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ABSTRACT

We describe a rapid, high-throughput method to scan for new RNA editing sites. This method is adapted from high-resolution melting (HRM) analysis of amplicons, a technique used in clinical research to detect mutations in genomes. The assay was validated by the discovery of six new editing sites in different chloroplast transcripts of *Arabidopsis thaliana*. A screen of a collection of mutants uncovered a mutant defective for editing of one of the newly discovered sites. We successfully adapted the technique to quantify editing of partially edited sites in different individuals or different tissues. This new method will be easily applicable to RNA from any organism and should greatly accelerate the study of the role of RNA editing in physiological processes as diverse as plant development or human health.

INTRODUCTION

RNA editing is a process reported in a wide range of organisms from viruses to mammals and plants where it has different functions such as regulating gene expression, increasing protein diversity or reversing the effect of mutations in the genome (1). RNA editing is defined as a site-specific modification of RNA molecules, occurring by nucleotide insertion/deletion, nucleotide substitution or nucleotide modification, usually by deamination of A to I or C to U. Occurring via several molecular mechanisms, different types of editing have been described generally involving a specificity factor (RNA or protein) that recognizes the editing site, an enzyme catalyzing the reaction and sometimes other accessory factors. RNA editing alters the sequence of different types of mRNAs but also tRNAs, rRNAs and small regulatory RNAs (2) (microRNAs). The number of site-specific editing sites varies considerably between organisms. While about a thousand have been reported in some early diverging land plants (3), only a few are known in humans. In many cases, RNA editing is essential for correct production of the protein encoded by the RNA, such as in trypanosomatid and plant organelles or in humans, where this process is essential for the absorption of dietary fats in small intestine by producing the lipid-carrying protein apolipoprotein B48 (4). In other cases, RNA editing modulates the functional properties of the encoded protein as in the case of the glutamate and serotonin receptors in the central nervous system (5,6).

RNA editing events result in a single nucleotide polymorphism between genomic DNA and the corresponding RNA sequence. Partial editing is common and results in a mixed edited and unedited RNA population. The unpredictability of RNA editing and the possibility of editing frequency varying with tissue, development and environmental conditions have made it extremely difficult to effectively screen systematically for editing events or for mutants that are affected in the RNA editing processes. Computational approaches have suggested that editing is much more prevalent than previously realized, particularly in primates (7–9), but few of the predicted sites have been experimentally verified. Previously used methods such as cDNA sequencing, primer extension or pyrosequencing are either too expensive, not sensitive enough or too labor intensive for high-throughput screens (10–12). A one-step, high-throughput method that allows both the scanning of transcripts for new editing sites (without any prior knowledge of their nature or location) and the quantification of editing would greatly accelerate RNA editing research.

We reasoned that techniques used in clinical and genetics research to detect mutations and determine allele frequency should be suitable for detecting new editing sites and quantifying editing. High resolution melting (HRM) of PCR amplicons is used as a closed-tube method for mutation scanning and genotyping that does not require probes or labeled oligonucleotides, and no purification step is needed (13–18). A PCR is performed

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using a fluorescent double-stranded DNA dye that can be used in fully saturating conditions. The amplicon is analyzed by melting—the change in fluorescence caused by the release of the intercalating dye from a DNA duplex as it is denatured by increasing temperature is precisely monitored. The presence of heteroduplexes (containing one or more mismatches), that melt at lower temperature, alter the shape of the melting curve.

Here, we report the successful adaptation of HRM analysis to scan transcripts for new editing sites and to quantify editing variability in different individuals under various conditions. Our model is the plant *Arabidopsis thaliana* which displays a moderate frequency of C-to-U editing in mitochondrial and chloroplast transcripts, but the approach would be easily applicable to RNA samples from any organism and to any type of nucleotide substitution or modification editing that results in a different base being incorporated by reverse transcriptase.

MATERIAL AND METHODS

Template preparation

DNA was isolated from leaves of Arabidopsis Col-0 as described in Edwards *et al.* (19). Total RNA was extracted with an RNeasy minikit (Qiagen Pty Ltd, Clifton Hill, VIC, Australia) and treated with a DNA-free kit (Ambion, Austin, TX, USA). DNA-free RNA (3 μg) was reverse transcribed for 1 h at 50°C with the SuperScript III Reverse Transcriptase (Invitrogen Australia Pty, Mount Waverley, VIC, Australia) using random hexamers. PCR and RT-PCR products were cloned in the pGEM-T easy vector (Promega, Madison, WI, USA).

HRM: amplification and melting conditions

The primers used to scan *Arabidopsis* plastid transcripts are given in Supplementary Table S1. They allow the amplification of fragments of an average size of 500 bp (ranging from 350 to 1330 bp). The intercalating dye used was LCGreen Plus (Idaho Technology Inc., Salt Lake City, UT, USA).

(i) One nanogram of gDNA, (ii) 25 pg of plasmid DNA and (iii) 3 μl of a 1/100 dilution of cDNA or a mixture of (i) and (iii) were amplified in a volume of 10 μl using the LightCycler 480 Probes Master mix (Roche Diagnostics GmbH, Mannheim, Germany) and 200 nM of each primer. All PCR reactions were performed in duplicate. PCR cycling and HRM analysis was performed on an LC480 machine (Roche). The exact amount of cDNA used was not determined, but was adjusted empirically in preliminary experiments to give similar rates of amplification for a wide range of amplicons to those measured using genomic DNA template. The approach is very robust to large differences in cDNA/gDNA ratio.

Short amplicons (60–200 pb) were produced with the following conditions; one cycle of 95°C for 10 min; 40 cycles of 95°C, 20 s; 60°C, 20 s and 72°C, 20 s. For longer amplicons, extension time was increased to 30 s. The amplicons were then denaturated at 95°C for 30 s, renaturated at 70°C for 30 s and melted from 70 to 90°C with 30 signal acquisitions per degree.

The cost per data point for the HRM results was approximately $0.79 AUD, including the cost of primers, amplification mix, fluorescent dye and multiwell plates.

Primer pairs which gave PCR fragments with candidate editing sites were used to re-amplify, clone and sequence cDNAs (sequencing by Macrogen Inc., Seoul, Korea), at a cost of approximately $5.60 AUD per sample.

Amplification and HRM analyses

Amplicons were analyzed with the LightCycler 480 software package. First, an absolute quantification analysis was performed to check the amplification curve and the Crossing Point (CP) value. Then, a *T*m calling analysis was done to generate melting curves representing the fluorescence signal (at 450–500 nm) with increasing temperature, melting peaks corresponding to the negative derivative ($-d/dT$) of the fluorescence signal, and to calculate *T*m values.

Transcript scanning. To detect sequence variation between genomic and cDNA we used the Gene Scanning Software. This software analyzes the high-resolution melting curve data to identify changes in the shape of the curve that indicate sequence polymorphisms. The melting curves of different samples are normalized using the fluorescence intensity before and after melting, and temperature shifted to superimpose the curves and better compare their shapes. The Gene Scanning Software generates a difference plot by subtracting the curves from a reference curve and automatically groups samples with similar melting curves. To detect editing events, we compared the amplification product obtained from gDNA and a mix of gDNA and cDNA, and used the Auto Group mode with the highest sensitivity value.

Quantitative assay using short amplicons. The editing efficiency of a particular site was determined by comparing the shape of the melting peaks and *T*m values of RT-PCR products to known dilution mixes of edited and unedited plasmids containing the same site.

Poisoned primer extension assay

PPE of RT-PCR products and determination of editing efficiency were performed essentially as described in Peeters and Hanson (11) except that the oligonucleotides used were fluorescently labeled at the 5′ end using 6-carboxyfluorescein (FAM) (Sigma Genosys, Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia). PCR templates were generated using the primers listed in Supplementary Table S1. Unincorporated primers and nucleotides were removed using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) treatment following the manufacturer's instructions.

The 5′ FAM oligonucleotide (0.5 pmol) was extended using 1U of Thermosequenase (ThermoSequenase Cycle Sequencing kit, USB) in a 20 μl reaction containing the thermosequenase buffer, 100 nM of 3 out of 4 dNTP and 100 nM of the fourth ddNTP in a thermocycler for 35 cycles (30 s at 94°C, 30 s at 55°C, 10 s at 72°C). PPE products were resolved on 12% acrylamide

sequencing gels (Sequagel, National Diagnostics, Atlanta, GA, USA) at 55W. Following electrophoresis, gels were scanned at 200 μm resolution using a Typhoon Trio imager (GE Healthcare, Amersham, UK) with the emission filter 520 BP 40. FAM fluorescence was collected at 488 nm with a photomultiplier tube voltage set at 500 V.

The cost of a single PPE reaction came to approximately $5.00 AUD including labeled primer, enzymes and sequencing gel.

Scans were analyzed using ImageQuantTL 1D gel analysis software (Amersham Biosciences, Piscataway, NJ, USA). The bands corresponding to edited or unedited products were manually defined. The software gives raw volume data corresponding to fluorescence intensity. A background volume was subtracted from edited and unedited band volumes, by defining as a band the location of either edited or unedited product in the unedited plasmid and edited plasmid control, respectively. The editing efficiency (% edited product) for every lane was calculated as 100*(edited band volume—background volume for edited band)/(edited band volume—background volume for edited band) + (unedited band volume—background volume for unedited band).

RESULTS

HRM detects single nucleotide polymorphisms between DNA and RNA with high throughput

Until recently, special apparatus was required to perform HRM analysis that was not designed for high-throughput analyses. However, the latest generation of real-time PCR machines (e.g. Roche LC480) offers the possibility of analyzing 384 samples in parallel on a machine not specifically designed for HRM but with sufficiently resolutive melting curves to attempt the analysis. We tested the ability of the LC480 to detect C to U editing events in transcripts. As we planned to compare amplification products from genomic DNA with a C at the editing sites and RT-PCR products with a T at the same position, we assayed the detection of C/T heteroduplexes, by mixing plasmid DNA containing a C and plasmid DNA containing a T at the same site in various ratios. We were able to convincingly detect single base-pair mismatch heteroduplexes that alter the melting curve shape in amplicons up to 700 bp (data not shown).

To scan transcripts for editing sites, we compared the melting curves of amplification products from genomic DNA (homoduplexes) to amplification products from a mix of genomic (gDNA) and cDNA (containing heteroduplexes if the cDNA has been reverse transcribed from an edited mRNA). The procedure is described in Figure 1a. Our interest was to gain a complete description of the editing sites in chloroplast transcripts of the model plant *A. thaliana* as an important step towards characterizing editing factors in plants. To generate amplicons to be analyzed by HRM, primers originally designed to screen chloroplast transcripts for transcription, processing and splicing defects were used (Charles Andrés, Andéol Falcon de Longevialle, ALCB, Claire Lurin and IS, in preparation). This primer set comprises 320 pairs spanning the entire Arabidopsis plastid genome with an average amplicon size of ~500 bp.

Among these primer pairs, 21 couples flank all 28 of the known editing sites (20). Four of these pairs flank multiple editing sites. All these pairs were tested and all 21 amplicons were flagged as containing heteroduplexes by the HRM assay, demonstrating the sensitivity of the technique (Table 1).

Discovery of six new editing sites in *Arabidopsis* chloroplasts

Out of the full set of 320 amplicons, besides the 21 known to contain editing sites, 18 other amplicons were found to give different melting curve shapes from gDNA alone versus a mix of gDNA and cDNA. The amplification products were checked by gel electrophoresis and those presenting more than one band (5 out of the 18) were not considered further as multiple PCR products obviously interfere with the melting analysis. To confirm the remaining 13 candidates, we sequenced cloned RT-PCR products. For 6 candidates, no difference in the sequence was found indicating that they are false positives. The remaining 7 candidate amplicons contained single C to T changes consistent with RNA editing, covering six new sites not previously identified in *Arabidopsis* (Figure 2, Table 1). One of these new sites was covered by two amplicons in the screen and detected in both. Five of the six new sites could be further confirmed by analysis of public EST databases that revealed sequences containing the same C/T polymorphisms. No publicly available ESTs exist for the sixth transcript, *accD*.

Identifying mutants impaired in editing

The factors involved in RNA editing in plants are still elusive. Two pentatricopeptide repeat (PPR) proteins have been reported to be essential for the editing of specific sites in the chloroplast transcript *ndhD* of Arabidopsis (21,22), raising the possibility that this large family of RNA-binding proteins could constitute the specificity factors recognizing the sequence around the target C and recruiting the enzyme to catalyze editing (23).

We are undertaking extensive HRM screening of *Arabidopsis* mutant lines lacking different proteins of the PPR family, to check for defects in RNA editing. One mutant, *clb19*, with an insertion in the gene At1g05750 was found to be impaired in the editing of two sites in the transcripts *rpoA* and *clpP* (Figure 3). This mutant will be described more fully elsewhere (Charles Andrés, ALCB, Maricela Ramos Vega, Arturo Guevara-García, María de la Luz Gutiérrez-Nava, Araceli Cantero, Luis Felipe Jiménez, Claire Lurin, IS and Patricia León, in preparation). The site in *rpoA* is one of the six new sites discovered in the screen developed during this study.

HRM to quantify RNA editing

HRM has been used to detect mutations in heterogeneous DNA populations and proved reasonably sensitive (15). We decided to adapt this type of sensitivity test to quantify editing (the procedure is shown in Figure 1b and differs primarily from the screens described previously in that the cDNA is not mixed with genomic DNA before

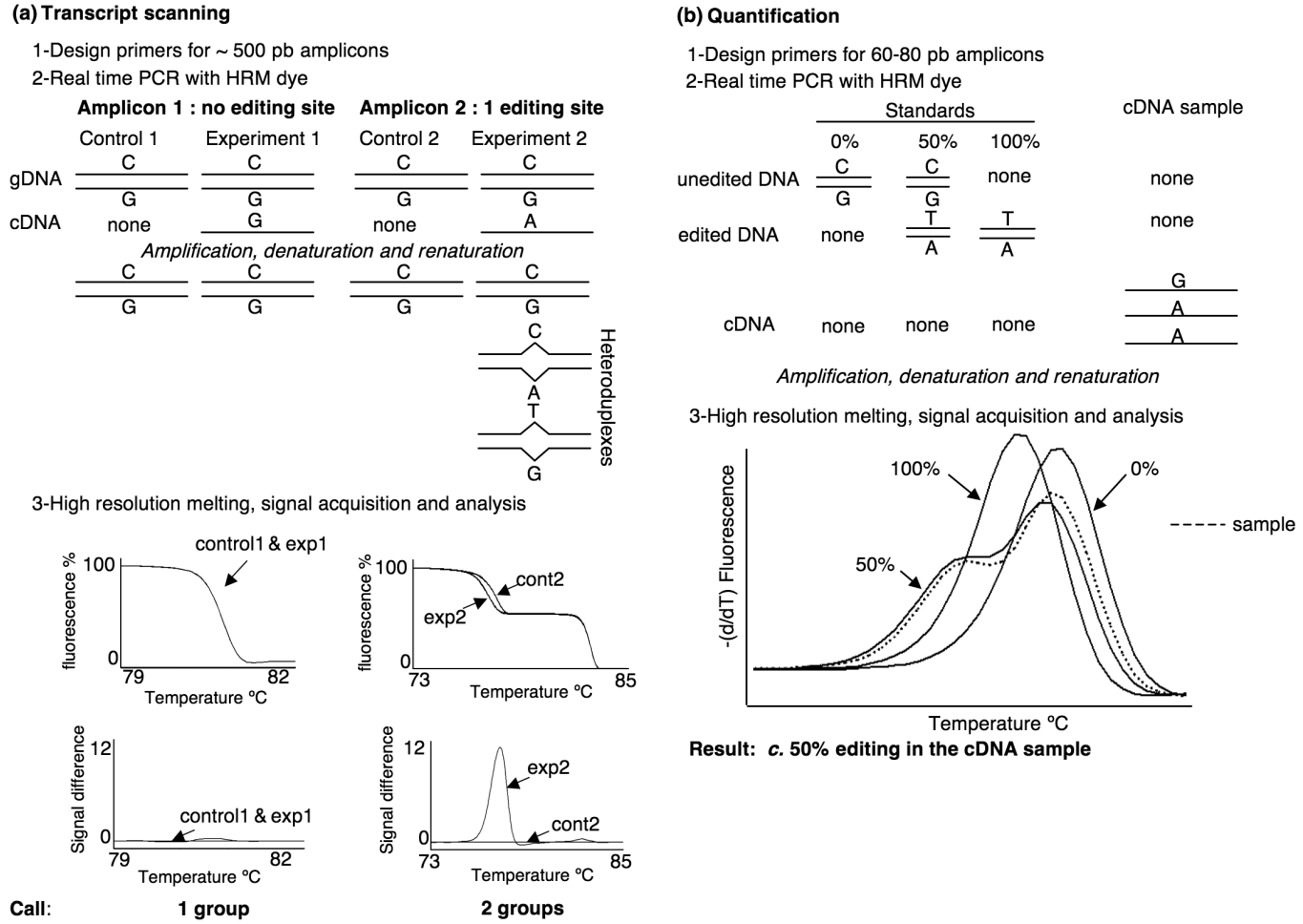

Figure 1. Procedures to scan transcripts for new editing sites (**a**) and quantify editing (**b**) by high-resolution melting analysis of amplicons. A real-time PCR is done in the presence of a fluorescent double-stranded DNA-specific dye. At the end of the amplification, amplicons are denatured, renatured and then a high-resolution melting is performed. The decrease in fluorescence is precisely monitored as the temperature increases causing the denaturation of the DNA molecules and the release of the fluorescent dye. The presence of less thermostable heteroduplexes in a sample alters the shape of the melting curves. (**a**) To scan transcripts, primers spanning the transcripts are designed to give PCR products with a maximum size of 600 pb. For each primer pairs, the shape of the melting curve of a control genomic DNA (cont) is compared to the melting curve of a mix of genomic DNA and cDNA (exp: experiment). If there is no editing site, both control and experiment (e.g. exp1 shown here, amplicon chloro96) exhibit the same curve shape and are called as a single group. If there is an editing site within the amplicon, the curve of the experiment will be different from the control [as shown in exp2; *rps14* (37161) within the chloro72 amplicon] and two groups of curves will be called. The differences in the curves are easily visualized by plotting the difference in signal between control and experiment (shown at the bottom of the panel). (**b**) To quantify editing in a sample, primers are designed to generate small amplicons flanking the editing site. Editing standards are produced by cloning amplicons from genomic DNA for the unedited standard (0%) and from an edited cDNA sample (100%) and by mixing unedited and edited plasmids with increments depending on the required degree of quantification accuracy. The derivative ($-dF/dT$) of the fluorescence signal is plotted to show the melting peak. The shape of the melting peak of a cDNA sample is compared to the shape of the melting peak of the standards, giving an estimate of the extent of editing in the sample (here around 50%).

amplification and that much shorter amplicons are required for the best results). We first calibrated the test with mixes of plasmid DNA containing a range of ratios of C to T at known editing sites. In short amplicons of 70 bp, as little as 2.5% T was easily detected and melting peak profiles for amplicons differing by only a few percent in C/T could be distinguished (Figure 4). In plant organelles, the extent of RNA editing of some sites can vary according to the genotype, the tissue or in different growth conditions (11,24–26). No systematic survey has been published so far concerning such changes, which may well have physiological relevance. To gauge the efficacy of the HRM assay for such a survey, we prepared cDNA samples from different *Arabidopsis* genotypes, different organs, or from plants grown in different conditions. The cDNAs were amplified, subjected to HRM analysis and their melting peak profiles were compared to the ones from plasmid mixes with increasing ratios of T as compared to C. Five primer pairs flanking four different sites in the transcripts *clpP*, *accD*, *ndhG* and *rpoA* were tested. The results obtained with the HRM assay were compared to those obtained by a widely used, but much more labor-intensive poisoned primer extension (PPE) assay (11). In all, 24 comparisons were made (6 samples × 4 editing sites). In 18 of the 24 comparisons, the editing efficiency measured by HRM matched that

Table 1. Editing sites in *A. thaliana* plastid genes

Locus (transcript)	Primer pair	HRM	Genome position	coding or non-coding	Codon	AA change	EST
AtCg00040 (matK)	Chloro 260	Yes	2931	CDS	236 Cau	H>Y	Yes
AtCg00130 (atpF)	Chloro 27	Yes	12707	CDS	31 cCa	P>L	Yes
AtCg00180 (rpoC1)	**Chloro 39F-21861R**	**yes**	**21806***	**CDS**	**170 uCA**	**S>L**	**Yes**
AtCg00170 (rpoB)	23581F-24093R	Yes	23898	CDS	811 uCa	S>L	No
AtCg00170 (rpoB)	Chloro 45	yes	25779	CDS	184 uCa	S>L	No
AtCg00170 (rpoB)	Chloro 45	yes	25992	CDS	113 uCu	S>L	Yes
AtCg00300 (psbZ)	**Chloro 68**	**yes**	**35800***	**CDS**	**17 uCa**	**S>L**	**Yes**
AtCg00330 (rps14)	Rps14.AT.rev-chloro71R	yes	37092	CDS	50 cCa	P>L	Yes
AtCg00330 (rps14)	Chloro 72	yes	37161	CDS	27 uCa	S>L	Yes
AtCg00500 (accD)	Chloro 112	yes	57868	CDS	265 uCg	S>L	No
AtCg00500 (accD)	**Chloro 113**	**yes**	**58642**	**3′UTR**			No
AtCg00570 (psbF)	Chloro 125	yes	63985	CDS	26 uCu	S>F	Yes
AtCg00580 (psbE)	Chloro 126	yes	64109	CDS	72 Ccu	P>S	Yes
AtCg00590) petL	Chloro 130	yes	65716	CDS	2 cCu	P>L	Yes
AtCg00065 (rps12)	**Chloro 140**	**yes**	**69553**	**intron**			Yes
AtCg00670 (clpP)	Chloro 141	yes	69942	CDS	187 Cau	H>Y	Yes
AtCg00740 (rpoA)	**Chloro 159, 286**	**yes**	**78691***	**CDS**	**67 uCu**	**S>F**	**Yes**
AtCg00840 (rpl23)	**Chloro 178**	**yes**	**86056**	**CDS**	**30 uCa**	**S>L**	**Yes**
AtCg00890 (ndhB)	Chloro 185	yes	94999	CDS	494 cCa	P>L	No
AtCg00890 (ndhB)	Chloro 186	yes	95225	CDS	419 Cau	H>Y	No
AtCg00890 (ndhB)	Chloro 187	yes	95608	CDS	291 uCa	S>L	No
AtCg00890 (ndhB)	Chloro 187	yes	95644	CDS	279 uCa	S>L	No
AtCg00890 (ndhB)	Chloro 187	yes	95650	CDS	277 uCa	S>L	No
AtCg00890 (ndhB)	Chloro 189	yes	96419	CDS	249 uCu	S>F	No
AtCg00890 (ndhB)	Chloro 189	yes	96579	CDS	196 Cau	H>Y	No
AtCg00890 (ndhB)	Chloro 189	yes	96698	CDS	156 cCa	P>L	No
AtCg00890 (ndhB)	Chloro 190	yes	97016	CDS	50 uCa	S>L	No
AtCg01010 (ndhF)	Chloro 204	yes	112349	CDS	97 uCa	S>L	No
AtCg01050 (ndhD)	Chloro 212	yes	116281	CDS	296 cCc	P>L	No
AtCg01050 (ndhD)	Chloro 212	yes	116290	CDS	293 uCa	S>L	Yes
AtCg01050 (ndhD)	Chloro 212	yes	116494	CDS	225 uCa	S>L	No
AtCg01050 (ndhD)	Chloro 213	yes	116785	CDS	128 uCa	S>L	Yes
AtCg01050 (ndhD)	Chloro 214	yes	117166	CDS	1 aCg	>M	No
AtCg01080 (ndhG)	Chloro 218	yes	118858	CDS	17 uCc	S>F	No

The current list of editing sites in *Arabidopsis* chloroplast transcripts. The transcript affected is given in the first column, and for each site the primers used to amplify the cDNA sequence containing the editing site are given. All 34 sites could be detected reproducibly by HRM, including the six new sites shown in Figure 2 and indicated in bold here. Asterisk indicates an editing site reported in other species [orchids (28) or pea (29)].

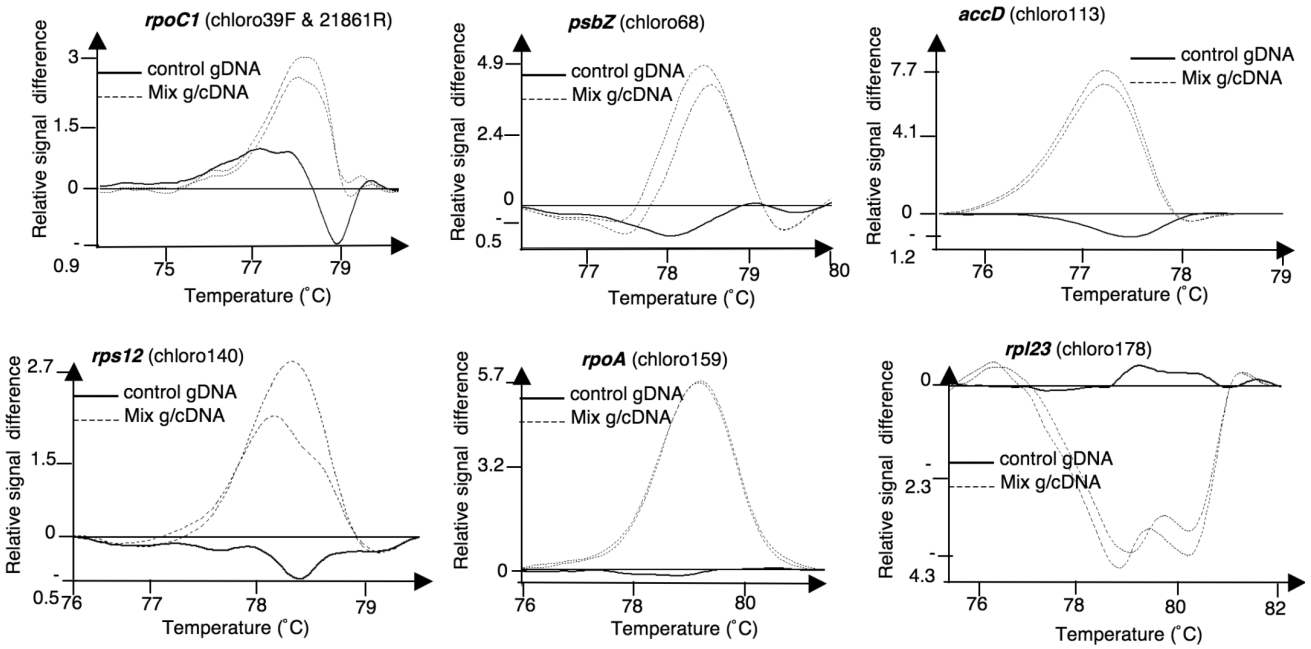

Figure 2. HRM screening of *Arabidopsis* chloroplast transcripts. Six new sites were identified by a high-throughput screen of all chloroplast transcripts. The duplicate fluorescence difference melting curves of controls (genomic DNA) and experiments (mix of gDNA and cDNA) are shown for the six new sites.

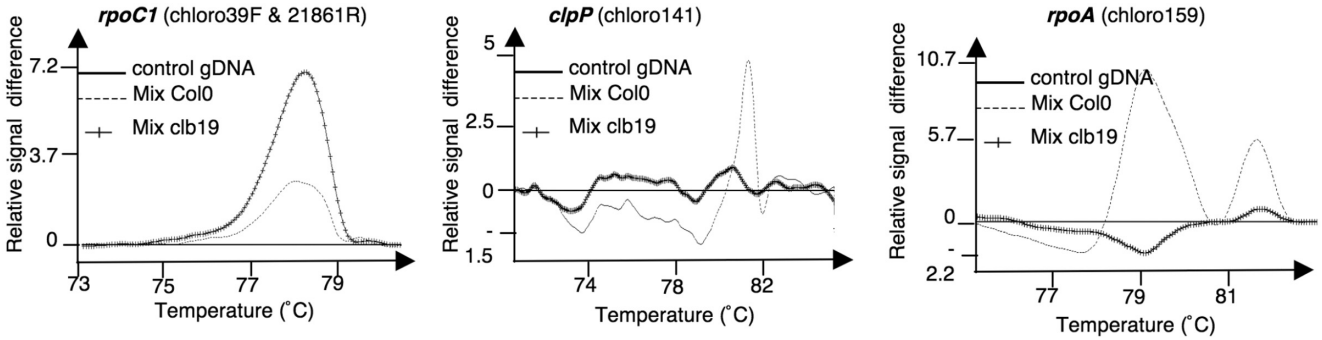

Figure 3. HRM screening of the *Arabidopsis clb19* mutant. The *clb19* mutant is altered in the editing of two sites: *rpoA* (genome position 78691) and *clpP* (genome position 69942). In both cases, the melting curve corresponding to *clb19* has the same shape as the unedited genomic control, indicating no detectable editing. A site from *rpoC1* is shown as a control where *clb19* and wild-type samples have similar curves.

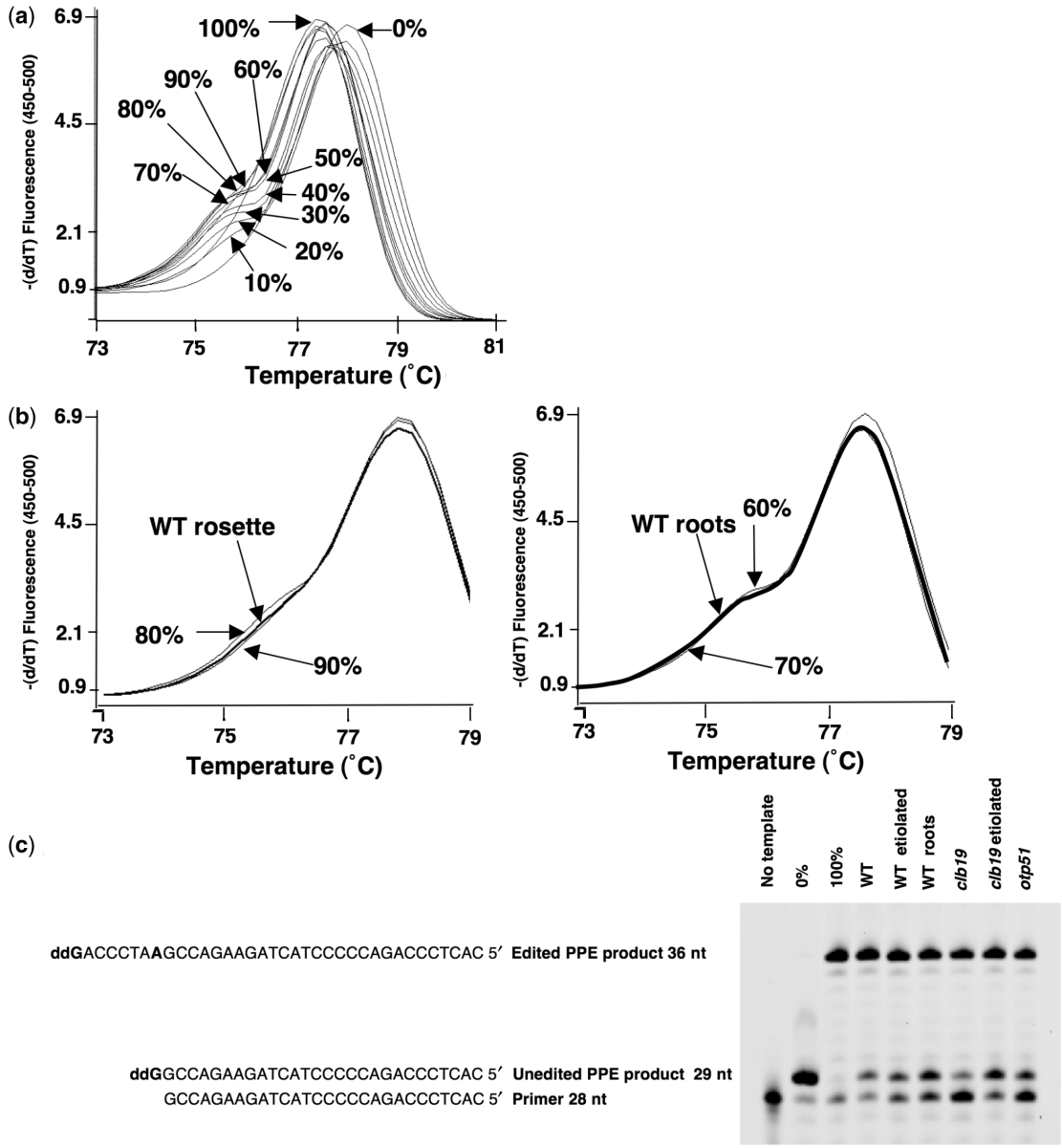

Figure 4. Comparative quantification of editing by HRM and PPE. (**a**) Standards used for the quantification: melting peaks ($-dF/dT$) of amplicons containing the sequence surrounding the editing site in *ndhG* (genome position 118858) with an increasing ratio of T as compared to C at the editing site (0–100%). (**b**) Determination of the extent of editing of *ndhG* (118858) in WT rosette and roots. The percentage of edited molecules is determined by comparison of the shape of the melting peak with the standards. It is given as a range between the values for the two standard curves on either side of the sample curve. (**c**) Poisoned primer extension assay to quantify editing of *ndhG* (118858). An RT-PCR is done with primers surrounding the editing site which then serves as a template for the extension reaction of a fluorescent oligonucleotide binding near the editing site. The extension is stopped by the incorporation of ddGTP at the editing site if the molecule is not edited or at the next C if it is edited. The extent of editing is determined by the ratios of fluorescence intensity between the two extension products in the gel.

Table 2. Comparative quantification of editing at four sites in different Arabidopsis lines, tissues and growth conditions using HRM and PPE

			WT Col-0					
			Light	Dark	Roots	*clb19* light	*clb19* dark	*otp51* light
accD	PPE	av.	97.5	87.4	96.9	72.1	60.2	91.2
57868		SD	0.3	0.3	0.9	1.3	0.7	0.8
	HRM	range	90–100	80–90	90–100	70–80	40–50	80–90
rpoA	PPE	av.	68.0	69.3	81.8	0	0	61.8
78691		SD	0.1	0.7	0.3	0	0	0.5
	HRM	range	50–60	60–70	80–90	0–10	0–10	50–60
clpP	PPE	av.	81.9	78.5	91.2	0	0	58.1
69942		SD	0.6	2.6	0.6	0	0	0.6
	HRM	range	80–90	80–90	90–100	0–10	0–10	60–70
ndhG	PPE	av.	84.2	80.5	66.8	82.3	68.6	72.8
118858		SD	1.0	1.2	1.7	0.3	1.7	1.5
	HRM	range	80–90	80–90	60–70	80–90	60–70	70–80

Comparative quantification of editing by HRM and PPE. Using the procedures described in Figs 1b and 4, the extent of editing in 24 samples differing by genotype, tissue origin and/or growth conditions was measured by HRM and by PPE. The table gives HRM results as ranges between the standard curves used to calibrate the assay and PPE results as mean values from three repetitions (with the SD also given). Results are given in% of edited molecules. Av.: average, SD: standard deviation of three repetitions.

measured by PPE (Table 2). In the cases where the two approaches gave different values, the values were reasonably close, generally within 10%.

DISCUSSION

Following optimization of the procedures, we employed HRM to analyze chloroplast transcripts from *A. thaliana*, already extensively analyzed by previous groups (20,27). This screen covered a total of 126 kb of *Arabidopsis* chloroplast transcripts in four amplification plates and took only 4 days to complete. The screen detected 34 editing sites in total, including all 28 known sites, whilst discovering 6 new editing sites (Figure 2 and Table 1). Four of these sites in are coding sequences (*rpoA*, *rpoC1*, *psbZ*, *rpl23*) and change the protein sequence translated from these mRNAs. The other two sites are the first to be identified in *Arabidopsis* chloroplast non-coding sequences (in the 3′ UTR of *accD* and the intron of *rps12*). Despite the sensitivity of the screen, only 11 false candidates needed to be eliminated (a false positive rate of 11/292 or 3.8%), and 5 of those could be ruled out simply by electrophoresis of the amplicons. This high sensitivity coupled with a low false positive rate, the simplicity of use and the affordability of the approach (6 times cheaper per data point than bulk sequencing of RT-PCR products) make HRM screening an extremely attractive new tool for studying RNA editing. We demonstrated that this method is highly suitable for systematically screening mutants for a defect in editing by discovering that the *Arabidopsis* mutant *clb19* fails to edit two sites, in *clpP* and *rpoA*.

Furthermore, using small amplicons, HRM is sensitive and accurate enough to detect $< 2.5\%$ editing and to easily quantify partially edited sites, the editing extent of which can vary in different genotypes, different organs and different conditions. Previous quantification techniques such as poisoned primer extension or pyrosequencing were much more labor intensive, low throughput and prohibitively expensive for large-scale surveys.

One of the few drawbacks of the approach is that HRM analysis alone cannot easily detect how many editing sites are present, or where within the amplified region the editing site is positioned. For this, sequencing of the amplified product is required.

In conclusion, we have demonstrated that a method originally designed to detect DNA mutations and genotype individuals in clinical research and diagnostics can be simply adapted to research on RNA editing. Currently research in this area is limited by the lack of cheap, effective approaches for screening for new editing sites or for mutants affected in the editing process. The approach described here can be simply and directly applied to samples from any organism, so this breakthrough should stimulate research in many laboratories.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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