5′
oNP190	CTGTGATGTTATCATCGACTATGATTATCTAATAG	PCR ndhA 5′
oNP191	ATCTCTATAATAAGTAAATGCCCTTTTTTCC	PCR ycf33′
oNP192	GGCTCAATCCGAAGGAAATTATG	PCR ycf35′
oNP193	CAGATCACGGCCATATTATTAAAAGC	PCR ycf3 3′
oNP195	GGCAGAATGTGAAAGATTACCCTTT	PCR ndhA 5′
oNP202	TTTTTGCCCTCCTACTGGGAATCACA	SYBR ndhA 5′
oNP203	GCCAGCATATTCAGGACCAATACGTTG	SYBR ndhA 3′
oNP204	TTCCTCCACTAGCAGGTTTCTTCGGAA	SYBR ndhB 5′
oNP205	AACGCTCGTAAGGAGTCCTATTGAAACCA	SYBR ndhB 3′
oNP206	ACTGGAGATTGGGAATCGATCCACTTTC	SYBR ndhD 5′
oNP207	ATTCCGAGTAACCGGCCAAGCC	SYBR ndhD 3′
oNP208	CCTGATGCTATGGAAGGACCCACTCC	SYBR ndhF 5′
oNP209	TCCAGGGTAGGGATATGAAAAGAGGAAGAA	SYBR ndhF 3′
oNP210	ACCCAATTTATTCTGCCTTTTCGCTGG	SYBR ndhG 5′
oNP211	AAGAAGTTGTGCGACAGCTACAAAGTAGGA	SYBR ndhG 3′
oNP212	CTAATGATGATCCTGCACGTATTTCGTGTG	SYBR petB 5′
oNP213	GTCAATACAGCCAAAACCACGCCTG	SYBR petB 3′
oNP214	CGGAGACGACGAACAAAAATGCGTT	SYBR rpl20 5′
oNP215	CCTGCCTCTATCTCGATGAGAAGAAACAAA	SYBR rpl20 3′
oNP216	AAGATCAATTCGGACTGGCTCTTGGC	SYBR rpoB 5′
oNP217	CAAAGTTTGTGGAGTCGGTTTCGATTGAC	SYBR rpoB 3′
oNP218	ATGGCCGTGATCTGTCATTAGAGGAGAA	SYBR ycf3 5′
oNP219	CTTGTTTCCAATACTCAGCAGCTTGATCAA	SYBR ycf3 3′
oNP230	ATTGTGATGGATTCAGGTGATGGTGTGA	SYBR actin 5′
oNP231	TCACGCCCCGCAAGATCCAA	SYBR actin 3′
oNP232	GTCGATACAAAGTCTGTTTTTTCGAAGA	PPE matK
oNP233	AGTCCTTGCAACTCCCCTTGTC	PCR matk 5′
oNP234	CCCAATCGTTGCATAAAAGTTCGTA	PCR matK 3′
oNP235	GCAATTCTTGCATCAAAAGGCACTCTTC	SYBR matK 5′
oNP236	GTTTAGACGAATCCTTTGTGGTTGAGTCCA	SYBR matK 3′

Oligonucleotides used to amplify around editing sites are called PCR, the ones used in a poisoned primer extension are noted PPE, and the ones used in the quantification of mRNAs are called SYBR. Each oligonucleotide can be positioned on the maize chloroplast genome (accession NC_001666) by performing a sequence search.

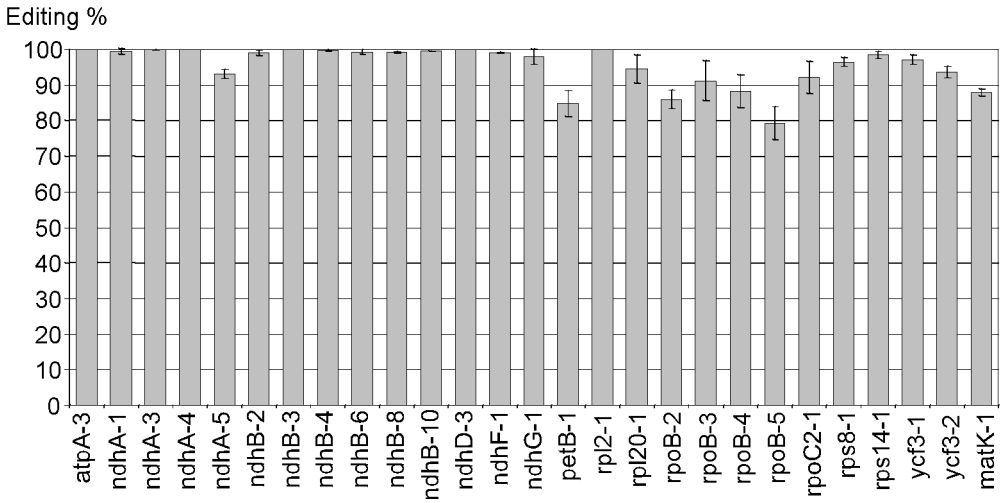

FIGURE 2. Editing extent of the 27 different sites in young green leaf chloroplasts. For each site, three different young green leaf samples were examined. Two samples correspond to greenhouse-grown seedlings (one of them is sample 4 of this study), the other is a Magenta box grown seedling (sample 1 of this study). For each site, the mean of the three independent experiments is given as well as the standard deviation. The X-axis represents the 27 different editing sites using the same descriptive name as in Table 1.

ples is: sample 2: 1.9, sample 3: 2.2, sample 4: 1.2, sample 5: −1.6, sample 6: −2.8, sample 7: 2.5, sample 8: −1.4, sample 9: −4.6, and sample 10: −1.9. These variations could be due to small inaccuracies in the total mRNA quantification or to small but genuine differences in α-actin mRNA abundance or both. We have followed the standard practice of comparing real-time RT-PCR data to a reference transcript rather than to estimates of total RNA.

Figure 6 displays the relative abundance of mRNA of the four genes (*ndhA*, *ndhB*, *rpoB*, and *ycf3*) that have several editing sites. Most of the tissues examined have a lower mRNA abundance than the green leaf tissue. *NdhA* and *ndhB* mRNAs are less abundant in roots, anthers, ovules, and callus. *RpoB* mRNAs are less abundant in roots, anthers, and callus, although the decrease is not as pronounced as for *ndhA* and *ndhB*. *Ycf3* mRNAs are 50 to 80 times less abundant in roots, anthers, and ovules than in leaves, and are extremely reduced in nongreen cultured cells (around 700 times less than in leaf).

We similarly examined the mRNA abundance in the six other genes that exhibit variable editing efficiency (Fig. 7). The ndh subunits *ndhD*, *F*, and *G* mRNAs have a pattern of relative abundance similar to *ndhA* and *ndhB*: roots, anthers, ovules, and callus accumulate less mRNA than the green leaf sample. *MatK* and *petB* have patterns of mRNA abundance similar to the ndh genes, although anther tissues contain more *petB* mRNA than root, ovules, or callus tissue. *Rpl20* mRNA abundance is less variable; the pattern of abundance is very similar to *rpoB*, with even an increase in relative abundance of mRNA in the etiolated leaf tissue (sample 3).

Editing efficiency does not invariably increase nor decrease with more target RNA relative to α-actin RNA. This point is illustrated in Figure 8 with respect to two editing sites, ndhF-1 and ycf3-2, but can be observed for a number of other editing sites by comparing the editing extent (Figs. 3–4) with the RNA abundance (Figs. 6–7). In Figure 8A, ndhF-1 editing frequency can be seen not to decrease directly in proportion to the decreases in RNA abundance, which range from 30 to 70 times less than in green leaves. Likewise in Figure 8B, we illustrate that editing efficiency of ycf3-2 in tissues with comparable RNA abundance ranges from 30 to 90%. *RpoB* transcripts are the only ones in which the editing efficiency of most sites is generally lower when the mRNA abundance is lower. Except for site rpoB-3, which remains unaffected throughout the different tissues, editing efficiency of other *rpoB* sites is lower in roots, anthers, and callus; likewise, the mRNA abundance relative to the green leaf tissue is lower in those same tissues. Increased RNA abundance of *rpoB* also correlates with higher *rpoB* editing efficiency in etiolated leaves (sample 3), as there the mRNA abundance is six times higher than in the calibrating leaf sample, and the editing of the four editing sites is also at higher efficiency than the young green leaves.

We detected only one gene whose product's activity could possibly be as severely affected by editing as by decrease in transcript abundance. NdhB-4 editing efficiency was assayed to be only 8% in roots and 1% in callus. The amino acid at this editing site is highly conserved not only in plants, but also in sequences of photosynthetic bacteria (Fig. 9). Although the amino acids encoded by the edited ndhB-3 and ndhB-4 sites

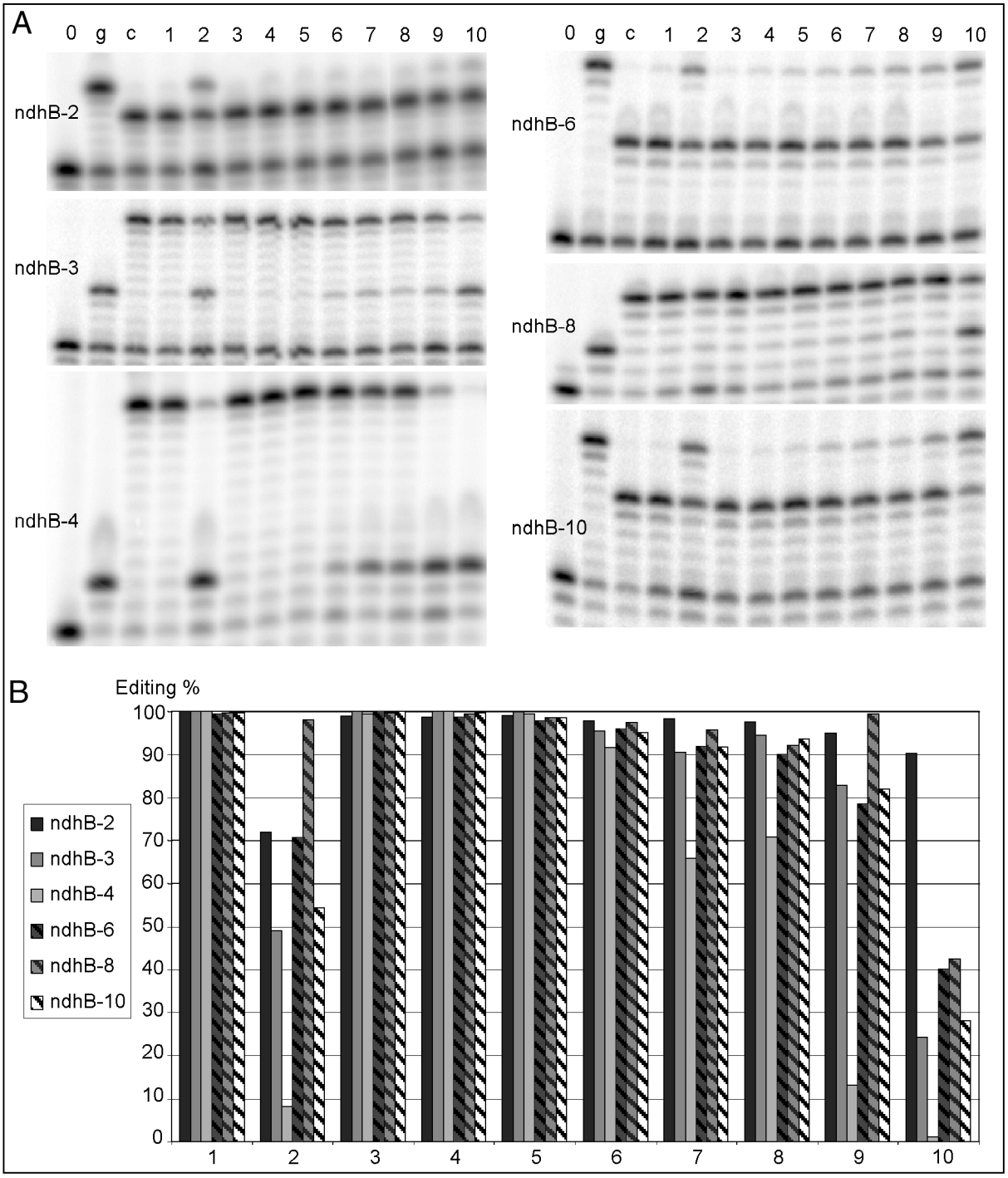

FIGURE 3. Poisoned primer extension assay on the six *ndhB* editing sites. **A**: The six editing sites, ndhB-2, ndhB-3, ndhB-4, ndhB-6, ndhB-8, and ndhB-10 (see Table 1) analyzed by PPE in 10 different plant tissue samples. 1: young green leaf grown in a Magenta box; 2: root; 3: etiolated young leaf; 4: young green leaf from a greenhouse seedling; 5: old green leaf; 6: anthers; 7: stem vascular tissue; 8: silks; 9: ovules; and 10: callus tissue. Lane 0 represent a PPE without template; this indicates the size of the radiolabeled oligonucleotide. Lane g is a PPE with a cloned unedited (genomic) RT-PCR product; lane c is a PPE with a cloned edited (cDNA) RT-PCR product. These two lanes, respectively, illustrate the PPE bands corresponding to the unedited or edited RT-PCR counterpart. In **B**, editing extent (%) of all six editing sites is shown as a function of the origin of the RT-PCR products. Note that the ndhB-2 and ndhB-10 PPE reactions were performed with ddATP instead of ddGTP.

are equally highly conserved, and only 24 nt apart, the ndhB-4 site is always less edited than ndhB-3 except in leaves and anthers. The combination of low transcript abundance and lack of editing of a highly conserved codon makes it unlikely that active NDHB subunit is present in roots and callus.

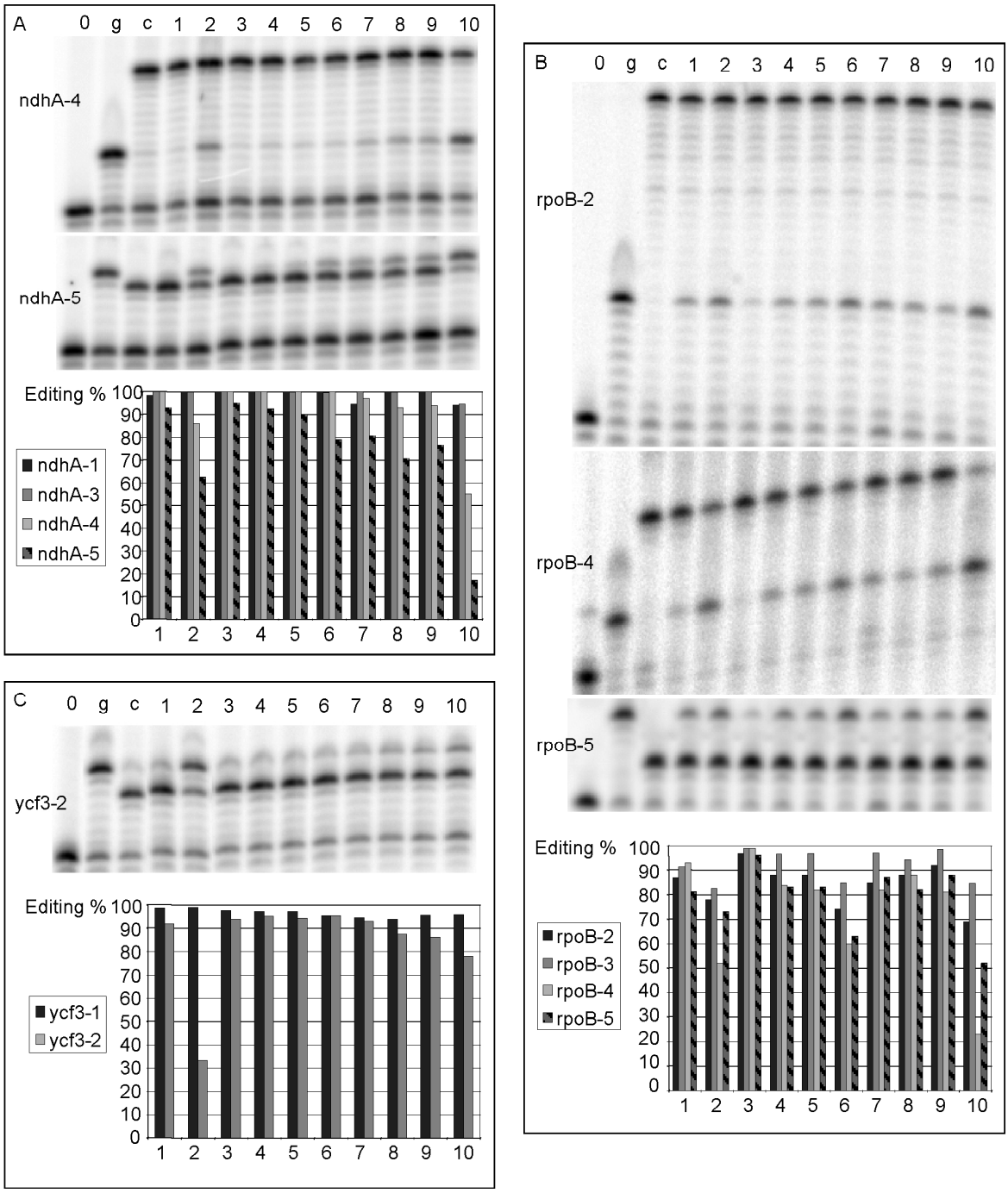

FIGURE 4. PPE on *ndhA*, *rpoB*, and *ycf3* editing sites. The different lanes correspond to those in Figure 3. Only the editing sites that are variable in the 10 plant tissue are displayed as PPE gels. **A**: PPE gel of the sites ndhA-4 and ndhA-5 and the editing efficiency of the four *ndhA* editing sites in each RNA sample labeled as in Figure 3. **B**: PPE gel for editing sites rpoB-2, rpoB-4, and rpoB-5, and the histogram for all four *rpoB* editing sites. **C**: PPE gel of the ycf3-2 editing site and the editing efficiency for the two editing sites of *ycf3*.

DISCUSSION

Models for the mechanism(s) of tissue-specific editing efficiency variation

We observed more variation in the maximum extent of editing than expected from the limited prior data available. Twenty-two of the editing sites have different editing efficiencies across the plant tissues surveyed, whereas five others (atpA-3, rpl2-1, rpoC2-1, rps8-1, and rps14-1) exhibit little variation according to the developmental status of the plastids and tissue. Little is known about the factors that influence the editing efficiency at a site. Certainly *cis*-acting elements are im-

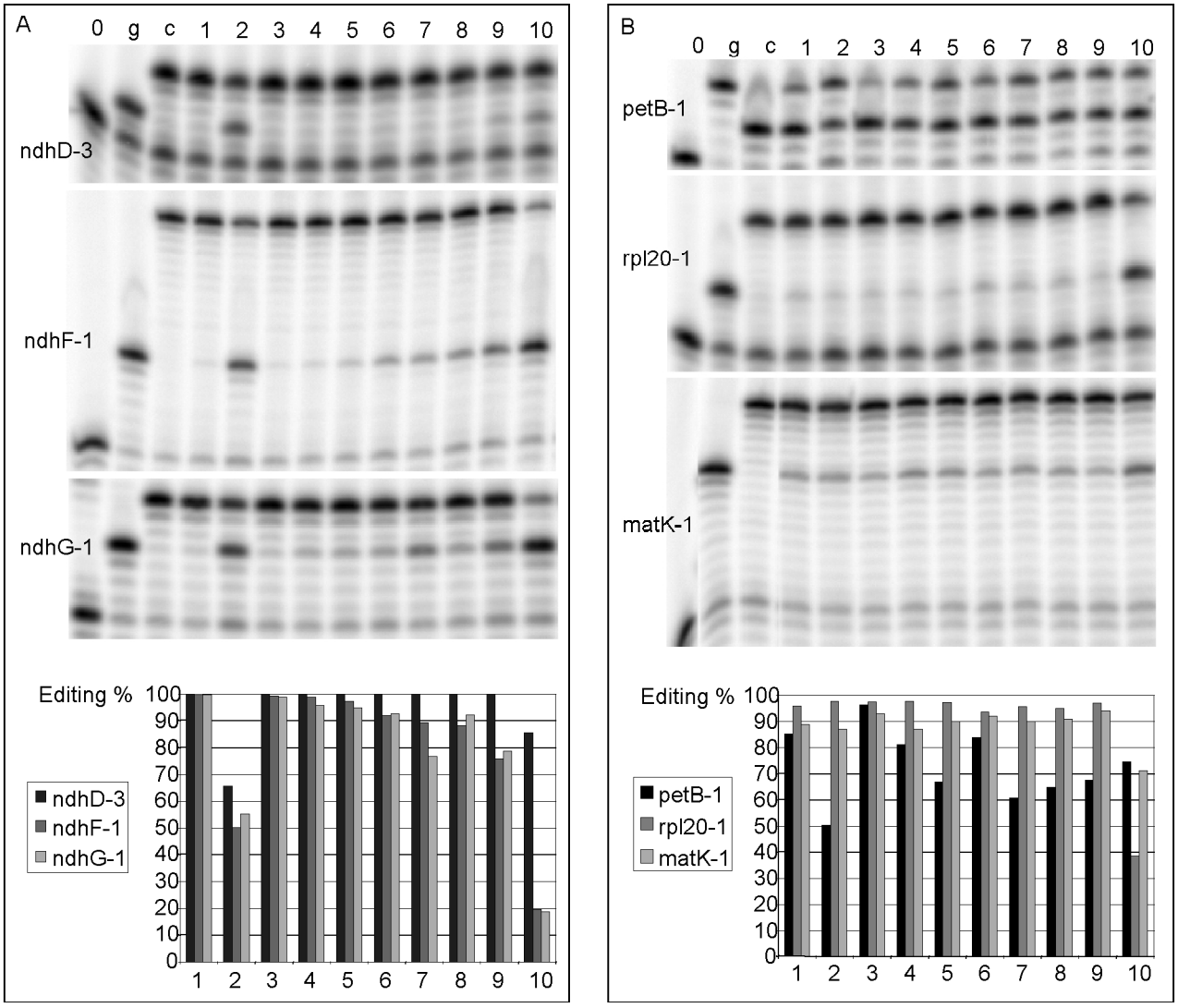

FIGURE 5. PPE assay on six other editing sites that exhibit variability in editing efficiency. The axes of the PPE gels and histograms are the same as in Figures 3 and 4. **A**: Editing of ndhD-3, ndhF-1, and ndhG-1. **B**: Editing of petB-1, rpl20-1, and matK-1.

portant; transgenics with altered sequences surrounding edited Cs are often edited less efficiently than the wild-type sequence (Bock et al., 1996; Chaudhuri & Maliga, 1996; Reed & Hanson, 1997). Possibly some sites do not have appropriate *cis*-acting elements for efficient editing.

A model consistent with our finding of site-specific developmental variation invokes the existence of nuclear-encoded site-specific *trans*-acting factors that are regulated by tissue-specific promoters. The precedent for this model is the prediction of site-specific factors from analysis of chloroplast transgenic plants that overexpress certain editing sites. When *psbL*, *rpoB*, and *ndhF* sites are overexpressed in transgenic tobacco chloroplasts, for example, the endogenous homologous transcripts exhibit a reduction in editing efficiency, implying that a site-specific *trans*-acting factor is limiting (Chaudhuri & Maliga, 1996; Reed & Hanson, 1997; Reed et al., 2001a). A recent study (Nakajima & Mulligan, 2001) shows that three editing sites (in *rps8*, *rps14*, and *rpl20* mRNAs) are less edited when maize plantlets are shifted from 20 °C to 37 °C. The authors suggest that the higher rate of transcription they detect for these genes could exceed the editing capacity, resulting in lower editing. Perhaps nuclear genes for editing factors are not expressed sufficiently under heat-shock conditions to compensate for a rapid increase in chloroplast gene transcript abundance. Some sites may not have adequate amounts of *trans*-factors even in wild-type nontransgenic maize, in normal growth conditions, particularly in certain tissues. The extent of editing in a particular tissue could be affected if a nuclear-encoded site-specific *trans*-acting factor is controlled by a tissue-specific promoter. In that

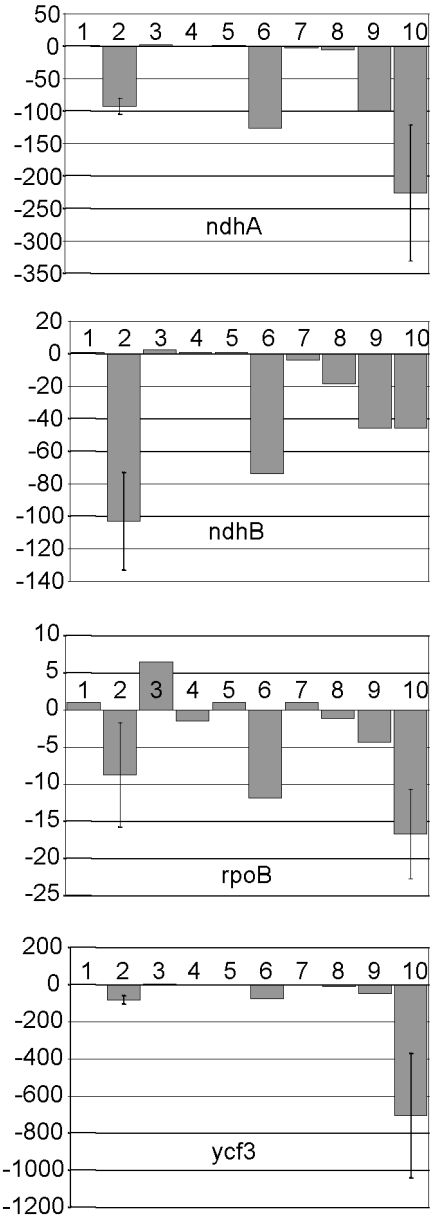

FIGURE 6. Relative quantitation of *ndhA*, *ndhB*, *rpoB*, and *ycf3* mRNA abundance. Abundance in mRNA is calculated relative to an endogenous reference, α-actin, and relative to a sample calibrator; sample 1, the young green leaf tissue. The X-axis shows the 10 different plant samples (see Fig. 3); the Y-axis displays the *n*-fold variation in reference to the calibrator (mRNA abundance for the calibrator is one). The scale of the Y-axis is different for each gene displayed. For samples 2 (root) and 10 (callus) the mean ± standard deviation for two independent experiments is displayed. The standard deviation for sample 10, gene *ndhB*, was equal to zero.

case, the amount of editing could be regulated by *trans*-factor's promoter's activity in each tissue, when promoter activity and amount of gene product are correlated. Stability or translatability of the *trans*-acting factor could also vary in different tissues. If a plastid polypeptide is not required for function of particular tissues, there would be no selective pressure to ensure the expression of a site-specific factor responsible for editing of the transcripts encoding the polypeptide.

Some sites never reach 100% editing in any of the 10 tissues analyzed. Perhaps there is never sufficient *trans*-acting factor, nor the required optimal *cis*-acting elements, to permit 100% editing. For these sites, it is also possible that the C-to-U editing reaction is not driven to 100% U at equilibrium, but the reverse U-to-C reaction may also be occurring. Though U-to-C editing events have not been detected in angiosperms, they have been reported in chloroplasts of hornworts (Yoshinaga et al., 1996). If a U-to-C reverse editing reaction can occur in angiosperms, then this reaction must also be site-specific to Cs that normally undergo editing to U, as no genomically encoded Us have yet been detected to be changed to C in transcripts in flowering plants.

Developmental variation in abundance of particular plastid transcripts

Though our quantitative comparisons of plastid transcript abundance were performed to evaluate the relationship of transcript abundance and RNA editing efficiency, the information by itself is interesting with regard to developmental regulation of plastid gene expression, as real time RT-PCR has not previously been used to analyze quantitatively the abundance of individual maize plastid genes' transcripts. The mRNA quantification we performed was normalized to the nuclear house-keeping gene, α-actin. All of the 10 genes exhibited significant decreases in plastid RNA/α-actin ratios in four tissues: root, anthers, ovules, and callus. Three *ndh* genes' mRNAs exhibited over a 100 times decrease relative to young leaf, suggesting that the NAD(P)H-plastoquinone-oxidoreductase is not likely to be very active in those tissues. The biological function of this complex is still under debate (Burrows et al., 1998; Kofer et al., 1998; Koop et al., 1998; Maliga & Nixon, 1998; Roldan, 1999), but has been postulated to function to reduce oxidative stress. In chloroplasts, the complex may function to reduce the plastoquinone pool using stromal reductant, allowing the production of ATP via the chlororespiration mechanism. The NDH complex has been reported to be active (Catala et al., 1997; Guera et al., 2000) in etiolated barley seedlings; this is consistent with our findings that *ndh* subunit transcripts are not decreased in abundance in etiolated leaves.

Not unexpectedly, transcripts of several genes encoding proteins important in photosynthesis exhibited large decreases in roots, callus, and reproductive tissues. Transcripts of *ycf3*, encoding a product needed for assembly of photosystem I (Ruf et al., 1997) and for *petB*, a subunit of the cytochrome b/f complex, were both greatly decreased in roots and callus. Transcripts of two genes encoding components of the plastid gene expression machinery, namely a ribosomal protein and a subunit of the plastid RNA polymerase, exhibited much less variation in abundance in roots, callus, and reproductive tissues, with the maximum decrease in the

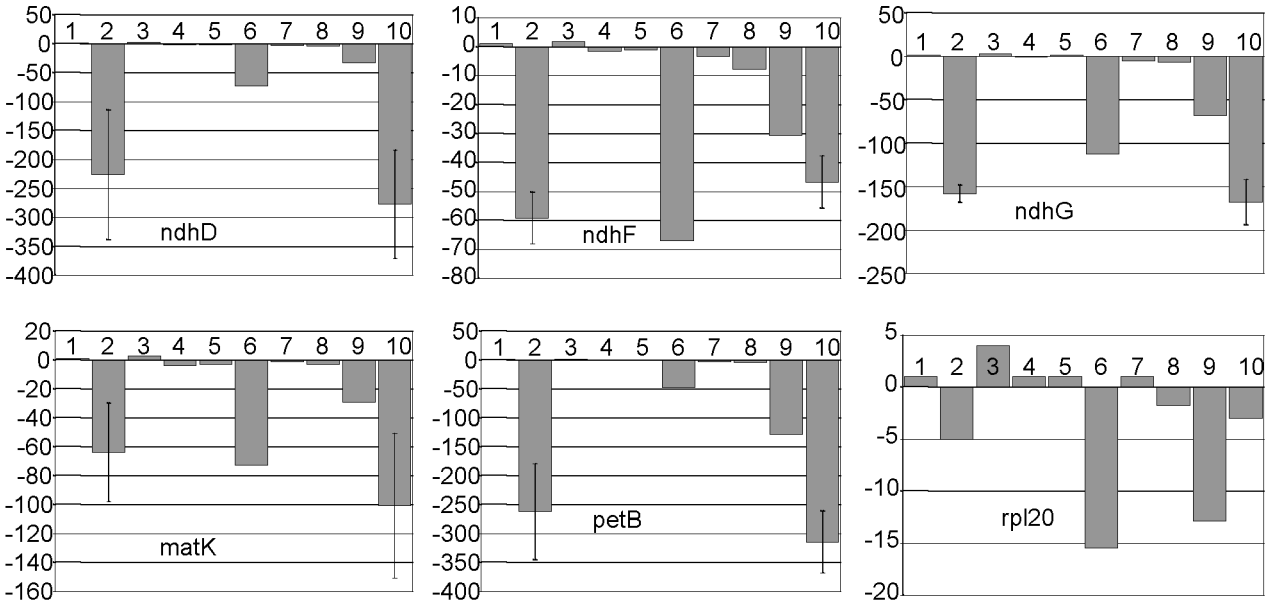

FIGURE 7. Relative quantitation of *ndhD*, *ndhF*, *ndhG*, *matK*, *petB*, and *rpl20* mRNA. Same axes as in Figure 6. Two independent experiments were performed on root and callus (except for *rpl20*); hence the error bars.

range of 10–20 times relative to α-actin, whereas the photosynthesis-related transcripts were decreased 200–700 times in such tissues.

Because we are comparing RNAs from different plastid and tissue types, the abundance of plastid RNA per cell could be affected not only by transcription rate and mRNA stability, but also by the number of chloroplast genomes per plastid and the number of plastids per cell. Because the volume of individual plastids and the total volume of all plastids vary between tissues, the concentration of individual transcripts per plastid may not always be reflected by the changes in abundance relative to α-actin. For example, plastids in young green leaves are larger and more numerous per cell on average than those in root cells. Thus, though *ycf3* transcript is 700 times more abundant in leaves than in roots, the leaf cell's average total plastid volume could easily be 10 times that of the root plastids, reducing the transcript concentration differential between a chloroplast and root plastid. Nevertheless, it is clear that editing efficiency can remain high over a large difference in transcript concentration. Over 80% of *ycf3* mRNAs are edited at site ycf3-2 in both chloroplasts and roots despite the large differential in transcript abundance.

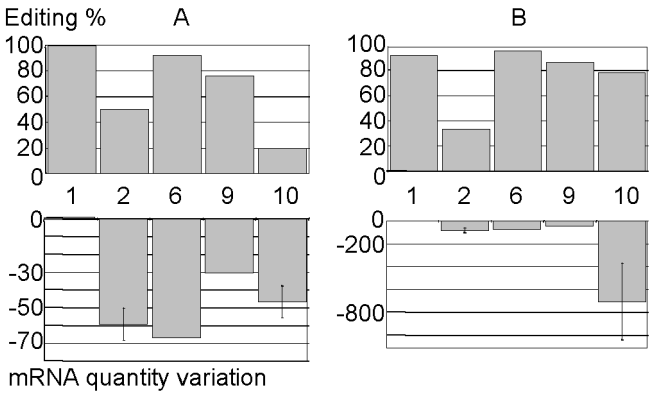

FIGURE 8. Comparison of editing and transcript abundance. **A**: ndhF-1 editing efficiency and *ndhF* mRNA abundance in lane 1: young green leaf tissue (calibrator for relative mRNA abundance); lane 2: root, lane 6: anther; lane 9: ovule; and lane 10: callus tissue. **B**: ycf3-2 editing efficiency and *ycf3* mRNA abundance in the same tissues. The Y-axis for editing efficiency and mRNA abundance are as in Figures 3–5 and 6–7, respectively.

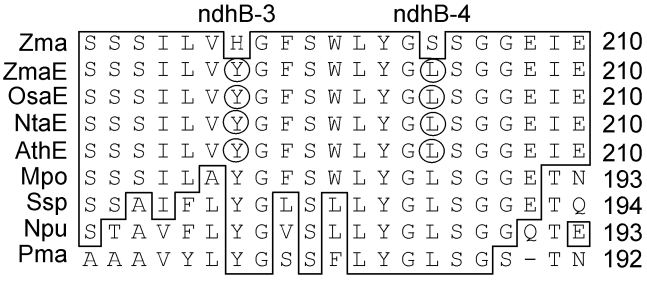

FIGURE 9. Alignment of a portion of *ndhB* predicted protein sequences illustrates the degree of conservation of the amino acid specified by the edited versus unedited transcript at the ndhB-3 and ndhB-4 editing sites. The different NDHB sequences are from Zma, *Zea mays* (P46619); Nta, *Nicotiana tabacum* (P06256); Osa, *Orysa sativa* (P12125); Ath, *Arabidopsis thaliana* (Q9T3G4); Mpo, *Marchantia polymorpha* (NP_039272); Ssp, *Synechoccystis sp.*(P72714); Npu, *Nostoc punctiforme* (NC_002791); and Pma, *Prochlorococcus marinus* (NC_002715). Zma is the sequence predicted from unedited transcripts, and ZmaE, NtaE, OsaE, and AthE are predicted from edited transcripts. The changes in predicted amino acid induced by editing are circled (Tsudzuki et al., 2001). This alignment was generated by the clustalX algorithm (MegAlign, DNAstar, Lasergene).

Biological significance of tissue-specific editing variations

When a codon is altered by RNA editing in chloroplasts, a more conserved amino acid is encoded than predicted from genomic DNA (Bock, 2000; Tsudzuki et al., 2001). Editing quite likely is required for production of functional proteins from chloroplast genes that carry editing sites. When editing creates the initiation codon, as in maize *rpl2* (Hoch et al., 1991), undoubtedly a lack of editing would be detrimental to production of the protein, but we detected little variation in *rpl2* editing efficiency. Experiments have been performed in which the coding sequences of several proteins were been altered so that the genomic DNA-encoded protein was synthesized. In the three genes reported to date, the protein encoded by unedited RNA was shown to produce a mutant phenotype or to be inactive (Bock et al., 1994; Zito et al., 1997; Sasaki et al., 2001).

Results of experiments to determine whether heterogeneity in editing status of chloroplast transcripts is reflected in variable protein composition have not yet been reported. In plant mitochondria, a partially edited transcript population encoding an ATPase subunit was shown to result in the accumulation of only one protein product (Lu & Hanson, 1994), but a heterogenous *rps12* transcript population resulted in accumulation of multiple protein products (Lu et al., 1996; Phreaner et al., 1996). Our identification of tissues where unedited transcripts of certain plastid genes represent 20% or more of the transcript population suggests appropriate targets for comparable comparisons of transcript versus protein variation.

Previous studies with transgenic tobacco plants that overexpress editing sites, with concomitant reduction in editing efficiency of endogenous transcripts, have shown that the plant can tolerate decreased editing in several different genes without obvious phenotypic effects. For example, we have previously observed that a 50% reduction in editing of a codon that specifies a conserved *rpoB* residue does not affect the growth and vigor of transgenic tobacco plants (Reed & Hanson, 1997). In maize, our analyses showed that the largest decreases in editing at individual sites occurred in tissues where the RNA abundance of the transcript was also decreased. A decrease in transcript abundance by 20 to 200 times would likely have a much larger impact on the amount of active protein than the 50 to 80% decreases in editing we detected in a number of transcripts in roots, callus, and reproductive tissues. Our genome-wide analysis of both editing efficiency and abundance of edited transcripts provides support for the hypothesis of Covello and Gray (1993) that editing arose as a correction mechanism for detrimental mutations. Our results imply that RNA-level correction of otherwise detrimental mutations remains the primary role of plastid RNA editing rather than generation of protein heterogeneity.

MATERIALS AND METHODS

Plant material

Maize plants (*Zea mays* B73, Maize Genetics Cooperation, Stock Center, University of Illinois at Urbana/Champaign) were grown to maturity in a greenhouse. Maize seedlings were also grown under sterile conditions on MS-agar medium (Murashige & Skoog, 1962) containing 30 g/L sucrose. Roots were harvested from maize kernels set to sprout in a petri dish on sterile filter paper soaked with sterile water and wrapped with aluminum foil to recreate the dark condition of soil-grown roots. Etiolated leaf material was harvested from sterile MS-agar cultures also wrapped in foil. Young green leaves samples were taken from either a MS-agar culture or from a greenhouse seedling, after 14 days of growth. The old leaf tissue corresponds to the third leaf set by the maize plant and was harvested after it has reached male maturity. Silks (stamens) and unfertilized ovules were harvested from a fully expanded ear before pollen shedding. A vascular tissue sample, white in appearance, was taken from a full-grown maize plant (at the same time as the silks and ovules were harvested), by a length cut in the stem several nodes under the male flower. Anthers were taken when the first male flowers started to open and shed their pollen. The three green and immature anthers were taken but not the pollen-shedding brown rusty anthers. Callus material was generated by cultivating W22 genotype kernels on MS medium supplemented with 2 mg/L of 2-4D and 0.2 mg/L 6-BAP, and left in the dark, in a growth room for several months.

RNA extraction and RT-PCR

RNA was extracted from the different plant tissues using the mini RNA extraction kit following the guidelines from Qiagen. To remove any traces of genomic DNA, three subsequent DNAse reactions were carried out using the DNAseout kit supplied by Ambion (Austin, Texas). Integrity of the total RNAs was always checked on agarose gel after the multiple rounds of DNAse treatments. Samples (1.5 μg) of DNAse-treated RNA were subjected to reverse transcriptase (Omniscript kit; Qiagen), using random hexamers to prime the reaction, for 1 h at 37 °C. The 20 μL final volume of the RT reaction were diluted by adding 180 μL of 10 mM Tris-HCl, pH 8; these cDNAs preparations were kept at 4 °C. To amplify around editing sites, PCRs reaction were carried out on 2-μL cDNA in a final volume of 30 μL, with reagents provided by Invitrogen-BRL (Platinum *Taq* polymerase), in a PTC-200 thermocycler (MJ Research). The cycles were as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. A minus RT control was included in each RT-PCR reaction, to check for any possible genomic DNA contamination. All the oligonucleotides used to amplify the chloroplasts mRNAs around editing sites are displayed in Table 3.

Cloning

For each of the 27 editing sites, a PCR reaction was performed on maize genomic DNA (same cycles as above). These PCR products and also the RT-PCR products for the green leaf tissue (sample 1) were cloned in the pCR2.1-TOPO vector

(Invitrogen). Single white colonies were cultured in LB-ampicillin and DNA was extracted following a classic alkaline lysis protocol. Several clones were sequenced (Cornell Bio-Resource Center) for each PCR or RT-PCR cloning, in order to have for each edited site a cloned fragment corresponding to an edited (RT-PCR) or nonedited (genomic PCR) mRNA.

Poisoned primer extension

The RT-PCR products surrounding an editing site were quantified on gels, and submitted to an enzymatic degradation of the primers and nucleotides present in the reaction, using the ExoSap reagents provided by USB corporation.

One hundred nanograms of oligonucleotide used for the primer extension was labeled with 30 μCi of [γ-^{32}P]ATP using 10 U of polynucleotide kinase (Fermentas) for 1 h at 37 °C. The oligonucleotide was separated from the nonincorporated [γ-^{32}P]ATP using a NucTrap column (Stratagene). The PPE reaction was carried out as described by Schiffer and Heinemann (1999) in a thermocycler (PTC-100; MJ Research). The reaction (20 μL) was stopped by adding 5 μL of stop solution (Thermosequenase kit, USB). Five microliters of each sample was loaded on a 12% acrylamide sequencing gel (sequagel, National Diagnostics), and electrophoresed in 1× TBE for 2 h. The gel was exposed to a phosphorimager screen (Molecular Dynamics) for 2 h and was then scanned using a Storm 840 (Molecular Diagnostics).

The quantification of the unedited and edited bands was performed with the ImageQuant software (Molecular Diagnostics, version 1.2) without background correction. For each gel, a TIFF version was exported from ImageQuant and displayed in Photoshop (Adobe, version 6) for illustration. No background settings were modified.

To corroborate the quantification data, the radioactive extension products of a PPE reaction were cut out from the gel and counted in a scintillation counter (Beckman Coulter LS5000 TD), using 5 mL of EcoLume scintillation cocktail (IGN).

Real-time SYBR-green RT-PCR

Chloroplast transcript accumulation was estimated by RT-PCR using the QuantiTect SYBR Green PCR kit, provided by Qiagen on a 7900HT Sequence Detection System (Applied Biosystems). The cDNA material used was prepared the same way as in the PPE assay, starting from the same RNA extractions. We took particular care in quantifying the RNA samples prior to retrotranscription, so as to start with the same amount of RNA for each sample. Each sample was analyzed by OD_{260} measurement and the concentration value was re-estimated by two subsequent agarose gel electrophoresis. Each quantitative RT-PCR was carried out on 2 μL of the cDNA preparation in a final volume of 20 μL following the protocol provided by Qiagen. After a first step of 15 min at 95 °C, the next 40 PCR cycles were as follows: 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The data acquisition is set to take place at each cycle during the 72 °C step. The oligonucleotides used in this PCR analysis were designed on the Primer Express Software (version 2.0) from Applied Biosystems, using the guidelines provided by Qiagen (oligonucleotides displayed in Table 3). After amplification, the melting point of the amplicon generated was verified by running a dissociation curve on the 7900HT to confirm the absence of primer dimers or other nonspecific PCR products. Quantitative RT-PCRs were repeated on young green leaf (sample 1, calibrator), root (sample 2), and callus (sample 10), starting from a new RNA extraction for the two first samples, and the existing callus RNA extraction.

Analysis of real-time RT-PCR data

Quantification of each mRNA was performed three times, using cDNA preparations including a minus RT control for each site and nontemplate controls. The real-time PCR data is analyzed as follows: For each gene, the Ct values of the triplicates were compared and a mean value was calculated (the standard deviation is not shown; it is negligible). The Ct value is the PCR cycle at which the fluorescence generated by the SYBR-green dye intercalated in the double-stranded DNA has reached a threshold value, calculated by the Sequence Detection System (SDS 2.0, Applied Biosystems) on the basis of the background fluorescence generated in the 15 first cycles of the PCR. Because the PCR performed is considered close to the theoretical efficiency of a PCR (i.e., doubling of amplicon quantity at each cycle), essentially because the amplicons are very short (100–110 bp), and because the SYBR-green fluorescence detects very low abundance of DNA, the nontemplate controls and the minus RT controls could exhibit an increase of fluorescence during the PCR cycles. Judging from the amplification curve and the high value of the Ct for those controls, a contaminated sample and a negative control are easily distinguished.

To quantify abundance of the different chloroplast mRNAs, the data is presented as a function of the abundance of a nuclear housekeeping gene α-actin. For each cDNA sample, the Ct value for α-actin is subtracted from the Ct value for each particular gene. We then present the data for each gene as a fold variation (increase or decrease) of the abundance of the mRNA of the same gene, in the young green leaf sample (tissue 1, called the calibrator). For that purpose, the Ct of the young green leaf sample (tissue 1) is subtracted from the calculated Ct values for a particular gene. This value is called the $\Delta\Delta$Ct, and is a variable in Ct cycles. To have a corresponding value in mRNA abundance, we calculate the value $2^{-\Delta\Delta Ct}$, as at each cycle the abundance of amplicon doubles (see Mills et al., 2001; Applied Biosystems, 2001).

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