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A convenient and efficient protocol for isolating high-quality RNA from latex of *Hevea brasiliensis* (para rubber tree)

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Abstract

Isolating high-quality RNA from latex of *H. brasiliensis* is a prerequisite to elucidating the molecular mechanisms of rubber biosynthesis and its regulation. Here, an improved protocol was developed for latex collection, transportation, storage, and RNA isolation. Compared with existing ones, our protocol eliminated liquid nitrogen for latex collection and subsequent low-temperature (−70 °C) condition for latex storage, making it more convenient and feasible when latex was collected in remote sampling sites, and latex storage and RNA isolation were conducted in poorly-equipped laboratories. Different methods (UV absorbance scans, denaturing gel electrophoresis, autoradiograph monitoring of cDNA synthesis) were used to confirm the high quality of the RNA prepared with this protocol, whose usefulness was further verified by several practical applications, including construction of one high-quality cDNA library, cloning of the full-length cDNAs of 3 novel *Hevea* sucrose transporter genes, and semi-quantitative RT-PCR analysis of two rubber-biosynthesis essential genes and one sucrose transporter gene.

Keywords: *Hevea brasiliensis*; Latex; RNA isolation; RNA quality; Liquid nitrogen; Ultra-low temperature

1. Introduction

Natural rubber (NR) (*cis*-1,4-polyisoprene) is a raw material of great importance in commercial, defense, and transportation industries. Due to its incomparable elasticity, resilience, and resistance to high temperature, NR is still the largest type of elastomers being consumed globally. Presently, more than 98% of commercial source of NR comes from a single tropical tree species—*Hevea brasiliensis* (para rubber tree), mainly due to its abundance, high quality, and the convenience for harvest. As the increasing demands of NR in the world market elucidating the molecular mechanisms of rubber biosynthesis and its regulation become more urgently [1,2].

In *H. brasiliensis*, rubber synthesis takes place on the surface of rubber particles suspended in the latex, which contains 30–50% (W/W) rubber, and constitutes the milky cytoplasm of the specialized cell type-laticifer. Due to the lack of cytoplasmic connections of plasmodesmata between the laticifer network and their neighboring cells [3], latex contains the complete sets of transcriptome and proteome required for rubber synthesis and latex metabolism. Therefore, latex has become an ideal material for investigating the molecular mechanisms of rubber biosynthesis [4–7].

Isolation of high-quality RNA from latex is the first step for unraveling the molecular events that occurred in the laticiferous system of *H. brasiliensis*. The presence of ribosomes and RNAs (ribosomal RNA, messenger RNA and transfer RNA) in latex has been demonstrated for a long time [8,9]. However, due to the problems of latex coagulation and presence of active RNase, the isolation of high-quality RNA had been unsuccessful until Kush et al. [4] modified the method of Prescott and Martin [10], and made it a successful protocol for extracting latex RNA. The protocol described by Kush et al. [4] and its modified versions had been used in many researches ranging from full-length cDNA isolation [5,11], gene expression and regulation analysis [12], to latex transcriptome analysis [6]. However, all of these protocols require using liquid nitrogen in latex collection and ultra-low temperature (−70 °C) for subsequent latex storage. The storage, utilization and transportation of liquid nitrogen are not only expensive and inconvenient, but hazardous when not being properly manipulated. It's especially the case when

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sampling is conducted in remote and underdeveloped regions, where some precious *Hevea* materials always are. On the other hand, maintaining the storage conditions of −70 °C is both costly, and cannot be met in some labs.

Based on existing protocols, especially that described by Kush et al. [4], we developed an efficient protocol for isolating RNA from latex without usage of liquid nitrogen and ultra-low temperature conditions. RNA quality was examined by several methods (e.g. UV absorbance scans, formaldehyde denaturing agarose gel electrophoresis, autoradiograph monitoring of cDNA synthesis) and further confirmed by several practical applications (semi-quantitative RT-PCR, cDNA cloning and cDNA library construction).

2. Materials and methods

2.1. Plant materials

Eight-year-old *H. brasiliensis* trees (clone Reyan7-33-97), growing at an experimental plantation of the Rubber Research Institute of Chinese Academy of Tropical Agricultural Sciences (Danzhou, Hainan), were used for latex collection. These trees were planted in 1998, opened in 2005, and tapped in a half spiral, every 3 d, and no stimulation system (1/2S, d/3).

2.2. Latex collection, storage, and total RNA extraction

To improve the existing protocol described by Kush et al. [4], we designed 4 protocols as follows.

2.2.1. C+PCI

All solutions except Tris buffer were treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved. Autoclaved 0.1% DEPC-treated water (AD-H_2O) was used for preparation of Tris buffers. Glassware was baked for 4 h at 180 °C, and plasticware was 0.1% DEPC-treated and autoclaved. The extraction buffer (EB) was prepared by adding solid Tris to the solution (300 mM LiCl, 10 mM disodium salt EDTA, 10% (W/V) SDS) at a final concentration of 100 mM, with no pH adjustment (with a final pH value of approx. 9.7). *Hevea* trees were tapped using a tapping knife, and 20 s after tapping, about 5.0 mL of latex was allowed to drop directly into a 50-mL polypyropylene Falcon® tube containing 5.0 mL of EB placed on ice. The tubes were briefly shaken and taken on ice to the laboratory for RNA extraction.

Ten milliliters of water-saturated phenol/chloroform/isoamyl alcohol (PCI) (25:24:1) was added to the latex mixture and vigorously shaken. The mixture was stored at 2–6 °C for different days (3, 7, 10, 14) prior to RNA extraction or for immediate use (0 d). For storage, the tubes were vigorously shaken once each day. The mixture was centrifuged at 12,000 ×*g* for 10 min at 4 °C. Collect the aqueous phase, extract with an equal volume of PCI (25:24:1), and centrifuge at 12,000 ×*g* for 5 min at 4 °C. The supernatant was then extracted with an equal volume of chloroform/isoamyl alcohol (24:1), and centrifuged at 12,000 ×*g* for 5 min at 4 °C. Removing the supernatant to a new tube, 8 M LiCl solution was added to a final concentration of 2 M. The tube was placed at −20 °C overnight and centrifuged at 12,000 ×*g* for 30 min at 4 °C. The pellet was dissolved in 0.5 mL TE (pH7.6). Repeating the LiCl precipitation step, the pellet was re-dissolved with 100 μL of AD-H_2O. 10 μL of 3 M NaAc (pH 5.2) and 300 μL of absolute alcohol were added, mixed and placed at −20 °C for 2 h. Centrifuge at 12,000 ×*g* for 30 min at 4 °C, and completely discard the aqueous phase. The pellet was washed with 1 mL of 75% ethanol, and collected by centrifugation at 12,000 ×*g* for 10 min at 4 °C. Repeating the ethanol wash step, the pellet was dried for 10 min at room temperature, and dissolved with 20 μL of AD-H_2O, and stored at −70 °C.

In this protocol, latex collection and storage were done under Cooling temperatures (0–6 °C), and the PCI was added into latex mixture for latex storage; therefore we named this protocol "C+PCI".

2.2.2. RT+PCI

This protocol was similar to "C+PCI", except latex was collected and subsequently stored at room temperature.

2.2.3. C

This protocol was similar to "C+PCI", except that for subsequent storage, no PCI was added to the latex mixture.

2.2.4. RT

This protocol was similar to "C", except that latex collection and subsequent storage were done at room temperature.

2.3. Determination of RNA purity and yield by UV absorbance

RNA absorption was scanned with a spectrophotometer at 230 nm, 260 nm, 270 nm, and 280 nm. The ratios of A260/A280, A260/A230, and A260/A270 were calculated to evaluate RNA purity. RNA concentration was determined by the empirical formula described by Glasel [13], and the RNA yield (μg/g latex) was calculated according to the following equation:

$$\text{RNA yield}\,(\mu g/g\ \text{latex}) = \frac{\text{RNA volume}\,(\mu L) \times \text{RNA concentration}\,(\mu g/\mu L)}{\text{Latex weight}\,(g)}.$$

2.4. Evaluation of RNA integrity by denaturing agarose gel electrophoresis

RNA sample of 10 μg was separated by gel electrophoresis using a 1.2% formaldehyde denaturing agarose gel [14]. The intactness of ribosomal RNA bands and the intensity ratio of 28S and 18S rRNA were determined to evaluate total RNA integrity [15].

2.5. Monitoring cDNA synthesis from total RNA by autoradiograph

cDNA synthesis was performed using the RevertAidTM First Strand cDNA Synthesis Kit of Fermentas (V.A.Graiciuno g.8, LT-2028 Vilnius, Lithuania) according to the manufacturer's