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Quantitative Analysis of RNA Editing at Specific Sites in Plant Mitochondria or Chloroplasts Using DNA Sequencing

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[Abstract] Cytidine-to-uridine (C-to-U) RNA editing is one of the most important post-transcriptional RNA processing in plant mitochondria and chloroplasts. Several techniques have been developed to detect the RNA editing efficiency in plant mitochondria and chloroplasts, such as poisoned primer extension (PPE) assays, high-resolution melting (HRM) analysis, and DNA sequencing. Here, we describe a method for the quantitative detection of RNA editing at specific sites by sequencing cDNA from plant leaves to further evaluate the effect of different treatments or plant mutants on the C to U RNA editing in mitochondria and chloroplasts.
Keywords: C to U RNA editing, DNA sequencing, Mitochondria, Chloroplasts, Editing extent

[Background] C to U RNA editing is one of the most important post-transcriptional modifications that occur in the plant mitochondrial or chloroplast genes, which usually changes the first or second positions of nucleic acid triplet codons leading to altered protein sequences and is essential for their normal functions (Takenaka *et al.*, 2013; Yan *et al.*, 2018). The RNA editing and processing in mitochondria or chloroplasts have been reported to function in plant male sterility, seed development, adaptations to the environment, and resistance to pathogens (Hammani *et al.*, 2011; Dahan and Mireau, 2013; Garcia-Andrade *et al.*, 2013; Barkan and Small, 2014; Ren *et al.*, 2020; Yang *et al.*, 2020). Several methods have been established for the detection of RNA editing sites or editing levels, such as poisoned primer extension (PPE) assays, high-resolution melting (HRM) analysis, and DNA sequencing (Roberson *et al.*, 2006; Chateigner-Boutin *et al.*, 2007; Hayes and Hanson, 2007). However, the PPE assays usually require radiolabeled oligonucleotides (Hayes and Hanson, 2007). It is hard to distinguish the editing levels at two very close editing sites using HRM assays (Chateigner-Boutin *et al.*, 2007). Currently, DNA sequencing has been an accurate, economic, and widely used method for the RNA editing assays (Bentolila *et al.*, 2012; Brehme *et al.*, 2015; Yang *et al.*, 2017; He *et al.*, 2018). Here, we describe a method for the RNA editing detection by DNA sequencing of cDNA to further evaluate the effects of different treatments or plant mutants on the C to U RNA editing in mitochondrial and chloroplast transcripts (Yang *et al.*, 2020).

Materials and Reagents

1. Pipette tips and tubes (Axygen, different sizes, and types)

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2. *Nicotiana benthamiana* leaves
3. TRIzol™ Reagent (Invitrogen, catalog number: 15596026)
4. PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, catalog number: DRR047A)
5. FastPfu DNA Polymerase (TransGen Biotech, catalog number: AP221-01)
6. Liquid nitrogen
7. Gel loading dye
8. TAE buffer (Dunker *et al*., 2021)
9. Agarose (Invitrogen, catalog number: 75510019)
10. Marker III (TIANGEN Biotech, catalog number: MD103)

Equipment

1. Thermal Cycler (Bio-Rad, model: S1000)
2. Refrigerated Centrifuge (Thermo Fisher, model: Legend Micro 17R)
3. Electrophoresis System (BEIJING LIUYI BIOTECHNOLOGY CO., LTD., DYY-7C)
4. PIPETMAN Pipettes (Gilson, models: P1000, P100, P20, P2, catalog numbers: F123602, F123615, F123600, F144801)

Software

1. BioEdit (https://bioedit.software.informer.com/)
2. Primer Premier 5 (https://primer-premier-5.software.informer.com/)

Procedure

A. Primer design and template generation

1. To detect the RNA editing at a specific or several RNA editing sites in mitochondria or chloroplast transcripts, the forward and reverse primers could be designed according to sequences ~100 bp upstream and downstream of the editing sites using Primer 5 or other primer designing software (Note 1), with a recommended primer length of ~20 nt and melting temperature of ~60°C.
2. Total RNAs from plant tissues are isolated with the TRIzol™ Reagent according to the manufacturer's instructions and the protocols (MacRae, 2007) (Note 2).
3. About 800 ng total RNAs were reverse-transcribed into cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa) according to the manufacturer's instructions with the final volume up to 20 μl (Note 3).

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B. PCR amplification and DNA sequencing of the targeted editing sites

1. Perform PCR amplification of the fragments that span the targeted RNA editing sites using high-fidelity DNA polymerase.

   Set up the PCR, adding the following components into a 0.2 ml PCR tube with the final volume up to 30 µl:

ddH_2O	18.4 µl
5× FastPfu Buffer	6 µl
2.5 mM dNTPs	2.4 µl
Forward primer (10 µM)	0.8 µl
Reverse primer (10 µM)	0.8 µl
FastPfu DNA Polymerase (2.5 U/µl)	0.6 µl
Template	1 µl

   Run PCR reactions:

1 cycle	95°C, 2 min
34 cycles	95°C, 20 s; 55°C, 20 s; 72°C, 20 s
1 cycle	72°C, 2 min

2. Perform gel electrophoresis (2% agarose) with half of the PCR products to confirm whether the amplification is specific and correct in size.
3. Perform DNA sequencing with the other half of the PCR products using the amplification primers by a DNA sequencing service provider (Note 4).

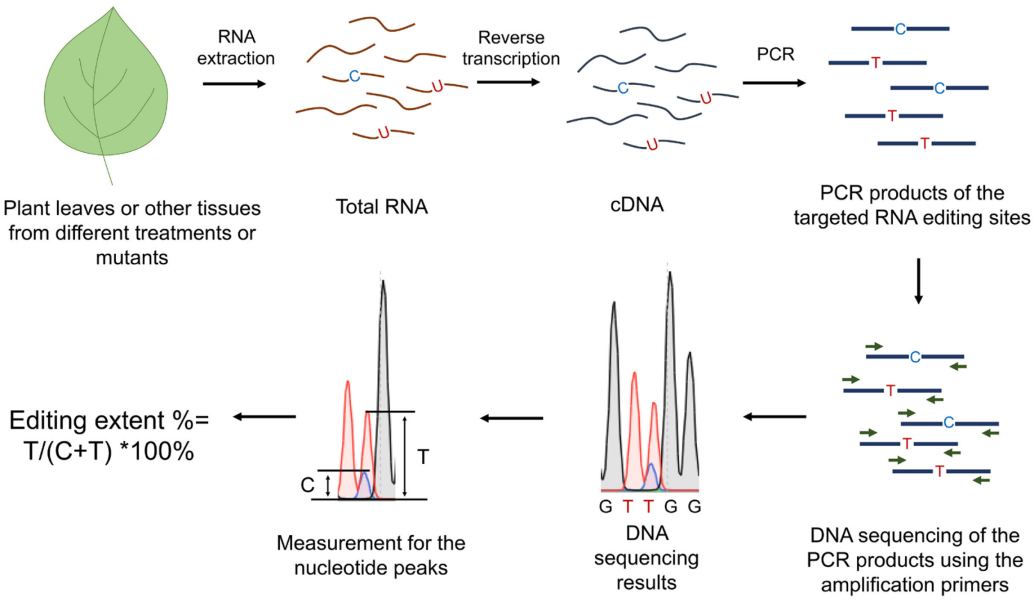

Figure 1. Workflow of the RNA editing analysis of specific sites in mitochondria and chloroplasts

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Data analysis

As shown in Figure 1, cDNA sequences were evaluated for their respective C to T differences. The extent of RNA editing was estimated by the relative height of the respective nucleotide peaks in the sequence analysis using the BioEdit software. The editing levels are calculated as editing efficiency %= T/(C+T) ×100%.

Here, we show an example of our previously published work (Yang *et al*., 2020). We analyzed the C to U RNA editing extent of several mitochondrial genes in *NbMORF8*-silenced *N. benthamiana* leaves using GFP silenced leaves as the control. We used one site as an example to indicate the quantitative measurement of the peaks using BioEdit (Figure 2).

1. Open the sequencing files by BioEdit software.
2. Put the black cross at the top of targeted nucleotide peaks (nucleotide T and C, respectively) and read the second number, which shows the peak height.
3. Calculate the editing extent as efficiency %= T/(C+T) ×100%

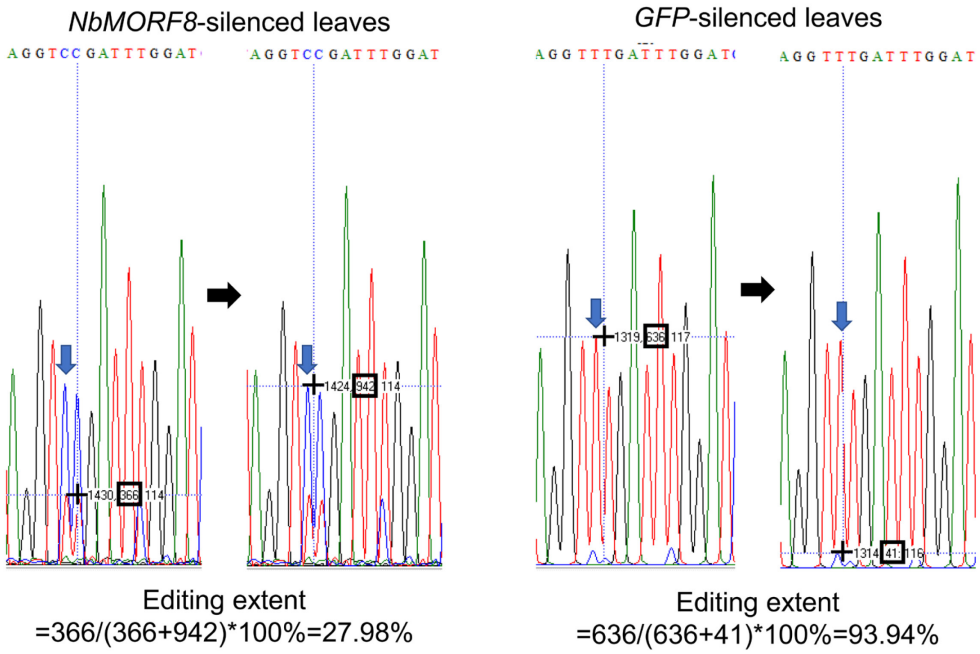

Figure 2. Analysis of RNA editing extent by Bioedit. The blue arrows mark the targeted nucleotide peaks, and the black hollow boxes mark the reads of the peak heights.

Notes

1. The size of the PCR fragments is recommended in the range of 150-300 bp.
2. The quality of the total RNA is crucial. Therefore, it is recommended to use the best RNA isolation method optimized for different plant species.
3. Since genomic DNA sequences represent an unedited version of the targeted transcripts, it is

important to erase the genomic DNA to avoid its interference with the RNA editing analyses.

4. The PCR products are recommended to be sequenced in both forward and reverse directions since the sequencing quality from one side sometimes is not sufficient for further analysis. In our case, the DNA sequencing was conducted by a sequencing company using Applied Biosystems 3730XL.

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Competing interests

The authors declare no conflict of interests.

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