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Arabidopsis chloroplast quantitative editotype

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ABSTRACT

Chloroplast C-to-U RNA editing is an essential post-transcriptional process. Here we analyzed RNA editing in *Arabidopsis thaliana* using strand-specific deep sequencing datasets from the wild-type and a mutant defective in RNA 3′ end maturation. We demonstrate that editing at all sites is partial, with an average of 5–6% of RNAs remaining unedited. Furthermore, we identified nine novel sites with a low extent of editing. Of these, three sites are absent from the WT transcriptome because they are removed by 3′ end RNA processing, but these regions accumulate, and are edited, in a mutant lacking polynucleotide phosphorylase.

1. Introduction

The expression of chloroplast genetic information requires several RNA maturation steps, including 30 to 40 C-to-U editing events in flowering plants. For example, RNA editing in *Arabidopsis thaliana* affects 34 sites [1,2]. In most cases, editing occurs in first or second codon positions, leading to a change in the amino acid encoded (reviewed in [3]). Editing is less common in third codon positions, introns, and other untranslated regions. Therefore, RNA editing is widely considered to be essential for the production of functional proteins (reviewed in [3, 4]).

Under standard growth conditions, most sites have been reported to be fully edited, with a minority being partially converted from C to U [1,5]. One example of partial editing is the first *ndhD* ACG codon in tobacco and *Arabidopsis*, whose conversion to AUG generates the translation initiation codon [6,7]. Moreover, some sites completely edited in leaves are far less edited in non-photosynthetic tissues [8,9]. Reductions in editing efficiency have also been observed under stress conditions, or when chloroplast development is disrupted genetically or pharmaceutically [10–12]. The chloroplast RNA editing machinery is only beginning to be understood. Recently, pentatricopeptide repeat (PPR) proteins have been demonstrated to recognize sequence elements immediately upstream of editing sites [reviewed in 3, 4]. PPR proteins associate with 10–20 nt RNA motifs [13,14] and are thus capable of conferring specific recognition of one or a few editing sites. A growing number of PPR proteins has been assigned genetically to specific editing sites [7,15].

Although the majority of PPR proteins are conserved between distantly related species [16], it has been demonstrated that editing sites evolve rapidly [17–19], in close association with their cognate *trans*-acting factors [20]. Despite our knowledge of editing sites and specificity factors, we still do not know how editing sites originate. Deep, strand-specific cDNA sequencing (RNA-seq) offers a new opportunity to analyze organellar RNA metabolism [21], including quantitative analysis of RNA editing, which should identify low efficiency sites that could represent emerging ones. Here we demonstrate that unedited messages can readily be detected for every editing site, and that editing efficiencies for many sites are lower than previously assumed. Also, novel sites with low editing efficiency are identified. A possible link to the promiscuous action of known editing factors is discussed.

2. Materials and methods

A. thaliana Columbia ecotype (Col-0) was used as WT in this study; PNPase mutants were also from a Col-0 background [22]. RNA-seq data were taken from [22]. The CLC Genomics Workbench was used for RNA-seq analysis: Adapters were trimmed and low

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quality bases were removed. RNA-seq reads were mapped strand specifically to the *Arabidopsis* chloroplast genome (NC_000932). Known editing sites were manually converted from C to Y in this reference sequence. For identification of novel RNA editing sites, SNP detection was performed. Only bases with a quality score of greater than 30 (Illumina) were taken into account. The minimum variant frequency was set to 3% and coverage under 10 reads was excluded.

2.1. Cloning and sequencing of individual cDNAs

Total RNA from 14-day-old seedlings was prepared using TRIzol (Invitrogen), digested with DNase I (Roche), and cDNA was synthesized with Superscript III reverse transcriptase using gene-specific primers (Suppl. Table 1). PCR was performed using an adapter primer introduced during reverse transcription, and gene-specific primers (Suppl. Table 1). PCR products were gel purified when necessary, cloned and sequenced by SMB (Berlin) using the primer pJet1.2rev (Suppl. Table 1). Only clones with individual barcodes introduced by reverse transcription were counted.

2.2. CAPS analysis

Strand-specific RT-PCR products containing the editing sites were purified, and 500 ng were digested with restriction endonucleases, then separated in 12.5% polyacrylamide/TBE gels.

2.3. RNA gel blot analysis

Strand-specific RNA gel blots were performed as previously described [22].

3. Results and discussion

3.1. Detection of unedited RNAs for all known Arabidopsis editing sites

Chloroplast RNA editing has been assessed by various methods including Sanger sequencing of RT-PCR products, high-resolution melting of amplicons and poisoned primer extension [1,2,23,24]. An important limitation is that each of these methods requires a priori knowledge of editing site position, because they rely on comparisons between selected RT-PCR products with the genomic DNA sequence. By contrast, RNA-seq has the power to identify any single nucleotide polymorphism (SNP) including editing sites. We therefore analyzed RNA-seq datasets that had been exploited to identify chloroplast non-coding RNAs in *Arabidopsis* [22]. The datasets were derived from four independent libraries constructed from total cellular RNA, from which most rRNA sequences had been removed by RNA capture, and RNAs <80 nt had been discarded [22]. Two of these libraries were created from wild-type (WT) plants, and two from plants lacking chloroplast polynucleotide phosphorylase (PNPase) due to a T-DNA insertion. PNPase is a 3′ → 5′ exoribonuclease whose activity is broadly required for 3′ end maturation [25].

In the WT, we mapped 10,448,101 (WT1) and 8,152,401 (WT2) reads to the chloroplast genome, which collectively represented 32% of total reads (reads mapped to the chloroplast in mutant *pnp1-1*: dataset 1 = 8,935,080, which corresponds to 30.4% of total reads; dataset 2 = 7,804,442; 28.1%). Read density differs widely between genomic regions, reflecting the known gene-specific differences in chloroplast RNA accumulation [26]. Similarly, coverage differences for individual genes between WT and *pnp-1* samples are expected [22]. The reference genome took into account editing at all previously identified sites, i.e. all sites appeared as Y in the sequence. This was done to assure equal mapping of edited and non-edited reads. Data quality was assessed by calculating standard deviations between replicate experiments. With few exceptions, variation was low, suggesting reliable quantifications across experiments (Suppl. Table 2).

33 of 34 known editing sites were covered by more than 20 reads, the exception being *rpoC1*-21806 (Table 1). Five additional sites were represented by less than 100 reads in the two combined WT samples (*accD*-58642 3′-UTR, *rpoB*-25779, *rpoB*-25992, *petL*-65716, and *ndhB*-95608; numbers refer to genome position). Overall the median read number at an editing site was 103 (WT samples not combined). The high read density at most sites allowed a robust quantification of editing events: The overall editing efficiency is 94%, but individual sites can vary drastically. For example, we found that the *ndhD* translation initiation codon is edited only to a 45% extent. This is consistent with an earlier study that estimated 40% editing at this position [7]. Even lower editing was measured at the only intronic site, in *rps12*, where <30% C-to-U conversion was observed. Editing extent at all other sites exceeded 74%, reaching over 99% for *psbE*-64109. Taken together, we conclude that there is no example of complete editing in the chloroplast transcriptome. In fact, if coverage of the transcriptome is deep enough, it is expected that nascent transcripts still attached to the RNA polymerase will be sequenced as well. Such transcripts are likely in part unedited and thus lead to the reduced calculation of editing in an RNA-seq dataset.

3.2. Nine novel editing sites in the chloroplast transcriptome

SNPs which are below the detection limit of conventional sequencing of bulked cDNA PCR products can be revealed by deep sequencing, given sufficient coverage. The RNA-seq datasets investigated here show coverage of between 13 and over 11 500 reads at known editing sites, with the variability reflecting both transcript abundance and inherent biases of the cDNA cloning method used (Table 1). We therefore sought evidence of so-far unknown editing sites on a genome-wide level.

To detect editing events, we ran a SNP detection script on WT and *pnp* datasets. SNPs were called when at a given position at least 3% of reads had a mismatch, with a lower coverage limit set to 10 reads. We took advantage of the two biological replicates and removed all SNPs which were only present in one replicate. All SNPs with a genome-encoded C were further investigated. For the WT we detected 50 such SNPs, and for *pnp* 51. About half of these SNPs were within tRNA or rRNA coding regions, and were ascribed to RNA modifications (e.g. methylation) that cause errors during reverse transcription (Suppl. Table 3). Given our focus on C-to-U editing, we disregarded these sites. Some of the remaining SNPs were situated at the end or within stretches of genomically-encoded T's. DNA and RNA polymerases exhibit increased rates of slippage in such tracts [27,28]. It is therefore likely that these apparent sites are artifacts generated during library preparation. Thus, these sites were also excluded from further analysis. Finally, we excluded sites with only one apparently edited read.

Following this quality assessment, we were left with six novel sites in WT-derived datasets, and three novel sites exclusively found in PNPase-deficient plants. Most of these sites are within non-coding transcripts or non-coding regions of mRNAs (Table 2). The two new editing sites within a coding region were both in *ndhB*, which brings the total number of editing sites to eleven for this gene. The new sites are edited at a low efficiency and both alter third codon positions and do not change the encoded amino acid. Our results thus yield a tentative cumulative total of 43 editing sites in the *Arabidopsis* chloroplast, a 26% increase over the previously described 34. An extrapolation of this ratio to *Arabidopsis* mitochondria suggests that more than one hundred mitochondrial sites remain to be identified. The number of mitochondrially-derived

Table 1
Extent of chloroplast RNA editing quantified by RNA-seq. Two independent WT samples and two *pnp1-1* samples were combined and the conversion of 34 known editing sites investigated by RNA-seq. The coverage at a given site is presented in reads. The amount of C's converted to U's is given in percent.

	WT		*pnp1–1*	
	Coverage [reads]	Editing (%)	Coverage [reads]	Editing (%)
matK(2931)	108	93	513	93
atpF(12707)	2724	95	5843	90
rpoC1(21806)	13	15	48	19
rpoB(23898)	161	85	321	98
rpoB(25779)	43	86	76	76
rpoB(25992)	47	94	112	94
psbZ(35800)	450	95	1202	98
rps14(37092)	4856	94	4075	90
rps14(37161)	7315	96	7103	92
accD(57868)	872	99	654	86
accD(58642)	23	83	11	100
psbF(63985)	1880	98	2382	94
psbE(64109)	11536	100	17403	100
petL(65716)	73	86	164	70
rps12(69553)	191	27	1282	18
clpP(69942)	436	81	513	62
rpoA(78691)	742	91	449	76
rpl23(86055)	2158	75	3374	45
ndhB(94999)	179	94	203	84
ndhB(95225)	297	99	343	98
ndhB(95608)	98	80	154	90
ndhB(95644)	150	81	241	61
ndhB(95650)	149	84	241	89
ndhB(96419)	323	92	468	88
ndhB(96579)	146	90	150	86
ndhB(96698)	186	82	254	76
ndhB(97016)	171	95	314	88
ndhF(112349)	173	96	1630	99
ndhD(116281)	433	92	463	92
ndhD(116290)	369	90	407	91
ndhD(116494)	103	93	113	97
ndhD(116785)	198	98	245	97
ndhD(117166)	139	45	408	35
ndhG(118858)	357	85	457	77
Total	37099	94	51616	88

Table 2
Novel chloroplast editing sites identified by RNA-seq. Nine novel C->U editing sites were discovered of which three are only present in PNP mutants (zero percent editing in the WT). The coverage at a given site is presented in reads. The amount of C's converted to U's is given in percent. The position of the editing site in the reference sequence NCBI: NC_000932 is indicated. The potential *cis*-element containing the edited C and 19 upstream bases is given. Confirmation of six editing sites detectable in the WT by cloning of individual cDNAs is shown on the right. Editing at three known editing sites, present in the PCR product for *ndhB* is shown in italics.

	Genome position	*cis*-Element	WT		*pnp1-1*		cDNA cloning	
			Coverage	Editing (%)	Coverage	Editing (%)	Coverage	Editing (%)
atpH 3′UTR	13210	GTAGTTTTTTTAATTCTATC	2702	4	4254	4	76	8
ycf3 Intron 2	43350	GACTAGATATGCCTAAATAC	390	12	1685	1	38	5
rps4 3′UTR	45095	ATTTTTCCTATTCATGTATC	69	10	205	1	35	3
ndhK-ndhJ	49209	CTTCATAAATTAGAATTAAC	1342	6	864	0	43	7
rps18 3′UTR	68453	ATTTCTACTCTACCTTCCCC	25	0	721	26		
ycf2 as	91535	TCATCAATATCGATATCATC	2	0	47	11		
ndhB 3′UTR	94622	CTACTTTTTACATATCTCTC	2	0	324	6		
ndhB	96439	TCACTGTAGGAATTGGGTTC	419	6	597	2	41	7
ndhB	96457	CAATTGCGCTTATATTCATC	518	5	820	2	41	5
ndhB	*96419*						*41*	*98*
ndhB	*96579*						*41*	*98*
ndhB	*96698*						*41*	*100*

reads in our datasets, however, was insufficient to test this hypothesis.

To confirm the six novel sites in WT material, we sequenced cDNA clones (Table 2, right columns). Two sites uniquely found in the PNPase mutant were confirmed using Cleaved Amplified Polymorphic Sequence (CAPS) analysis (Fig. 1A). For each of the six new sites in the WT, editing efficiencies are below 10%. The novel editing site in the *rps18* 3′ UTR is >20% edited in *pnp,* which is reflected in the CAPS analysis. The results from traditional cDNA analysis parallel quantifications based on RNA-seq. It has to be noted, however, that the low clone numbers preclude a robust quantification of editing efficiency (Table 2).

The finding of new, low-efficiency events in *Arabidopsis* non-coding regions is in agreement with an RNA-seq survey of grape mitochondrial editing, which pointed out that the number of events in non-coding regions could be higher than anticipated, as their low efficiency makes only deep sequencing methods amenable to their discovery [29]. Low efficiency additionally suggests that these editing events are not essential, and that selection does not work towards increasing their efficiency. These assumptions

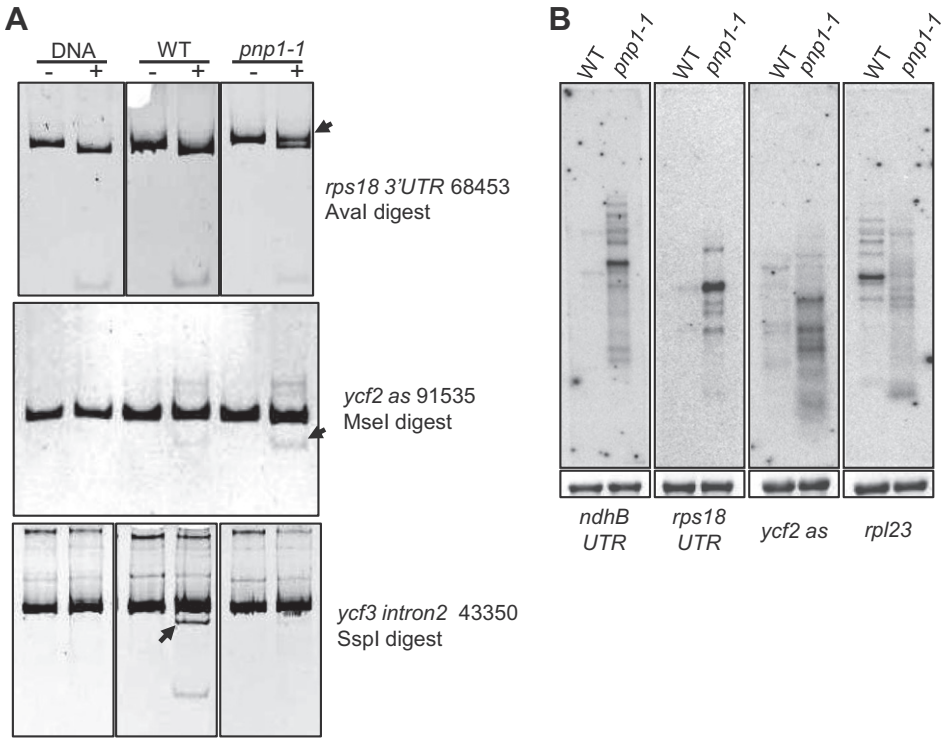

Fig. 1. The absence of PNPase influences RNA editing. (A) RT-PCR was used to amplify cDNA from the editing regions shown underneath each gel. The same region was amplified from total WT DNA as a control. Products were digested (+) or not (−) with the enzymes shown below each gel and separated in polyacrylamide gels. The AvaI and MseI sites are destroyed after editing of sites 68453 and 91535, whereas the SspI site is created by editing of site 43350. Black arrows indicate PCR products diagnostic of editing. (B) Strand-specific probes were used with RNA gel blots to detect four transcripts bearing editing sites influenced by PNPase. The probes were designed to hybridized to the region were editing occurs.

are supported by the fact that the editing sites uncovered in the PNPase mutant are in RNA regions that do not normally accumulate in the WT (see below).

Finally, we note that our initial SNP screen included all possible base conversions. We found a large number of various SNPs within tRNAs and rRNAs (Suppl. Table 3). By contrast, no non-C-to-T SNPs were found in mRNAs in WT RNA-seq data, while three non-C-to-T SNPs in mRNAs were found in PNPase datasets (Suppl. Table 3). The significance of these SNPs remains unknown at present.

3.3. The absence of PNPase affects RNA editing

PNPase deficiency leads to a virescent phenotype, although plants can grow to maturity on soil [25]. In these plants, editing efficiencies were overall only slightly altered, with a 6% decrease, although some sites had increased editing. The greatest change for a site with good coverage was observed for *rpl23*-86055, whose efficiency is 75% in the WT but 45% in *pnp*. The transcript structure for *rpl23* is severely altered in *pnp* mutant (Fig. 1B) which may contribute to the observed editing variation.

The situation is more diverse for the new editing sites we discovered. As mentioned above, three of the new sites (*rps18* 3′UTR, *ycf2* antisense [as], and *ndhB* 3′UTR) are only present in the mutant. The near-absence of WT coverage for these positions suggested that they are located in regions normally absent in WT transcripts. This hypothesis seemed reasonable given that many chloroplast transcripts in *pnp* mutants have 3′ extensions [25], and that two of the three sites are in 3′ UTRs. To test this possibility, RNA gel blots were used to test the sites (Fig. 1B), which confirmed their significant overexpression in the mutant. WT RNA amplified by RT-PCR also reflects a small amount of editing (Fig. 1A).

Four other novel sites (*atpH* 3′UTR, *ycf3* intron 2, *rps4* 3′UTR, *and ndhK-ndhJ*) are less edited in the mutant compared to WT, akin to *rpl23* (Fig. 1A and Table 2). All of these sites are present in both WT and *pnp* genotypes. This raises the question of how the presence of PNPase affects editing efficiency. One explanation would be a direct influence of PNPase, which forms trimers but has not been detected in other stable macromolecular complexes [25]. This does not exclude, however, that PNPase has transient interactions with RNA-binding proteins, as has been suggested in a recent study of chloroplast 3′ end processing [30]. An alternative possibility is that editing efficiency is affected by impaired RNA processing, perhaps through alteration of RNA secondary structure. Also, increased transcript abundance in the mutant could exceed the capacity of the editing machinery. Finally, the virescent phenotype of *pnp1-1* may be associated with pleiotropic effects on RNA editing related to chloroplast dysfunction. This is supported by several other mutants with pronounced loss of chlorophyll that display reduction of editing at multiple sites [10–12].

3.4. Emergence of new editing sites

One of the sites we discovered through RNA-seq is located in the *atpH* 3′ UTR, and was suggested, but not confirmed, to be a target of the PPR protein CRR22 based on target site predictions [15]. CRR22 is required for editing of *rpoB*-25779, *ndhB*-96419, and *ndhD*-116281 [15], all of which are >85% processed (Table 1). By contrast, *atpH* 3′UTR-13210 is edited poorly. Most parsimoniously, it can be concluded the three previously identified sites are the primary targets of CRR22, while *atpH* 3′UTR-13210 editing is the result of promiscuous and weaker RNA binding. The previously identified site in the *accD* 3′ UTR (*accD*-58642) might also be an off-target effect of a PPR protein. An alternative explanation for inefficient editing might be that additional factors like the MORF/RIP proteins [31,32] or other chloroplast RNA-binding proteins [33], which were shown to be required for efficient editing, do

not act at these sites. Further analysis of RNA-seq datasets will give greater insight into promiscuous editing and in general, a broader perspective on organellar transcriptomes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.03.022.

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