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Dear editor,

Thank you very much for the comments on the manuscript, JOVE60019R1 “Discrimination and mapping of the primary and processed transcripts in maize mitochondrion using a circular RT-PCR-based strategy”. The MS is revised according to the editorial comments, and a rebuttal letter addressing each of the comments has been uploaded individually.

Thanks very much for all your help to make this work better, and we are looking forward to hearing from you soon.

Best regards,

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TITLE:

Discrimination and Mapping of the Primary and Processed Transcripts in Maize Mitochondrion Using a Circular RT-PCR-Based Strategy

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KEYWORDS

circular RT-PCR, Northern blot, quantitative RT-PCR, RNA 5’ polyphosphatase treatment, RNA normalization, primary and processed transcripts, maize mitochondrion

SUMMARY

We present a circular RT-PCR-based strategy by combining circular RT-PCR, quantitative RT-PCR, RNA 5’ polyphosphatase-treatment, and Northern blot. This protocol includes a normalization step to minimize the influence of unstable 5’ triphosphate, and it is suitable for discriminating and mapping the primary and processed transcripts stably accumulated in maize mitochondrion.

ABSTRACT

In plant mitochondria, some steady-state transcripts have 5’ triphosphate derived from transcription initiation (primary transcripts), while the others contain 5’ monophosphate generated post-transcriptionally (processed transcripts). To discriminate between the two types of transcripts, several strategies have been developed, and most of them depend on presence/absence of 5’ triphosphate. However, the triphosphate at primary 5’ termini is unstable, and it hinders a clear discrimination of the two types of transcripts. To systematically differentiate and map the primary and processed transcripts stably accumulated in maize mitochondrion, we have developed a circular RT-PCR (cRT-PCR)-based strategy by combining

cRT-PCR, RNA 5’ polyphoshpatase treatment, quantitative RT-PCR (RT-qPCR), and Northern blot. As an improvement, this strategy includes an RNA normalization step to minimize the influence of unstable 5’ triphosphate.

In this protocol, the enriched mitochondrial RNA is pre-treated by RNA 5’ polyphosphatase, which converts 5’ triphsophate to monophosphate. After circularization and reverse transcription, the two cDNAs derived from 5’ polyphosphatase-treated and non-treated RNAs are normalized by maize *26S* mature rRNA, which has a processed 5’ end and is insensitive to 5’ polyphosphatase. After normalization, the primary and processed transcripts are discriminated by comparing cRT-PCR and RT-qPCR products obtained from the treated and non-treated RNAs. The transcript termini are determined by cloning and sequencing of the cRT-PCR products, and then verified by Northern blot.

By using this strategy, most steady-state transcripts in maize mitochondrion have been determined. Due to the complicated transcript pattern of some mitochondrial genes, a few steady-state transcripts were not differentiated and/or mapped, though they were detected in a Northern blot. We are not sure whether this strategy is suitable to discriminate and map the steady-state transcripts in other plant mitochondria or in plastids.

INTRODUCTION

In plant mitochondria, many mature and precursor RNAs are accumulated as multiple isoforms, and the steady-state transcripts can be divided into two groups based on the difference at their 5’ ends[1-4]. The primary transcripts have 5’ triphosphate ends, which are derived from transcription initiation. By contrast, the processed transcripts have 5’ monophosphate generated by post-transcriptional processing. Discrimination and mapping of the two types of transcripts are important to unravel the molecular mechanisms underlying transcription and transcript end maturation.

To distinguish between the primary and processed transcripts in plant mitochondrion, four major strategies have been developed. The first strategy is to pre-treat the mitochondrial RNAs with tobacco acid pyrophosphatase (TAP), which converts 5’ triphosphate to monophosphate and enables primary transcripts to be circularized by RNA ligase. The transcript abundances of TAP-treated and non-treated RNA samples are then compared by rapid amplification of cDNA ends (RACE) or circular RT-PCR (cRT-PCR)[2-4]. In the second strategy, processed transcripts are firstly depleted from mitochondrial RNAs using terminator 5’-phosphate-dependant exonuclease (TEX), and the primary transcripts left are then mapped by primer extension analysis[5,6]. The third strategy is to pre-cap the primary transcripts using guanylyl transferase, and then the position of triphosphated 5’ termini is determined by primer extension together with ribonuclease or S1 nuclease protection analysis[7-9]. Different from those depending on the presence/absence of 5’ triphosphate, the fourth strategy combines *in vitro* transcription, site-directed mutagenesis, and primer extension analysis to characterize the putative promoters and determine the transcription initiation sites[8,10,11]. By using these strategies, many primary and processed transcripts have been determined in plant mitochondria.

However, several studies have reported that the 5’ triphosphate of primary transcripts were unstable, and they were easily converted to monophosphate for unknown reason[2,4,12,13]. This problem hinders a clear discrimination of the two types of transcripts by using techniques depending on the presence/absence of 5’ triphosphate, and previous efforts to systematically discriminate between the primary and processed transcripts in plant mitochondria failed[2,12].

In this protocol, we combine cRT-PCR, RNA 5’ polyphosphatase treatment, RT-qPCR, and Northern blot to systematically distinguish the primary and processed transcripts stably accumulated in maize (*Zea mays*) mitochondrion (**Figure 1**). cRT-PCR allows simultaneous mapping of 5' and 3' extremities of a RNA molecule, and it is usually adapted to map transcript termini in plants[2,12,14,15]. RNA 5’ polyphosphatase could remove two phosphates from the triphosphated 5’ termini, which makes the primary transcripts available for self-ligation by RNA ligase. Previous studies showed that mature *26S* rRNA in maize had processed 5’ terminus, and it was insensitive to RNA 5’ polyphosphatase[1,16]. To minimize the influence of unstable triphosphate at primary 5’ termini, the 5’ polyphosphatase-treated and non-treated RNAs are normalized by mature *26S* rRNA, and the primary and processed transcripts are then differentiated by comparing the cRT-PCR products obtained from the two RNA samples. The cRT-PCR mapping and discrimination results are verified by Northern blot and RT-qPCR, respectively. Finally, alternative primers are used to amplify those transcripts detected in Northern blot but not by cRT-PCR. By using this cRT-PCR-based strategy, most steady-state transcripts in maize mitochondrion have been differentiated and mapped[1].

PROTOCOL

1. Primer design

1.1. Design gene-specific primers for reverse transcription (RT) using PCR primer design software (**Table of Materials**) based on the general rules of primer design[17].

NOTE: RT primers are highly specific to the target transcripts, and they are generally anchored on the 5’ part of coding sequences (mature mRNAs and precursor RNAs), or ~500–600 nt downstream of the anticipated 5’ end (*18S* and *26S* rRNAs).

1.2. Design pairs of divergent primers to amplify the circularized transcripts by cRT-PCR.

NOTE: The paired divergent primers flank the 5’-3’ junction of the circularized transcripts, and their positions vary among the target transcripts analyzed (**Figure S1**). Some transcripts have long UTRs; for example, *nad2*-1 5’ UTR and *rps4*-1 3’ UTR are 1,985 and 1,826/1,834 nt, respectively[1]. If both primers are anchored on coding region, it will be hard to amplify the target transcripts. By contrast, some UTRs are short; for example, the 3’ UTRs of *nad2*-1 and *nad4*-1 are 34/35 and 29–31 nt, respectively[1]. If the paired primers are located far away from the coding sequences, the PCR will fail. Generally, two pairs of divergent primers are designed for each target transcripts, while multiple pairs may be necessary to map those whose transcript patterns are complicated and/or UTRs are very long.

1.3. Design pairs of convergent primers to prepare RNA probes for Northern blot.

NOTE: The paired primers are located on coding region of the target gene, and the size of the PCR product should be in the range of 100 to 1,000 bp. Each forward primer should contain a restriction enzyme site to minimize vector sequences in the resulting probes.

2. Preparation of crude mitochondrion from maize developing kernels

2.1. Sterilize pestles, mortars, glass funnels, tubes, and tips by autoclave, and dry them in oven.

2.2. Perform all procedures at 4 °C or on ice, and pre-cool all solutions.

2.3. Prepare 100 mL of extraction buffer (EB), composed of 0.3 M sucrose, 5 mM tetrasodium pyrophosphate, 10 mM KH_2PO_4, 2 mM EDTA, 1% [w/v] polyvinylpyrrolidone 40, 1% [w/v] bovine serum albumin, 5 mM L-cysteine, and 20 mM ascorbic acid. Adjust to pH 7.3 with KOH and sterilize by filtration.

2.4. Prepare 100 mL of wash buffer (WB) consisting of 0.3 M sucrose, 1 mM EGTA, and 10 mM MOPS (3-(N-morpholino)propanesulfonic acid). Adjust to pH 7.2 with NaOH and sterilize by filtration.

NOTE: It is suggested to prepare EB and WB using DEPC-treated deionized H_2O.

2.5. Collect 20 g developing kernels at 11–20 days after pollination (DAP) to a 50-mL tube placed on ice, and then transfer the kernels to pre-cooled mortars.

NOTE: Use a ratio of 100 mL of EB to 20 g of maize kernels.

2.6. Add 10–20 mL of ice-cold EB to each mortar, and grind the kernels completely.

2.7. Add more EB, and filter the ground tissues through two layers of filter cloth (**Table of Materials**).

2.8. Centrifuge the filtrate at 8,000 x *g* for 10 min, and discard the pellet.

2.9. Transfer the supernatant to a new tube, and centrifuge it at 20,000 x *g* for 10 min.

2.10. Pour off the supernatant, and resuspend the pellet in 6 mL WB.

2.11. Aliquot the suspension to five 1.5-mL RNase-free tubes, and centrifuge them at 14,000 x *g* for 5 min.

2.12. Discard the supernatant, freeze the mitochondrial pellet in liquid nitrogen, and store at -80 °C.

3. Extraction of mitochondrial RNA

3.1. Extract mitochondrial RNA with a commercial reagent (**Table of Materials**) according to the manufacture's instructions.

CAUTION: This reagent contains phenol and guanidine isothiocyanate. Work with it in a fume hood, and wear lab coat and gloves.

3.2. Dissolve the isolated mitochondrial RNA in DEPC-treated deionized H_2O, and estimate RNA concentration and purity with a spectrophotometer (**Table of Materials**).

NOTE: Generally, ~250 µg mitochondrial RNA is obtained from 20 g of 15-DAP maize kernels.

3.3. Prepare one agarose gel composed of 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1mM EDTA), 1.5% agarose (**Table of Materials**), and 1x nuclear staining dye (**Table of Materials**).

3.4. Add an appropriate volume of 10x loading buffer (0.5% bromophenol blue, 0.5% xylene cyanol FF, and 50% glycerol), and load mitochondrial RNA/Loading buffer mixture on the 1.5% agarose gel.

3.5. Run the gel in 1x TAE buffer at 5 – 6V/cm for 20 – 25 min, and evaluate mitochondrial RNA integrity by imaging the gel with a gel documentation system (**Table of Materials**).

NOTE: The presence of two distinct bands (~3,510 and ~1,970 nt for maize mitochondrial *26S* and *18S* rRNAs, respectively) is an acceptable standard for intact mitochondrial RNA. To exclude possible degradation, RNA integrity should be investigated during the multiple steps of circularized RNA preparation (**Figure 2**).

4. RNA 5' polyphosphatase treatment

4.1. Set up RNA 5' polyphosphatase (**Table and Materials**) treatment (**Table 1**), and incubate at 37 °C for 30–60 min.

4.2. Recover the 5' polyphosphatase-treated RNA with an RNA purification kit (**Table of Materials**) according to the manufacturer's instructions.

4.3. Repeat steps 3.3 to 3.5.

5. RNA circularization

5.1. Prepare two circularization reactions using the same amounts of 5' polyphosphatase-treated and non-treated mitochondrial RNAs (**Table 2**), and incubate both reactions at 16 °C for 12–16 h.

5.2. Recover the two sets of self-ligated RNAs using the same kit as in step 4.2.

NOTE: It should be noted that only a fraction of mitochondrial RNA will be self-ligated, and the recovered RNA will be a mixture of linear and circularized transcripts.

5.3. Repeat steps 3.3 to 3.5.

6. Reverse transcription

6.1. Synthesize two sets of cDNAs from the same amounts of circularized 5' polyphosphatase-treated and non-treated RNAs (200 ng).

6.2. Prepare a primer mixture by adding an equal ratio of 26S-CRT and up to 7 other RT primers.

NOTE: The final concentration of the primer mixture should be 1 µM.

6.3. Prepare two pre-mixtures by combining the reagents listed in **Table 3**, incubate at 65 °C for 5 min, and then chill on ice for 2 min.

6.4. Assemble two RT reaction systems (**Table 4**), and incubate them at 42 °C for 50 min.

6.5. Heat both RT reactions at 70 °C for 5 min, and then chill them on ice.

7. Normalization

7.1. Prepare two PCR reactions by adding the same volume of template cDNAs derived from 5' polyphosphatase-treated or non-treated RNAs, divergent primers flanking the 5'-3' junction of circularized *26S* rRNA (i.e. 26S-CF1 and -CR1), etc. (**Table 5**).

7.2. Run the two reactions in a thermal cycler (**Table of Materials**) under conditions described in **Table 6**.

7.3. Prepare one agarose gel composed of 1x TAE buffer, 1.0% agarose, and 1x nuclear staining dye.

7.4. Add 2 µL 10x loading buffer (0.5% bromophenol blue, 0.5% xylene cyanol FF, and 50% glycerol) to each of the two PCR products, and load them on the gel.

7.5. Run the gel in 1x TAE buffer at 5–6 V/cm for 30–40 min, and image it using the same gel documentation system as step 3.5.

7.6. Compare the abundance of the two PCR products using computer software (**Table of Materials**, **Figure 4A**), and optimize the normalization by changing the amounts of template cDNAs if necessary.

8. PCR amplification

8.1. Prepare pairs of PCR reactions by adding appropriate volume of the normalized cDNAs and a pair of divergent primers flanking 5'-3' junction of the target transcripts (**Table 7**).

NOTE: The amount of template cDNAs used for PCR amplification of the target transcripts are determined by the *26S* rRNA normalization results.

8.2. Perform the PCR reactions according to the program described in **Table 8**.

8.3. Repeat steps 7.3 to 7.5.

8.4. Change to nested divergent primers, and verify the first round PCR results by repeating steps 8.1 and 8.2.

8.5. Repeat steps 7.3 to 7.5.

8.6. Recover the prominent bands that could be repeated in two rounds of PCR amplification using a gel DNA recovery kit (**Table of Materials**).

9. Determination of transcript termini

9.1. Clone the gel-purified PCR products into a blunt-end vector (**Table of Materials**) using standard techniques.

9.2. Perform colony PCR to select positive clones containing the target inserts, and sequence them commercially.

NOTE: Positive clones containing inserts with variable size are usually detected from a single recovered band because many steady-state transcripts in plant mitochondrial have heterogeneous 5' and/or 3' ends[1,12].

9.3. Align the sequencing data with maize mitochondrial genome using basic local alignment search tool (BLAST) of national center for biotechnology information (NCBI). Choose organism "maize (taxid:4577)", and search database "Nucleotide collection (nr/nt)".

9.4. Find the 5'-3' junction of the circularized transcript, and determine the positions of 5' and 3' transcript termini.

9.5. Calculate the size of target transcripts.

10. Verification of the cRT-PCR mapping results by RNA gel blot hybridization

NOTE: RNA gel blot hybridization is performed by using a commercial kit (**Table of Materials**), which contains the reagents for transcription-labeling of RNA with digoxigenin (DIG) and T7 RNA polymerase, hybridization, and immunological detection. Please refer to the protocols provided in this kit for more details. Make sure that only RNase free equipment is used for the whole procedure.

10.1. Amplify the DNA fragment used to prepare RNA probe, and clone it to the same vector as in step 9.1, which contains a T7 promoter 17 bp upstream of the insertion site.

10.2. Linearize the construct using proper restriction enzyme, recover the linearized plasmid by a DNA purification kit (**Table of Materials**), and dissolve it in DEPC-treated deionized H_2O.

10.3. Label RNA probes with DIG-11-UTP by a commercial kit (**Table of Materials**) according to the manufacturer's instructions.

10.4. Prepare 500 mL of 10x MOPS buffer (0.2 M MOPS, 50 mM sodium acetate, and 10 mM EDTA) using DEPC-treated deionized H_2O. Adjust to pH 7.0 by NaOH, and sterilize by filtration.

10.5. Add 2–3 volumes of loading buffer (50% formamide, 6.2% formaldehyde, 1x MOPS, 10% glycerol, and 0.1% bromophenol blue) to the mitochondrial RNA prepared in steps 3.1–3.3.

10.6. Denature the RNA sample/Loading buffer mixture at 65 °C for 10 min, and then chill on ice for 1 min.

10.7. Prepare a denatured agarose gel (2% formaldehyde, 1.2% agarose, and 1x MOPS), and load the RNA sample/Loading buffer mixture to the gel (1–2 μg mitochondrial RNA per well).

10.8. Run the gel in 1x MOPS at 3–4 V/cm for ~4 h.

10.9. Prepare 2 L of 20x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). Treat it with 0.1% DEPC overnight, and sterilize by autoclave.

10.10. Rinse the gel twice in 20x SSC (15 min each time), and transfer gel RNA to a nylon membrane (**Table of Materials**) by capillary transfer with 20x SSC for 10–16 h.

10.11. Fix the RNA to membrane by baking at 120 °C for 30 min.

10.12. Perform RNA hybridization and immunological detection with a commercial kit (**Table of Materials**) according to the manufacturer's instruction.

11. Discrimination of primary and processed 5' ends

11.1. Discriminate the primary and processed 5' ends by comparing the cRT-PCR products obtained from the normalized 5' polyphosphatase-treated and non-treated RNAs.

NOTE: To primary transcripts, the abundance of PCR products from 5' polyphosphatase-treated RNA is much higher than that from non-treated counterpart (**Figure 1**, 'Gene A,' and **Figure 4A**). However, to processed transcripts, a comparable level of PCR products would be amplified from the two sets of mitochondrial RNAs (**Figure 1**, 'Gene B').

11.2. Design RT-qPCR primers based on the cRT-PCR mapping results.

11.3. Verify the cRT-PCR discrimination results by RT-qPCR.

NOTE: The two cDNA samples derived from 5' polyphosphatase-treated and non-treated RNAs are normalized by the RT-qPCR products of *26S* mature rRNA.

REPRESENTATIVE RESULTS

Estimation of mitochondrial RNA circularization efficiency

In a previous study, both total and mitochondrial RNAs were used for cRT-PCR mapping of mitochondrial transcript termini in *Arabidopsis* (*Arabidopsis thaliana*), and the two types of RNAs gave similar mapping results[12]. Initially, we also used total RNAs for cRT-PCR mapping of mitochondrial transcript termini in maize. After many trials, we found the target transcripts were hard to detect. As an improvement, we enriched the mitochondrial RNA from maize developing kernels, and it made the amplification of the circularized target transcripts in one round of PCR possible.

To explain the easy amplification of target transcripts after the enrichment, we estimated RNA circularization efficiency by performing RT-PCRs on representative mitochondrial gene *nad5* (**Figure 3** and **Figure S2**). The maize *nad5* gene contains two *trans*- and two *cis*-splicing introns. After self-ligation of total and mitochondrial RNAs, first strand cDNAs were independently synthesized using *nad5*-RT1 and -RT2 primers. The two primers could reverse transcribe both linear and circularized *nad5* mRNAs (**Figure S2**). Four pairs of convergent primers are used for PCR amplification: sqF1&sqR1 and sqF2&sqR2 for semi-quantitative RT-PCR (RT-sqPCR) and qF1&qR1 and qF2&qR2 for RT-qPCR. For cDNAs transcribed by *nad5*-RT1, all four pairs of primers detected both circularized and linear *nad5* mature mRNAs; for the *nad5*-RT2 reverse transcription reaction, sqF2&sqR2 and qF2&qR2 surveyed both forms of mRNA, while sqF1&sqR1 and qF1&qR1 PCR products were derived from circularized *nad5* only. Based on this analysis, we used the sqF2&sqR2 (for RT-sqPCR) and qF2&qR2 (for RT-qPCR) PCR products to normalize the *nad5*-RT1 and -RT2 reverse transcription reactions, and estimated the ratio of circularized *nad5* by comparing the sqF1&sqR1 (for RT-sqPCR) or qF1&qR1 (for RT-qPCR) PCR products. Prior to self-ligation, RNAs were pre-treated by RNA 5' polyphosphatase to make all transcripts available for circularization by RNA ligase. The RT-sqPCR analysis showed a very small fraction of *nad5* mRNA was circularized when total RNA was used, but the ratio was dramatically increased when the enriched mitochondrial RNA was used (**Figure 3B**). RT-qPCR results showed that the ratio of self-ligated *nad5* mRNA increased from 3.7% to 32% after the enrichment (**Figure 3C**). Analysis of *nad1* gave similar results, and the circularization efficiency of *nad1* mRNA increased from 0.7% to 30% (**Figure 3D–F**).

Per these analyses, about 30% maize mitochondrial RNAs were self-ligated after the improvement, and the increased circularization efficiency explains the easy detection of the target transcripts by cRT-PCR.

Determination of maize *cox2* mRNA termini using the cRT-PCR-based strategy

We used the *cox2* gene as an example to introduce the mapping and discrimination of maize mitochondrial transcripts by using the cRT-PCR-based strategy (**Figure 4**).

In the beginning of mapping *cox2* mRNA, three outward-facing primers CF1, CF2, and CR1 were designed on the gene coding region (**Figure 4D**). CF1 and CF2 were nested primers, and CF2 was 69 bp downstream of CF1. Using the template cDNAs synthesized in steps 6.1–6.5 and normalized at steps 7.1–7.6, the CF1&CR1 primer pair amplified two prominent bands, and the amplification results were repeated by nested primers CF2&CR1 (**Figure 4A**). Moreover, the two bands were strongly amplified from the 5’ polyphsophatase-treated RNA, while they were hard to detect in the non-treated counterpart, suggesting that they are sensitive to RNA 5’ polyphsophatase and have primary 5’ termini.

The two candidate bands were named *cox2*-1 and -2, and they were recovered independently from the agarose gel and cloned into vectors. Colony PCR results showed that the positive clones contain inserts with variable size, implying heterogeneous 5’ and/or 3’ termini of the transcripts (**Figure 5**). The positive clones were sequenced commercially, and the sequencing data were aligned with maize mitochondrial genome as described in step 9.3.

The sequencing and alignment results showed that the two transcripts were identical at 3’ ends, but different in the length of 5’ UTRs (**Figure 4D**). The 5’ ends of *cox2*-1 and -2 were enriched at 992–1,030 and 1,276–1,283 nt upstream of AUG, respectively, while their 3’ end was 39 nt downstream of the stop codon. Deduced from the cRT-PCR results, the calculated sizes of *cox2*-1 and -2 were 1,804–1,832 and 2,088–2,095 nt, respectively.

To verify the cRT-PCR mapping results, RNA gel blot hybridization was performed by using the probe derived from *cox2* gene coding region, and two major bands with similar size as *cox2*-1 and -2 were detected (**Figure 4C**). Additionally, two larger bands about 2,500 and 2,800 nt were detected in the Northern blot, but they were not amplified by cRT-PCR. The two larger bands were named as *cox2*-3 and -4, respectively. To map them, another two outward-facing primers (i.e. CR2 and CF3), anchored on the 5’ and 3’ UTRs of *cox2*-2, were designed, and their positions were close to the expected transcript ends of *cox2*-3 and -4. Using the primer pair CF3&CR2, two major bands were strongly amplified from cDNAs derived from 5’ polyphsophatase-treated RNA but not from the non-treated counterpart (**Figure 4A**). The sequencing results showed that they had identical 5’ termini while variable 3’ ends. The calculated size of the two transcripts were 2,512/2,513 and 2,833–2,835 nt, respectively, and they were close in size with the two larger bands detected in the Northern blot. Moreover, the cRT-PCR results suggested that *cox*2-3 and -4 have primary 5’ termini.

To confirm the discrimination results, five outward-facing primers were designed according to the cRT-PCR mapping results, i.e. qCF1, qCF2, qCR1, qCR2, and qCR3, and the primer pairs qCF1&qCR1, qCF1&qCR2, qCF1&qCR3, and qCF2&qCR3 were used for RT-qPCR analysis of *cox2*-1, -2, -3, and -4, respectively. The relative abundance of all four RT-qPCR products in 5' polyphsophatase-treated sample were much higher that those in the non-treated counterpart, which confirms the cRT-PCR discrimination results.

In summary, the transcript pattern of maize *cox2* gene is complicated, and the cRT-PCR-based strategy effectively discriminates and maps the multiple isoforms of *cox2* mRNA.

FIGURE AND TABLE LEGENDS

Figure 1: Overview of the cRT-PCR-based strategy. Illustration of the major steps of the cRT-PCR-based strategy. 5'+ and 5'- = the two RNA samples treated and non-treated by RNA 5' polyphosphatase, respectively. The two cDNA samples derived from 5' polyphosphatase-treated and non-treated RNAs are normalized by *26S* mature rRNA in maize, and the primary and processed transcripts are discriminated by comparing the cRT-PCR products. For gene A, the corresponding RNA has a primary 5' end because the PCR product abundance from 5' polyphosphatase-treated RNA is much higher than that from the non-treated counterpart; for gene B, the PCR products from the two sets of mitochondrial RNAs are amplified at a comparable level, implying a processed 5' terminus of the corresponding RNA. The coding region and UTRs are represented by gray box and bold lines, respectively. CRT = primer for reverse transcription; CF1, CF2, CR1, and CR2 = PCR primers flanking the 5'-3' junction of the circularized transcript; qCF and qCR = divergent primers for RT-qPCR.

Figure 2: Evaluation of mitochondrial RNA integrity by agarose gel electrophoresis. (**A**) Mitochondrial RNAs prepared from 15-DAP maize kernels. M = DNA molecular marker. (**B**) Kernel mitochondrial RNA after the treatment by 5' polyphosphatase and/or circularization by T4 RNA ligase. 5'+ = mitochondrial RNA after the treatment by 5' polyphosphatase; T4&5'+ = mitochondrial RNA after the treatment by 5' polyphosphatase and circularization by T4 RNA ligase; T4&5'- = mitochondrial RNA after circularization only.

Figure 3: Circularization efficiency of *nad5* and *nad1* mRNAs estimated by RT-PCRs. (**A**) and (**D**) Schematic diagram of *nad5* and *nad1* genes, respectively. Ex = exon. Exons and introns are shown as gray boxes and curved lines, respectively. The position of reverse transcription primers RT1 and RT2 are indicated by arrows. Black dots indicate the position of PCR primers, and the predicted size of the PCR products is shown. sqF1, sqF2, sqR1, and sqR2 are primers for RT-sqPCR; qF1, qF2, qR1, and qR2 are primers for RT-qPCR. (**B**) and (**E**) RT-sqPCR analysis of the circularization efficiency of *nad5* and *nad1* mRNAs, respectively. M = DNA molecular marker. (**C**) and (**F**) RT-qPCR analysis of the circularization efficiency of *nad5* and *nad1* mRNAs, respectively. The two cDNA samples reverse transcribed by RT1 and RT2 primers are normalized by sqF2&sqR2 (for RT-sqPCR) or qF2&qR2 (for RT-qPCR) PCR products. The circularization efficiency of *nad5* and *nad1* mRNAs is estimated by comparing the PCR products of sqF1&sqR1 (for RT-sqPCR) or qF1&qR1 (for RT-qPCR). circularized/(circularized+linear) = qF1&qR1 over qF2&qR2 in RT2 reaction/ qF1&qR1 over qF2&qR2 in RT1 reaction. Values are means and SDs of three biological

replicates. To exclude the potential influence by 5' triphosphate, the RNAs were treated by 5' polyphosphatase prior to self-ligation. Total and Mito = total and mitochondrial RNAs, respectively. RT1 and RT2 = cDNAs reverse transcribed by RT1 and RT2 primers, respectively

Figure 4: Mapping of *cox2* mature mRNA termini by using the cRT-PCR-cased strategy. (**A**) Gel separation of *cox2* cRT-PCR products. + and - = cDNAs derived from 5' polyphosphatase-treated and non-treated mitochondrial RNAs, respectively. The two cDNA samples were normalized by amplification of *26S* cDNA (*26S*). Five primers are used for cRT-PCR analysis of circularized *cox2* mRNA: CF1, CF2, and CF3 are nested primers, and CF2 and CF3 are 69 and 138 bp downstream of CF1, respectively; CR1 and CR2 are nested primers, and CR2 is 1,288 bp upstream of CR1. The bands indicated by '1' and '2' were amplified by both CF1&CR1 and CF2&CR1, while the '3' and '4' bands were amplified by CF3&CR2. All four of the bands were sequenced by cloning. The bands marked by asterisks could not be repeated, and they were excluded from the results. M = DNA molecular marker. (**B**) RT-qPCR analysis of the relative abundance of circularized *cox2* mRNA after 5' polyphosphatase treatment. The two cDNA samples were synthesized by a primer mixture containing 26S-CRT, cox2-CRT, nad5-CRT, nad6-CRT, nad7-CRT, nad9-CRT, cob-CRT, and cox1-CRT at equal ratio, and the cDNA derived from *26S* mature rRNA was used for normalization. +/- = *cox2* over *26S* in treated sample / *cox2* over *26S* in non-treated sample. Values represent means and SD of three biological replicates. The primers used for RT-qPCR are indicated. (**C**) Northern blot analysis of *cox2* transcripts. 2 μg mitochondrial RNA was loaded. The bands corresponding to different isoforms of *cox2* mature mRNA are marked. (**D**) Transcript termini of *cox2* mRNA deduced from cRT-PCR results. UTRs and open reading frames are shown as bold lines and gray boxes, respectively. The positions of 5' and 3' termini relative to AUG (+1) and UAA (-1), and numbers of single clones sequenced at those positions are shown. The positions of reverse transcription and PCR amplification primers are shown as closed and open arrow heads, respectively. Outward-facing primers used for RT-qPCR are shown as open squares, and the position of *cox2* probe is as indicated.

Figure 5: Selection of positive clones containing *cox2*-4 inserts by colony PCR. (**A**) Colony PCR to screen single clones containing *cox2*-4 inserts. The size of *cox2*-4 insert is about 1,165 bp, and that of the vector sequences is 100 bp. The calculated size of colony PCR products is about 1,265 bp, and those clones containing small size inserts were not sequenced, i.e., numbers 11, 14, 22, and 23. (**B**) Sequencing results of the positive clones screened in (A). 5'/3' ends = the 5' and 3' ends relative to AUG (+1) and UAA (-1), respectively. The sequencing results of numbers 12 and 20 were excluded because they were much smaller than the others.

Table 1: Reaction components of RNA 5' polyphosphatase treatment.

Table 2: Reaction components of mitochondrial RNA circularization.

Table 3: Pre-mixture for reverse transcription reaction. [a]The primer mixture contains equal ratio of 26S-CRT and up to 7 other RT primers, and the final concentration is 1 μM. [b]Two pre-mixtures are prepared side-by-side, and they contain the same amount (200 ng) of circularized 5' polyphosphatase-treated or non-treated mitochondrial RNA.

Table 4: Reaction components of reverse transcription. *The template RNA/primer/dNTP pre-mixtures prepared at step 6.3.

Table 5: Reaction components for PCR amplification of *26S* cDNA. *Initially, equal volume of template cDNAs (0.6 µL) are used for normalization. If necessary, change the amounts of template cDNAs to ensure the same abundance of *26S* PCR products between the two PCR reactions.

Table 6: PCR conditions to amplify *26S* cDNA for normalization.

Table 7: Reaction components for PCR amplification of the circularized target transcripts. *The volume of template cDNAs used in these reactions are determined by the *26S* rRNA normalization results.

Table 8: PCR conditions to amplify the target transcripts. [a]Exact annealing temperate depends on the melting temperature of the PCR primers. [b]Elongation time depends on the length of target to be amplified. Recommended time is 1 min per 1 kb of the PCR fragment. [c]In general, 30~35 cycles are enough to produce an adequate amount of PCR product. For low abundant transcripts, increase the number of cycles up to 40 cycles.

Figure S1: Position of primers for representative maize mitochondrial transcripts. CRT = reverse transcription primer; CF1, CF2, CR1, and CR2 = divergent primers for PCR amplification; qCF and qCR = divergent primers for RT-qPCR. The transcript termini of maize *nad2*-1, *rps4*-1, and *nad4*-1 have been determined previously (Zhang et al., 2019[1]). Positions of 5'- and 3'-ends relative to AUG (+1) and the last nucleotide of stop codon (-1) are shown. The coding regions and UTRs are indicated as gray boxes and bold lines, respectively.

Figure S2: Principle to estimate circularization efficiency of *nad5* mRNA in maize. In a self-ligation reaction, only a fraction of mitochondrial RNAs is circularized. To calculate the ratio of circularized *nad5* mRNA, two gene-specific primers are used to synthesize the first strand cDNAs, i.e., *nad5*-RT1 and -RT2. In the *nad5*-RT2 reverse transcription (RT) reaction, the PCR products amplified by sqF2&sqR2 (for RT-sqPCR) and qF2&qR2 (for RT-qPCR) are derived from both linear and circularized *nad5,* while the sqF1&sqR1 (for RT-sqPCR) and qF1&qR1 (for RT-qPCR) PCR products are derived from circularized *nad5* only; in *nad5*-RT1 reaction, all four pairs of PCR primers could amplify both forms of *nad5*. To calculate the ratio of circularized *nad5* mRNA, the two reactions are normalized by sqF2&sqR2 or qF2&qR2 PCR products. By comparing the abundance of sqF1&sqR1 or qF1&qR1 PCR products between the two RT reactions, the circularization efficiency of *nad5* mRNA is roughly estimated. ex = exon. Exons and introns are shown as gray boxes and curved lines, respectively. The positions of *nad5*-RT1 and -RT2 primers are indicated by arrows. Black dots indicate the positions of PCR primers, and the predicted size of the PCR products is shown.

Table S1: Primer information.

DISCUSSION

In a previous study, total and mitochondrial RNAs from cell suspension culture of *Arabidopsis* were used to map mitochondrial transcript termini by cRT-PCR, and similar results were obtained[12]. However, only enriched mitochondrial RNA was used to map mitochondrial transcript termini in many other studies[1-3,9]. We found that the enrichment of mitochondrial RNA is an important step for cRT-PCR mapping of mitochondrial transcript termini in maize. After this enrichment, the ratio of circularized mitochondrial RNA is dramatically increased from 0.3%–3.7% to ~30% (**Figure 3** and **Figure S2**), which makes the amplification of circularized target transcripts possible in one round of PCR.

The circularization efficiency of mitochondrial RNA was estimated by RT-PCR analysis on maize *nad1* and *nad5*, both of which are divided into independent precursors by *cis*-splicing introns. Because the potential influence from the presence of precursor RNAs as well as the variation of RT efficiency could not be ruled out, this is only a rough estimation of the real mitochondrial RNA circularization efficiency.

We altered the self-ligation conditions by altering the concentration of mitochondrial RNA and PEG8000, as well as by elongating the incubation time, but these changes did not seem to affect mitochondrial RNA circularization efficiency. Although we are not sure whether further Percoll gradient purification could increase the circularization efficiency, the quality of crude mitochondrion prepared in section 2 is good enough for cRT-PCR mapping of mitochondrial transcript termini in maize. Since multiple rounds of PCRs may cause false positive results, the amplification of target transcripts in one round PCR makes the mapping results more reliable.

The 5’ triphosphate of primary transcripts is unstable, and it could be converted to 5’ monophosphate for unknown reasons. This problem hinders a clear differentiation between the primary and processed transcripts by using approaches depending on the presence/absence of 5’ triphosphate[1,2,4]. The mature form of maize *26S* rRNA has a monophosphated 5’ end, and it is insensitive to RNA 5’ polyphosphatase. To minimize the influence of the unstable 5’ triphospahte, mature *26S* rRNA is used to normalize the two RNA samples, treated and untreated by 5’ polyphosphatase, and it is shown to be an important step to differentiate the two types of transcripts in maize mitochondrion. After normalization, the primary and processed transcripts could be discriminated by comparing cRT-PCR and RT-qPCR results obtained from 5’ polyphosphatase-treated and non-treated RNA samples. The primary transcripts are sensitive to 5’ polyphosphatase, and they are strongly amplified from the 5’ polyphosphatase-treated sample but not (or at a very low level due to the unstable 5’ triphosphate) from the non-treated counterpart. By contrast, the processed transcripts are detected at comparable levels between the two samples.

In plants, the transcript patterns of many mitochondrial genes are rather complicated[1,2]. For example, the maize *nad6* and *atp6* genes are expressed as both monocistrons and dicistrons, and *nad6*, *atp6*, and *atp6-na6* mature mRNAs have two, three, and two isoforms, respectively.

Moreover, the transcript population of one mitochondrial gene is actually a mixture of precursor, mature, and degradation RNAs. PCRs are prone to amplify small size molecules, and it is hard to detect all transcript isoforms using one pair of primers. Therefore, RNA gel blot hybridization is required to verify the cRT-PCR mapping results, and alternative primers may be necessary to amplify those transcripts detected in Northern blot but not by the first time cRT-PCR.

This protocol includes a RT-qPCR step to verify the cRT-PCR discrimination results. Because the multiple isoforms of some mature mRNAs are very close in size[1], it is impossible to confirm of all of the discrimination results by RT-qPCR, and some steady-state transcripts were determined by comparing the cRT-PCR results between the treated and non-treated RNAs only.

In this protocol, the 5' and 3' termini of the target transcripts were mapped by cloning and sequencing of the cRT-PCR products, and it limits the number of single clones to be sequenced. As an alternative method, the cRT-PCR products could be sequenced by next generation sequencing, which sequences thousands of molecules for one set of steady-state transcripts, and the mapping results would be more accurate.

Besides the discrimination of primary and processed 5' termini by cRT-PCR and RT-qPCR, the monophosphate 5' termini could be determined by identification of the surrounding RNA secondary structures[1,12]. It is well known that RNA secondary structures such as tRNA and t-element could mediate mitochondrial transcript end formation by directing endonucleolytic cleavages of RNase P and/or RNase Z[12,18-20]. Therefore, those 5' termini adjacent to tRNA or t-element should be derived from post-transcriptional processing and contain monophosphates.

By using this protocol, a great part of steady-state transcripts have been determined in maize mitochondria[1]. However, a few were not differentiated and/or mapped for unknown reason[1]. Moreover, we think the position of 5' transcript termini is better to be confirmed by primer extension analysis, though it is included in this protocol. Due to the lack of experimental data, we are not sure whether this strategy is suitable for other plant species, such as rice (*Oryza sativa*) and *Arabidopsis*. Besides plant mitochondria, both primary and processed transcripts are stably accumulated in plastids[21], and it is uncertain whether this strategy could be used to map and discriminate plastid transcripts.

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1

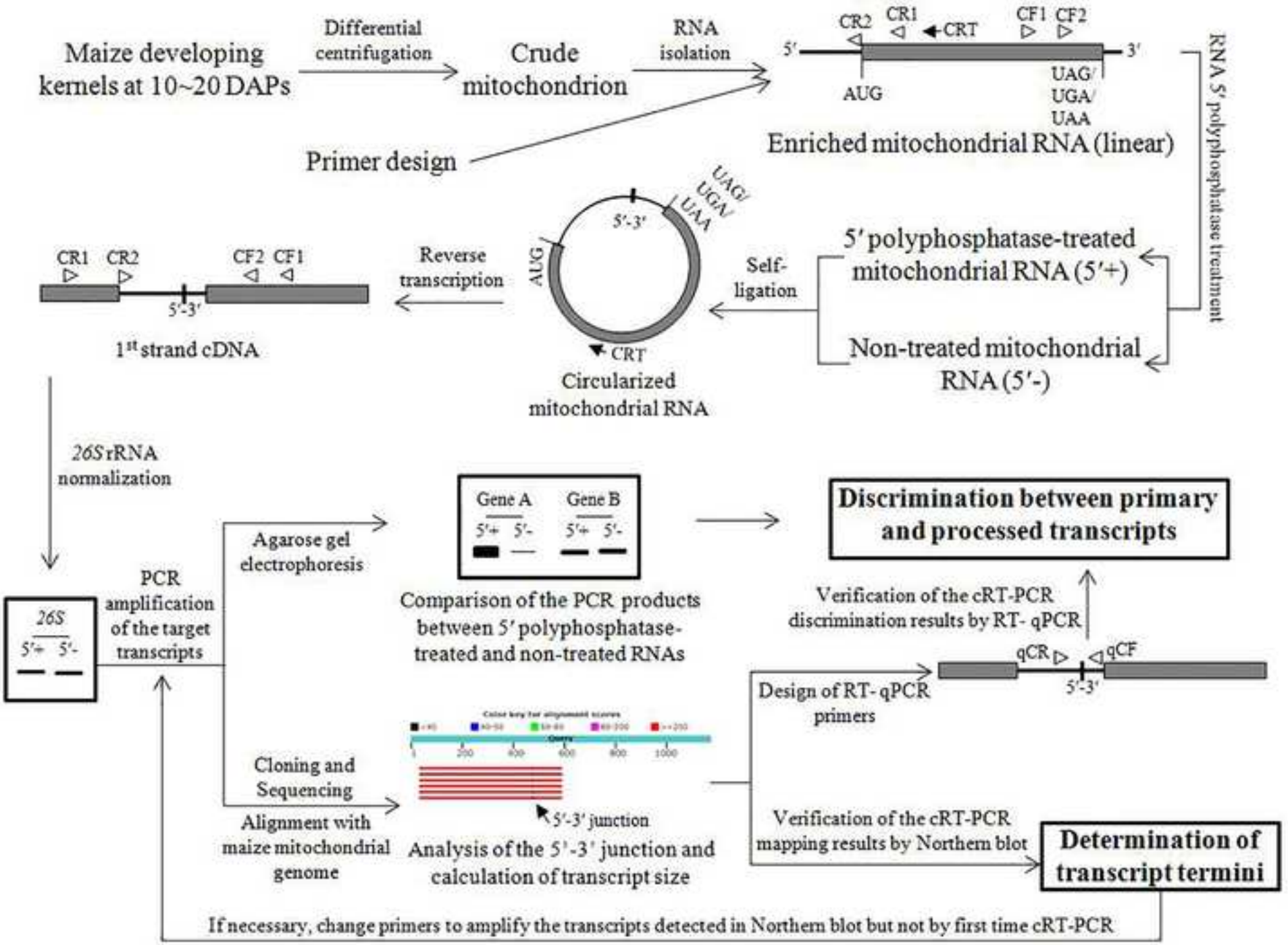

Figure 2

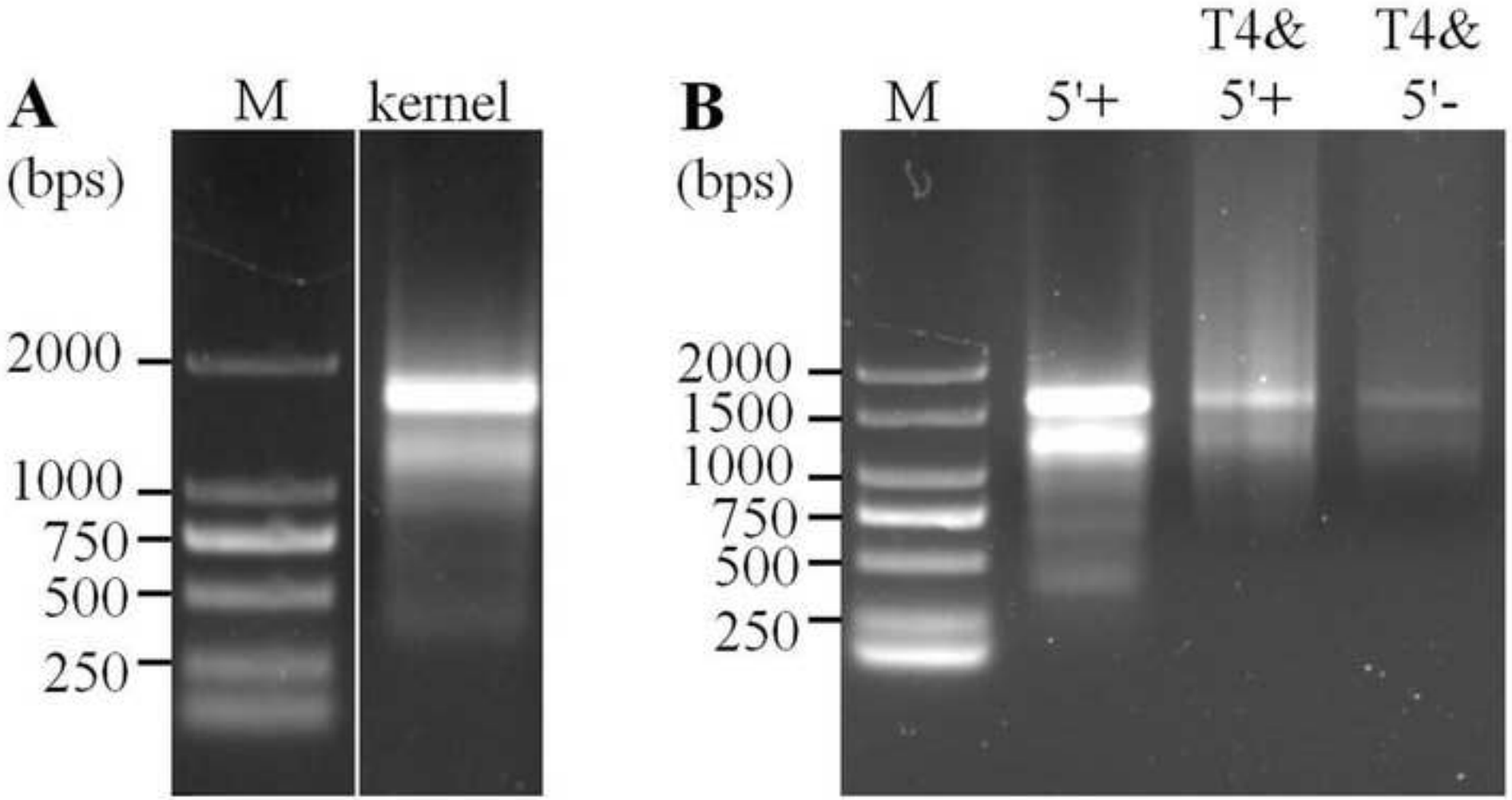

Figure 3

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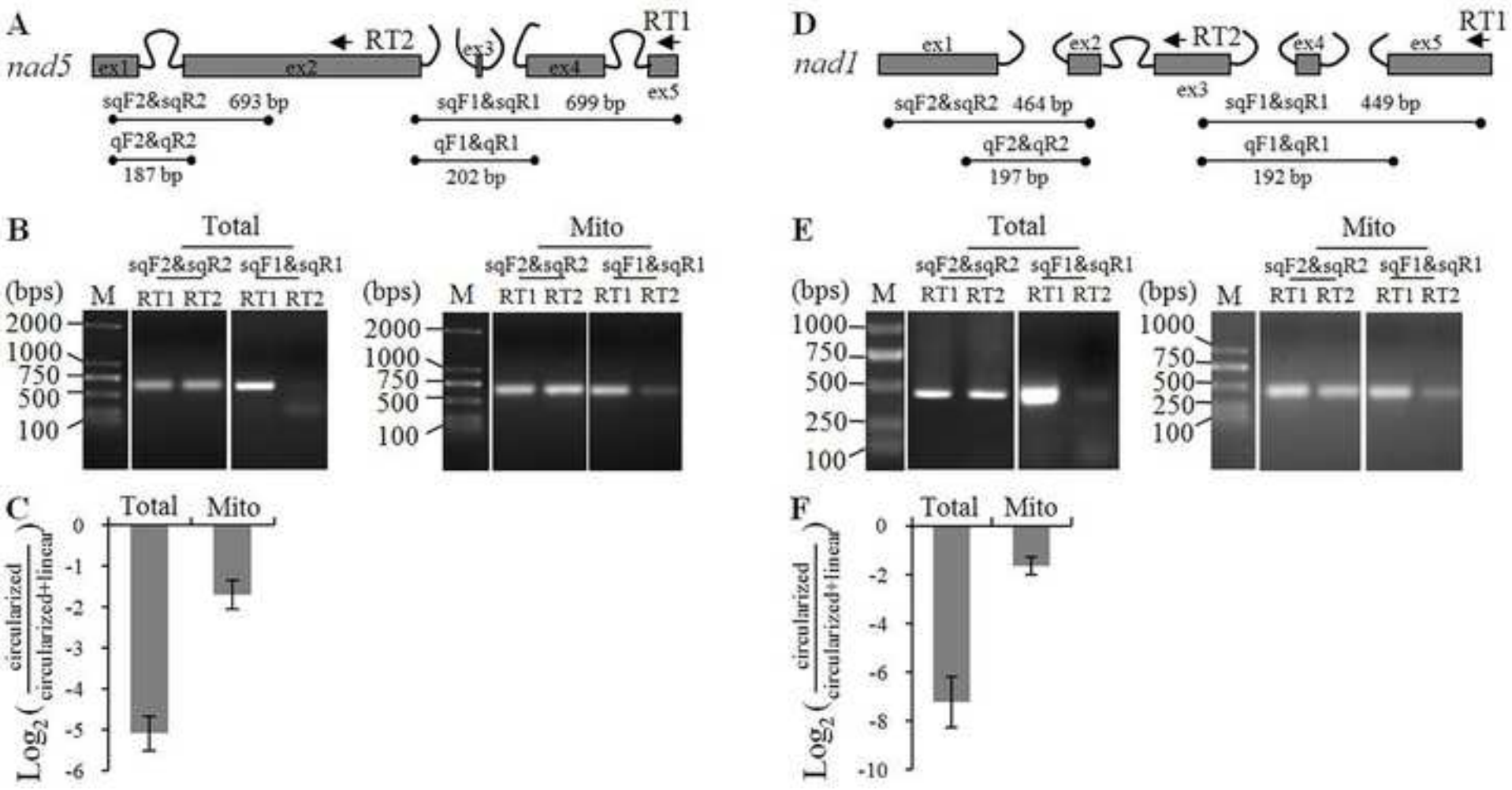


Figure 4

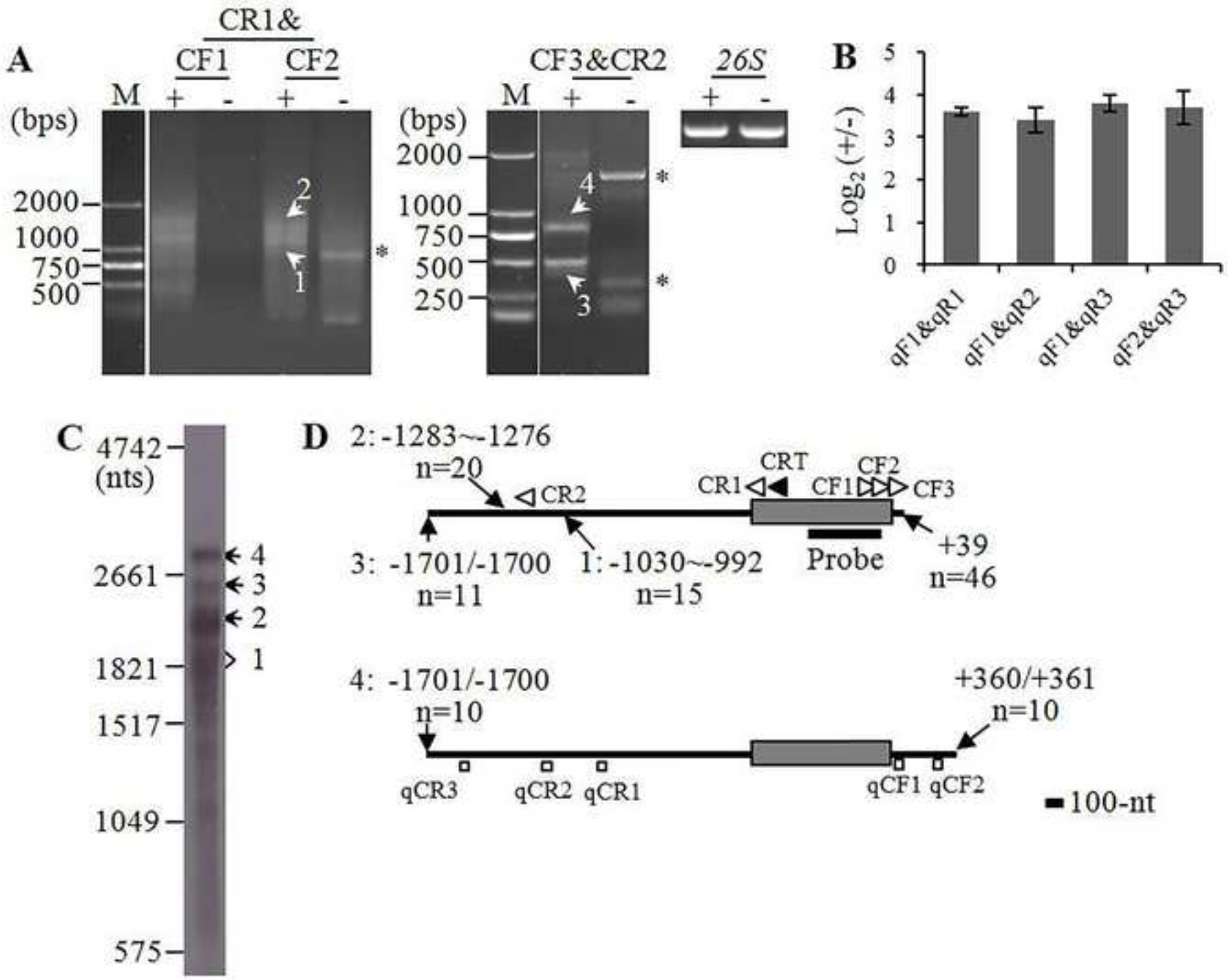
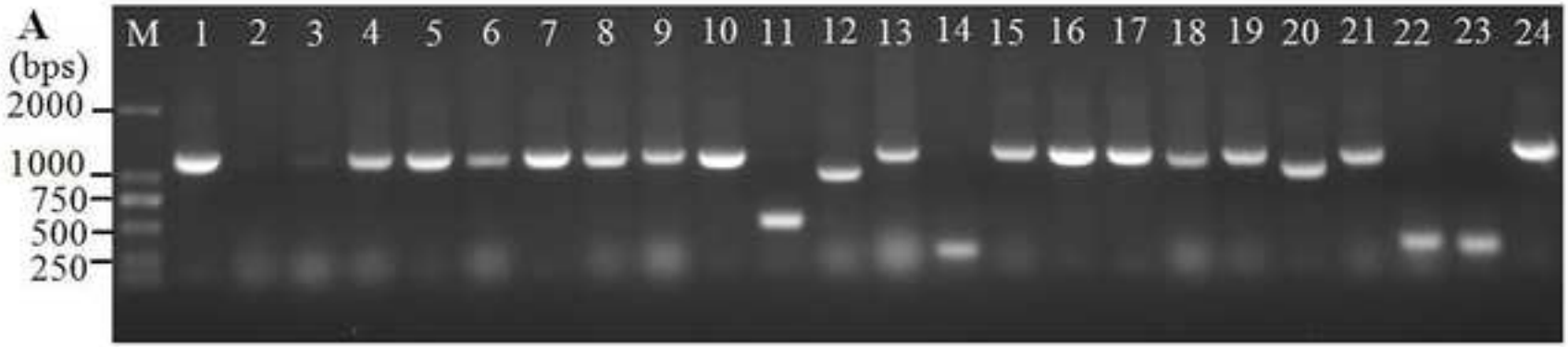


Figure 5

A



B

Lane No.	5'/3' ends	Lane No.	5'/3' ends	Lane No.	5'/3' ends	Lane No.	5'/3' ends
1	-1130/+39	8	-1014/+39	15	-1130/+39	**20**	**-844/+39**
4	-1023/+39	9	-1130/+39	16	-1026/+39	21	-1130/+39
5	-1010/+39	10	-996/+39	17	-1130/+39	24	-1130/+39
6	-1023/+39	**12**	**-726/+39**	18	-992/+39		
7	-1026/+39	13	-1130/+39	19	-1130/+39		

Table 1

Component	Volume for 50-µL reaction (µ	Final concentration
10x RNA 5' polyphosphatase buffe	5	1x
RNase inhibitor (40 U/µL)	0.75	0.6 U/µL
Mitochondrial RNA	x	0.2 µg/µL
RNA 5' polyphosphatase (20 U/µL)	2.5	1 U/µL
DEPC-treated deionized H2O	to 50 µL	-

Table 2

Component	Volume for 20-µL reaction (µ	Final concentration
10x T4 RNA ligase I buffer	2	1x
dATP (1 mM)	1	50 µM
PEG8000 (50%)	6	15%
RNA inhibitor (40 U/µL)	0.5	1 U/µL
5' polyphosphatase-treated or non-treated mitochondrial RNA	x	0.1~0.2 µg/µL
T4 RNA ligase I (30 U/µL)	0.4	0.6 U/µL
DEPC-treated deionized H2O	to 20 µL	-

Table 3

Component	**Volume for 10-µL pre-mixture (µL)**
Primer mixture (1 µM total)[a]	2
dNTP mixture (10 mM each)	1
Circularized 5' polyphosphatase-treated or non-treated mitochondrial	x
DEPC-treated deionized H2O	to 10 µL

Table 4

Component	**Volume for 20-µL reaction system (µL)**
Template RNA/primer/dNTP*	10
5x RTase buffer	4
RNase inhibitor (40 U/µL)	0.5
RTase (200 U/µL)	1
DEPC-treated deionized H2O	to 20 µL

Final concentration
-
1x
1 U/µL
10 U/µL
-

Table 5 Click here to access/download;Table;Table 5.xlsx

Component	Volume for 20-µL reaction system (µL)	Final concentration
Deionized H2O	7	-
2x reaction buffer	10	1x
dNTP mixture (10 mM each)	0.4	0.2 mM each
26S-CF1 (10 µM)	0.8	0.4 mM
26S-CR1 (10 µM)	0.8	0.4 mM
Templates cDNA derived from circularized 5' polyphosphatase-treated or	0.6	-
DNA polymerase (1 U/µL)	0.4	0.02 U/µL

1

Table 6

Step	Temperature	Time	Cycle number
Initial denaturation	95 °C	3 min	1 cycle
Denaturation	95 °C	15 sec	22-25 cycles
Primer annealing	56 °C	15 sec	
Extension	72 °C	30 sec	
Final extension	72 °C	5 min	1 cycle
Hold	4 °C	∞	-

Table 7

Component	**Volume for 20-µL reaction system (µL)**
Deionized H2O	to 20 µL
2x reaction buffer	10
dNTP mixture (10 mM each)	0.4
Divergent primer-forward (10 µM)	0.8
Divergent primer-reverse (10 µM)	0.8
Template cDNA derived from the circularized 5' polyphosphatase-treated or non-treated RNAs *	x
DNA polymerase (1 U/µL)	0.4

Final concentration
-
1x
0.2 mM each
0.4 mM
0.4 mM
-
0.02 U/µL

Table 8

Step	Temperature	Time	Cycle number
Initial denaturation	95 °C	3 min	1 cycle
Denaturation	95 °C	15 sec	22-40 cycles [c]
Primer annealing [a]	50-65 °C	15 sec	
Extenstion [b]	72 °C	0.5-1 min	
Finatl extension	72 °C	5 min	1 cycle
Hold	4 °C	∞	-

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Acetic acid	Aladdin, China	A112880	To prepare 1x TAE buffer
Applied Biosystems 2720 Thermal Cycler	Thermo Fisher Scientific, USA	4359659	Thermal cycler for PCR amplification
Ascorbic acid	Sigma-aldrich, USA	V900134	For preparation of extraction buffer
Biowest Agarose	Biowest, Spain	9012-36-6	To resolve PCR products and RNAs
Bovine serum albumin	Sigma-aldrich, USA	A1933	For preparation of extraction buffer
Bromophenol blue	Sigma-aldrich, USA	B8026	For preparation of loading buffer for agarose gel electrophoresis and Northern blot
DEPC	Sigma-aldrich, USA	V900882	Deactivation of RNase
DIG Northern starter kit	Roche, USA	12039672910	For DIG-RNA labeling and Northern blot. This kit contains the reagents for transcription-labeling of RNA with DIG and T7 RNA polymerase, hybridization and chemiluminescent detection.
EDTA	Sigma-aldrich, USA	V900106	For preparation of extraction buffer and 1x TAE buffer
EGTA	Sigma-aldrich, USA	E3889	For preparation of wash buffer
Gel documentation system	Bio-Rad, USA	Gel Doc XR+	To image the agarose gel
Glycerol	Sigma-aldrich, USA	G5516	For preparation of loading buffer for agarose gel electrophoresis
GoldView II (5000x)	Solarbio,. China	G8142	DNA staining
Hybond-N+, Nylon membrane	Amersham Biosciences, USA	RPN119	For Northern blot
Image Lab	Bio-Rad, USA	Image Lab 3.0	Image gel, and compare the abundance of PCR products.
KH_2PO_4	Sigma-aldrich, USA	V900041	For preparation of extraction buffer
KOH	Aladdin, China	P112284	For preparation of extraction buffer
L-cysteine	Sigma-aldrich, USA	V900399	For preparation of extraction buffer
Millex	Millipore, USA	SLHP033RB	To sterile extraction and wash buffers by filtration
Miracloth	Calbiochem, USA	475855-1R	To filter the ground kernel tissues
MOPS	Sigma-aldrich, USA	V900306	For preparation of running buffer for Northern blot
NanoDrop	Scientific, USA	2000C	For RNA concentration and purity assay

NaOH	Sigma-aldrich, USA	V900797	For preparation of wash buffer
pEASY-Blunt simple cloning vector	TransGen Biotech, China	CB111	Cloning of the gel-recovered band. It contains a T7 promoter several bps upstream of the insertion site.
Phanta max super-fidelity DNA polymerase	Vazyme, China	P505	DNA polymerase for PCR amplification
Polyvinylpyrrolidone 40	Sigma-aldrich, USA	V900008	For preparation of extraction buffer
Primer Premier 6.24	PREMIER Biosoft, USA	Primer Premier 6.24	To design primers for reverse transcription and PCR amplification
PrimeScript II reverse transcriptase	Takara, Japan	2690	To synthesize the first strand cDNA
PureLink RNA Mini kit	Thermo Fisher Scientific, USA	12183025	For RNA purificaion
RNA 5' polyphosphatase	Epicentre, USA	RP8092H	To convert 5' triphosphate to monophosphate
RNase inhibitor	New England Biolabs, UK	M0314	A component of RNA self-ligation and 5' polyphosphatase treatment reactions, and it is used to inhibite the activity of
Sodium acetate	Sigma-aldrich, USA	V900212	For preparation of running buffer for Northern blot
Sodium chloride	Sigma-aldrich, USA	V900058	To prepare 20x SSC
SsoFas evaGreen supermixes	Bio-Rad, USA	1725202	For RT-qPCR
T4 RNA Ligase 1	New England Biolabs, UK	M0437	For RNA circularization
Tetrasodium pyrophosphate	Sigma-aldrich, USA	221368	For preparation of extraction buffer
TIANgel midi purification kit	Tiangen Biotech, China	DP209	To purify DNA fragments from agarose gel
Tris	Aladdin, China	T110601	To prepare 1x TAE buffer
TRIzol reagent	Invitrogen, USA	15596026	To extract mitochondiral RNA.
Universal DNA purification kit	Tiangen Biotech, China	DP214	To recover linearized plastmids from the restriction enzyme digestion reaction
Xylene cyanol FF	Sigma-aldrich, USA	X4126	For preparation of loading buffer for agarose gel electrophoresis

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Date:	May 05, 2019

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Dear Dr. Zhang,

Your manuscript, JoVE60019R1 "Discrimination and mapping of the primary and processed transcripts in maize mitochondrion using a circular RT-PCR-based strategy," has been editorially reviewed and the following comments need to be addressed. Please track the changes to identify all of the manuscript edits. After revising the submission, please also upload a separate document that addresses each of the editorial comments individually with the revised manuscript.

Your revision is due by **May 21, 2019**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Phillip Steindel, Ph.D.
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Editorial comments:

1. There are still some writing errors. Please proofread, ideally by a native English speaker.
R: The MS was revised carefully, and the errors have been corrected.

2. The PCR steps are still fairly vague, apart from step 7.2. Did you always use the conditions in Table 6?
R: More details are added to the steps of Normalization (step 7) and PCR amplification (step 8). The PCR conditions for 26S rRNA normalization are different from that for PCR amplification of the target transcripts. In the revised MS, the PCR components and conditions for *26S* rRNA are shown in Tables 5 and 6, while those for the target transcripts are given in Tables 7 and 8.

3. 6.2: You mention 5-7 gene specific primers here, but only demonstrate one gene in the results. Should this be 'up to 5-7' primers?
R: Actually, the cDNAs used to map *cox2* were reverse transcribed by a primer mixture containing 26S-CRT, cox2-CRT, nad5-CRT, nad6-CRT, nad7-CRT, nad9-CRT, cob-CRT, and cox1-CRT. The composition of the primer mixture is added in the legend of Figure 4, and the primer sequences are added in Table S1. To avoid any confusion, '5-7 gene specific primers' is changed to "up to 7 RT primers".

4. 8.2: This is vague-how exactly is this used to verify the results?
R: Nested PCR is used to verify the first round PCR results, and more details are given in steps 8.1 to 8.6.

5. 9.1: Which vector did you use?
R: The vector used at step 9.1 is the same as that used at step 10.1. This information is added.

6. After revisions, please ensure the highlighted portion of the protocol is no more than 2.75 pages.
R: Less than 2.75 pages of essential steps in Protocol are highlighted for the video, and we think these steps form a cohesive story of the Protocol.

7. Figure 1: You mention qF1 and qR1 in the legend (as well as Table S1), but it looks to be qCR and QCF in the figure itself; please clarify.
R: We are sorry for the mistake. 'qF' and 'qR' were changed to 'qCF' and 'qCR', respectively.

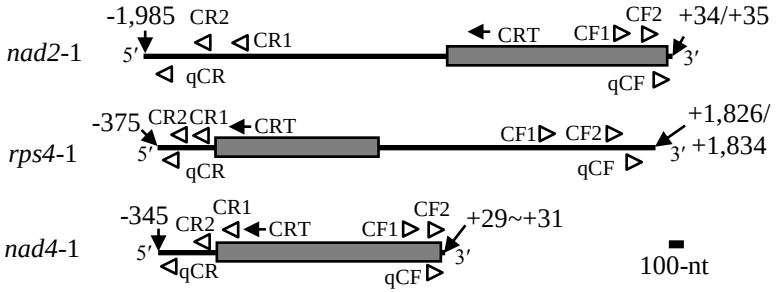

Figure S1: Position of primers for representative maize mitochondrial transcripts. CRT: reverse transcription primer; CF1, CF2, CR1, and CR2: divergent primers for PCR amplification; qCF and qCR: divergent primers for RT-qPCR. The transcript termini of maize *nad2*-1, *rps4*-1, and *nad4*-1 have been determined previously (Zhang *et al*., 2019). Positions of 5'- and 3'-ends relative to AUG (+1) and the last nucleotide of stop codon (-1) are shown. The coding regions and UTRs are indicated as gray boxes and bold lines, respectively.

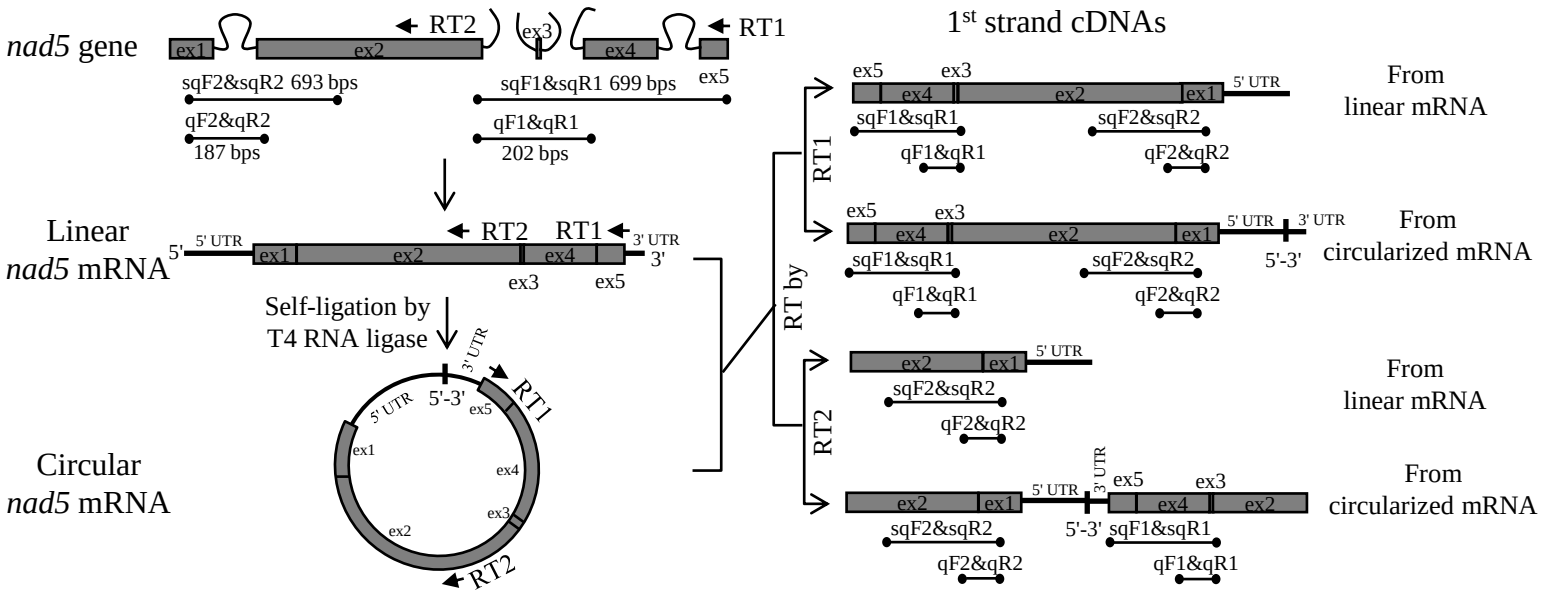

Figure S2: Principle to estimate circularization efficiency of *nad5* mRNA in maize. In a self-ligation reaction, only a fraction of mitochondrial RNAs is circularized. To calculate the ratio of circularized *nad5* mRNA, two gene-specific primers are used to synthesize the first strand cDNAs, i.e. *nad5*-RT1 and -RT2. In *nad5*-RT2 reverse transcription (RT) reaction, the PCR products amplified by sqF2&sqR2 (for RT-sqPCR) and qF2&qR2 (for RT-qPCR) are derived from both linear and circularized *nad5,* while the sqF1&sqR1 (for RT-sqPCR) and qF1&qR1 (for RT-qPCR) PCR products are derived from circularized *nad5* only; in *nad5*-RT1 reaction, all four pairs of PCR primers could amplify both forms of *nad5*. To calculate the ratio of circularized *nad5* mRNA, the two reactions are normalized by sqF2&sqR2 or qF2&qR2 PCR products. By comparing the abundance of sqF1&sqR1 or qF1&qR1 PCR products between the two RT reactions, the circularization efficiency of *nad5* mRNA is roughly estimated. ex: exon. Exons and introns are shown as gray boxes and curved lines, respectively. The positions of *nad5*-RT1 and -RT2 primers are indicated by arrows. Black dots indicate the positions of PCR primers, and the predicted size of the PCR products is shown.

Table S1: Primer information.

Primer name	Primer sequences (5'-3')	Use of Primer
cox2-CRT	TCATAGGTGTTGCTGCGTC	RT primers to prepare the cDNAs for normalization by *26S* rRNA and mapping of *cox2* transcript termini
26S-CRT	GAGGAATACTTAGGCTTAGAGG	
nad5-CRT	TCACTACGGTCAGGCTATC	
nad6-CRT	ATTGAACATCATAACCACGA	
nad7-CRT	GCTGAAGAATGAGCGTGTTC	
nad9-CRT	GATCGAAACTTGAACCCTTG	
cob-CRT	ACAACTCCGAGACACCAAAC	
cox1-CRT	TCCACGCATGTTGAAGATAG	
cox2-CF1	GTCGTTCAAATCTTACCTCCAT	cRT-PCR amplification of cox2-1 and -2
cox2-CF2	GCCTATCGTCGTAGAAGCAG	cRT-PCR amplification of cox2-1 and -2
cox2-CF3	CTGAAGCGGAAATGCA	cRT-PCR amplification of cox2-3 and -4
cox2-CR1	CAAAGAGCGATTGTGAGG	cRT-PCR amplification of cox2-1 and -2
cox2-CR2	AGCCAGGGTCCCATAAC	cRT-PCR amplification of cox2-3 and -4
cox2-qCF1	CTGAAGCGGAAATGCA	RT-qPCR amplification of cox2-1 to -3
cox2-qCF2	TTTAAGGCCGACCACTAC	RT-qPCR amplification of cox2-4
cox2-qCR1	GGGCTCGTCCTGTATCA	RT-qPCR amplification of cox2-1
cox2-qCR2	CTTTGTATCTGTGCTATTTCG	RT-qPCR amplification of cox2-2
cox2-qCR3	GGAGACTGAACACCGACAC	RT-qPCR amplification of cox2-3 and -4
26S-CF1	TCGCCGATGAAAGTGG	cRT-PCR amplification of *26S* mature rRNA
26S-CR1	CCAATCCACAACAAATCGA	
26S-qCF1	TGGTATGGAAGAACTGCTG	RT-qPCR amplification of *26S* mature rRNA
26S-qCR1	CAAAGAGCGCAGACTAGC	
cox2-probeF	GAATTCCAGCCATTACTATCAAAGC	To amplify the DNA fragment for preparation of *cox2* RNA probe
cox2-probeR	CCAATCCGCATAATCTTTC	
M13F	TGTAAAACGACGGCCAGT	Vector primers for colony PCR to screen positive clones containing the target inserts
M13R	CAGGAAACAGCTATGAC	
nad5-RT1	GTCCTGGCAAGCTCCTACA	To calculate the circularization efficiency of *nad5* mature mRNA by RT-sqPCR and RT-qPCR
nad5-RT2	CCGAACCCGCACTCAG	
nad5-sqF1	CATTCGGGCGAGACAG	
nad5-sqF2	GGATCTGAAGGAACCGCT	
nad5-sqR1	GTCCTGGCAAGCTCCTACA	
nad5-sqR2	GCCAACCTCCTGGAAAGAG	
nad5-qF1	CATTCGGGCGAGACAG	
nad5-qF2	GGATCTGAAGGAACCGCT	
nad5-qR1	GTTGGAGCAGCAAACTCG	
nad5-qR2	GATAGCCTGACCGTAGTGA	
nad1-RT1	GCCCCCTTCAGAAGAAACTT	To calculate the circularization efficiency of *nad1* mature mRNA by RT-sqPCR and RT-qPCR
nad1-RT2	CTCGAATTACAGGGACCTAC	
nad1-sqF1	GGTTATGTTCCTTATTCCTCGTC	
nad1-sqF2	GGCCCGATCATGAGTGAATA	
nad1-sqR1	GCCCCCTTCAGAAGAAACTT	
nad1-sqR2	ACCATTTGAGCTGCAGATCG	
nad1-qF1	GGTTATGTTCCTTATTCCTCGTC	
nad1-qF2	GCTACATTTATGTTAAGTCTGG	
nad1-qR1	GCCAACCTCCTGGAAAGAG	
nad1-qR2	ACCATTTGAGCTGCAGATCG	