Efficient protocol for isolation of high-quality RNA from tea without using liquid nitrogen for molecular analysis

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A good quality RNA is an important prerequisite for any downstream molecular analysis. However, isolation of quality RNA from tea is difficult due to presence of high amount of phenols and polysaccharides. We describe here an efficient protocol for total RNA isolation without liquid nitrogen from apical buds and tender leaves of tea. This protocol yielded RNA 1248±23 µg/g of fresh leaf tissue with high purity. The RNA was also found to be suitable for mRNA purification, cDNA synthesis and cloning through RT-PCR (reverse transcription-polymerase chain reaction) and cDNA library construction.

Keywords: cDNA library, polyphenols, RNA, mRNA, RT-PCR, Tea

Introduction
Tea, one of the world’s cheapest and most popular non-alcoholic beverage, is an important source of revenue for the major tea growing countries of the world including China, India, Sri Lanka and Kenya. Isolation of high quality RNA is crucial for gene transcript analysis. Therefore, it is essential that RNA preparation methods must produce RNA free of ribonuclease and DNA. Tea leaves contain high levels of polyphenols1, which are released on disruption of cells. These polyphenolic compounds when oxidized give rise to the formation of quinones2, which bind to the nucleic acids and thus making them unsuitable for molecular techniques. This is the major bottleneck in exploring the transcription profile in tea crop. Though RNA isolation has been previously reported on tea3,4, but all these protocols involved the use of liquid nitrogen. Moreover, the availability of liquid nitrogen all the time at all places can be under question.

Since tea being high polyphenol containing plant, the isolation of RNA for downstream application demands the formulation and evaluation and of a new optimized protocol. Therefore, we report here a reliable and reproducible protocol without liquid nitrogen to produce good quality RNA from leaf tissues of tea that can be used for downstream applications like mRNA isolation, RT-PCR, cDNA library construction and screening etc. This protocol would also save cost as it does not require the use of liquid nitrogen.

Materials and Methods

Plant Material
The apical shoot buds and tender leaves of Assam type clone TV-1 were collected from the Experimental Tea Garden, Assam Agricultural University, Jorhat, Assam, India.

Solutions and Reagents
Solutions and reagents used in the study were: extraction buffer with pH 7.0 [8 M guanidine hydrochloride, 25 mM EDTA, 20 mM MES, 2% PVPP (w/v), 5% BME (v/v), 2.0 M NaCl]; Tris saturated phenol:chloroform:isoamyl alcohol (PCI) (25:24:1, v/v/v); chloroform:isoamyl alcohol (CI) (24:1, v/v); 8 M LiCl; absolute ethanol and 70% ethanol; 3 M sodium acetate (pH 5.2); 0.1% DEPC treated autoclaved water. Glasswares, mortar and pestle were baked overnight at 180°C. Pipette tips and the plastic wares were treated with 0.1% DEPC-treated double-distilled water and autoclaved. The electrophoresis apparatus was

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Abbreviations: BME, β-Mercapto ethanol; CI, Chloroform: Isoamyl alcohol; DEPC, Diethylpyrocarbonate; H2O2, Hydrogen peroxide; PCI, Phenol:Chloroform:Isoamyl alcohol; PVPP, Polyvinyl polypyrrolidone; RT-PCR, Reverse transcription-polymerase chain reaction
treated with 3% H$_2$O$_2$ and rinsed with DEPC-treated autoclaved water.

**Protocol**

The present protocol is optimized by modifying the protocol of two previous methods$^{3,4}$ and developed for specifically purifying only RNA with selective precipitation using LiCl and grinding the plant tissue in presence of large volume of extraction buffer. Presence of high phenol in the tea leaves has been given special attention while optimizing the method by modifying the component of extraction buffer.

The plant material (1 g) was ground with 10 volumes of homogenization buffer containing 5% BME and 2% PVPP until a homogenous mixture was obtained. An equal volume of CI was added to the mixture, which was subsequently vortexed followed by centrifugation for 10 min at 9000×g at 4°C. The upper aqueous phase was then transferred to a new Oak Ridge tube and an equal volume of PCI was added, which was subsequently vortexed followed by centrifugation for 10 min at 9000×g at 4°C. The upper aqueous phase was then transferred to a cortex tube and 0.2 volume of 8 M LiCl was added to it to specifically precipitate RNA. The mixture was then incubated overnight at –20°C or at –80°C for 3 h. RNA was pelleted down by centrifugation for 10 min at 9000×g at 4°C. Washing with 3 M sodium acetate was repeated twice. Further washing of the pellet was done with 70% ethanol (v/v), followed by centrifugation for 5 min at 9000×g at 4°C. Washing with 3 M sodium acetate was repeated twice. Finally, the RNA pellet was dried at room temperature and dissolved in 100 µL of DEPC-treated autoclaved water.

The present protocol for isolation of RNA was also compared with other standard protocols, viz., Trizol (Invitrogen, California) method, Plant RNA Kit (Genei, India) and RNA-X Press™ Kit (HiMedia Laboratories, Mumbai, India). The efficiency of the present protocol was compared with the other standard methods as per manufacturer’s instruction provided in the kits with respect to yield, purity and integrity of the isolated RNA.

**Determination of RNA Quantity and Quality**

The purity and concentration of the isolated RNA was assessed by monitoring the absorbance at 230, 260 and 280 nm in a Nanodrop Spectrophotometer (NanoDrop 1000, Thermo Scientific, USA). Integrity of the isolated RNA was evaluated by electrophoresis with 5 µg RNA at 5-6 V/cm on a 1% denaturing agarose gel containing formaldehyde. Samples were stored at –80°C for further use. Polyadenylated (polyA$^+$) mRNA was purified from the total RNA preparation using PolyATract mRNA Isolation System (Promega, USA) following manufacturer’s instruction. The yield and purity of the isolated mRNA was also calculated by measuring the absorbance at 260 nm and 280 nm. Applicability of the isolated mRNA was further evaluated by RT-PCR.

**cDNA Synthesis**

First strand cDNA synthesis was carried out using 1 µg of mRNA using BluePrint First Strand cDNA Synthesis Kit (Clontech Laboratories inc., USA) according to manufacturer’s instructions. First strand cDNA was then subsequently subjected to RT-PCR with degenerate oligonucleotide primers for actin genes. The primers were designed using Primer3 tool (Forward primer: GCCGCCCTTAACGGGGACTGG, and Reverse primer: AGTACGACGAGTCCGGCCCCT). The PCR profile used was initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 5 min. Amplified products from RT-PCR were then resolved in 1% agarose gel. Subsequently, double stranded cDNA was also synthesized.

**cDNA Library Construction**

A full length cDNA library was constructed using SMART cDNA Library Construction kit (Clontech Laboratories Inc., CA, USA) following the manufacturer’s instruction. The cDNA thus produced was then ligated into λTriplEx2 vector and converted to pTriplEx2 by site specific recombination.

**Results**

**Assessment of Quality and Quantity of Extracted RNA**

The RNA isolated according to the present modified protocol was tested for yield and purity, and its ability to be amplified by RT-PCR and used for cDNA library construction. The yield of RNA using the present protocol was found to be approx 1248±23.115 µg/g of fresh tissue. The RNA yield with the present protocol was found to be much higher compared to that reported earlier.$^{3,4}$ A$_{260}$/A$_{280}$ ratio of isolated RNA was also within the acceptable
range of 2.06±0.008, indicating RNA purity without any contamination of proteins. A$_{260}$/A$_{230}$ ratio was also found to be 1.92±0.15, indicating it was free of polysaccharide and polyphenolic contamination.

The comparison of present modified protocol with those of other methods revealed that present method exhibited more yield of RNA with high purity. RNA yield using Trizol method, RNA Xpress Kit and Plant RNA kit was found to be 748±6.324, 445±8.147 and 321±4.711 µg/g of tissue, respectively (Table 1). Integrity of the RNA band in the gel using the present method was also high as compared to other tested protocols. Although extraction efficiency decreases in absence of liquid nitrogen but the present protocol could yield RNA with high yield and purity. Denaturing agarose gel electrophoresis of RNA sample showed clear distinct bands at 23S and 16S corresponding to mol wt 5 and 1.8 kb, respectively, suggesting no apparent RNA degradation (Figs 1a & b).

mRNA Purification and RT-PCR

The yield of poly (A)$^+$ RNA was found to be 36 µg/1.248 mg of RNA sample. A$_{260}$/A$_{280}$ ratio was found to be 1.84, indicating good quality of the isolated mRNA. The clearest evidence for RNA quality and purity was provided by RT-PCR conducted on first strand cDNA designed from mRNA using oligonucleotide primers for the actin gene. An expected 350 bp DNA fragment was detected in the RT-PCR when RNA isolated without liquid nitrogen was used as a template for subsequent mRNA purification and first strand cDNA synthesis (Fig. 1c).

cDNA Synthesis and cDNA Library Construction

To evaluate the suitability of isolated RNA in downstream enzymatic procedures, the isolated RNA from tea was used for 1st strand and 2nd strand cDNA synthesis (Fig. 2a & b) and subsequently for cDNA library preparation. An efficiency of 1.4x10$^6$ pfu/mL of packaged cDNA library was obtained, in which recombinant clones comprised of about 80.71%, as determined by colour selection with IPTG and X-gal, which can be considered as a good representational primary library size for gene expression analysis study.

Discussion

RNA yield (1248±23.115 µg/g) and quality (2.06±0.008) recorded using the present modified method was comparable to earlier report$^3$ of RNA yield in tea, wherein yield of 1304 µg/g was recorded using liquid nitrogen and 200 mM BME. In another report of RNA isolation from tea using liquid nitrogen, the yield was found to be 1220±0.80 µg/g$^7$, which is at par with the present protocol. Other methods tested in the present study failed to recover good quality intact RNA as revealed by agarose gel

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Table 1—Comparison of total RNA yield and purity by different methods

<table>
<thead>
<tr>
<th>Methods</th>
<th>A$<em>{260}$/A$</em>{280}$</th>
<th>A$<em>{260}$/A$</em>{230}$</th>
<th>Mean RNA yield (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current method</td>
<td>2.06±0.008</td>
<td>1.92±0.15</td>
<td>1248±23.115</td>
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<td>Trizol method</td>
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<td>1.24±0.23</td>
<td>748±6.324</td>
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<td>RNA-X Press Kit</td>
<td>1.26±0.006</td>
<td>1.11±0.34</td>
<td>445±8.147</td>
</tr>
<tr>
<td>Plant RNA kit</td>
<td>1.74±0.017</td>
<td>1.66±0.42</td>
<td>321±4.711</td>
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Fig. 1 (a-c)—Electrophoretic pattern of RNA isolated from leaf tissues of tea using different protocols: (a) Present modified method without liquid nitrogen [M: RNA ladder (Fermentas); Total RNA isolated (lanes 1 & 2)]; (b) Trizol method [Total RNA isolated (lane 1); Plant RNA kit (Genei, India) [Total RNA isolated (lane 2)]] & RNA-Xpress Kit (HiMedia, India) [Total RNA isolated (lane 3)]; & (c) Agarose gel electrophoresis of RT-PCR product from first strand cDNA synthesized from mRNA following total RNA isolation using present modified method without liquid nitrogen [lane M1, 100 bp DNA ladder (Genei, India); lane 2, amplified actin gene 350 bp; & lane M2, 500 bp DNA ladder (Genei, India)].

Fig. 2—Synthesis of cDNA using LD-PCR (Long Distance PCR): (a) 1st strand cDNA (lane 1) & ladder 100 bp (Invitrogen) (lane 2); (b) ladder 100 bp (Genei, India) (lane 1) & 2nd strand cDNA (lane 2).
analysis (Figs 1a & b). Use of large volume of extraction buffer in our modified protocol helped in better extraction and maceration of the tissue in the absence of liquid nitrogen. Effect of increased volume of extraction buffer was found to be suitable in extraction of RNA from secondary metabolite rich plants as reported in earlier studies\textsuperscript{1,8}. BME and PVPP have a great role to play during isolation of nucleic acid either alone or in combination especially in plants with high phenolics. In the present protocol, use of both in combination resulted in higher recovery of RNA as tea plant contains high polyphenols. Addition of BME and PVPP during crushing and extraction was found to be useful in preventing oxidation of phenolics as reported in Jute\textsuperscript{8}. Protocol for extraction of RNA without liquid nitrogen has been used for Mangrove\textsuperscript{1}, wherein they have recorded total RNA 20-40 µg/g fresh tissue.

In conclusion, our results showed that RNA prepared by the present modified method was of good quality and served as a robust template for RT-PCR amplification from cDNA for the actin gene and high titer cDNA library. The protocol described here was convenient, fast and simple method for isolating RNA without liquid nitrogen from tea leaf-tissues with high phenol content.

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References