An Improved Method of Isolation of High Quality Total RNA from Purple-Fleshed Sweet Potato, *Ipomoea batatas* (L.) Lam

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Abstract: To isolate high quality total RNA from plants of purple-fleshed sweet potato (*Ipomoea batatas* L. Lam) is difficult because these plants contain high levels of carbohydrates and pigment compounds. In this study, we developed a quick and reliable method for total RNA extraction. Using this extraction protocol, which was derived from the traditional CTAB method with modification, we obtained high quality total RNA from all the tested tissues including leaf blade, petiole, stem, fibrous root, thick root, and storage root. Spectrophotometric analysis, together with agarose gel electrophoresis analysis of extracted total RNA, indicated that the total RNA was perfectly intact and the yields were all more than 0.2 mg/g fresh weight of different tissues. A metallothionein-like protein gene (*G14*) fragment was successfully amplified by reverse-transcription-PCR and cDNAs of two target genes (*F3′H* and *DFR*) were isolated by the method of 3′-RACE and 5′-RACE using the first-strand 3′-RACE-ready and 5′-RACE-ready cDNA as templates which were synthesized from the total RNA extraction, indicating that the total RNA extraction was suitable for further molecular analysis.

Keywords: Isolation, Purple-fleshed, RNA, RT-PCR, Sweet potato (*Ipomoea batatas* L. Lam)

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INTRODUCTION

Sweet potato (*Ipomoea batatas* L. Lam.), the world’s seventh most important crop, is valuable. Purple-fleshed sweet potato is a special new kind of cultivar containing rich anthocyanins that can be used as edible pigment for food industry.[1]

In addition, the anthocyanins have multiple physiological functions, such as strong antioxidative activity,[2] antimutagenicity,[3] antihyperglycemic effect,[4] and hepatoprotective effects.[5] Because of these characteristics, purple-fleshed sweet potato has attracted worldwide interest in biomedical and plant science research. However, molecular research on this plant is relatively limited, because tissues of purple-fleshed sweet potato contain considerable amounts of polysaccharides, starch, and many other pigment compounds that have negative impact on the isolation and reduce the quality of the total RNA. You et al.[6] and Sun-Hyuntec K et al.[7] reported that total RNA had been isolated from different tissues of sweet potato by using guanidinium/SDS lysis buffer-CsCl gradient and a modified CTAB method, respectively. But, these methods were not satisfactory enough for high quality total RNA isolation from the purple-fleshed cultivars which contained even higher levels of polysaccharides and pigmented compounds than the common sweet potato. In the purple-fleshed sweet potato, polysaccharides and starch are usually co-precipitated with RNA in the alcohol precipitation process, resulting in a viscous RNA-polysaccharide mixture solution. In addition, phenolic compounds from the tissues of the purple-fleshed sweet potato are readily oxidized to form covalently linked quinines that avidly bind to nucleic acids.[8]

In this study, we report a modified procedure for RNA isolation from the purple-fleshed sweet potato cultivars. The results indicate that this approach is a quick, efficient, and reliable method for total RNA isolation from this species.

EXPERIMENTAL

Plant Material

Plants of the purple-fleshed sweet potato (*Ipomoea batatas* L. Lam. cv. Yamakawamurasaki) were grown in an experimental field at South China Normal University. Different tissues, including leaf blade (LB), petiole (PT), stem (ST), fibrous root (FR, Maximum diameter <2 mm), thick root (TR, 2 mm < Maximum diameter <5 mm), and storage root (SR, Maximum diameter >5 mm), were separately harvested for RNA isolation. All samples were snap-frozen in liquid N2 and stored at −80°C until use.
Extraction Buffer and Solutions

The extraction buffer used in this study included 2% (W/V) cetyltrimethylammonium bromide (CTAB), 100 mM Tris/HCl (pH 9.5), 1.4 M NaCl, 20 mM EDTA, 5% PVP (W/V), and 4% (V/V) β-mercaptoethanol (added just before use); chloroform/isoamyl alcohol (24:1 V/V); 10 mM LiCl; 3 M NaAc (pH 5.6); ethanol (70% and 100% V/V). All solutions were prepared with 0.1% (v/v) diethylpyrocarbonate (DEPC) and incubated at 37°C overnight before autoclaving (121°C, 40 min), except the Tris/HCl, which was prepared with DEPC-treated water.

RNA Extraction Procedure

The fresh tissues (1 g for each tissue) were ground to very fine power with a pestle and added to 10 mL extraction buffer (preheated at 65°C). After incubation at 65°C for 15 min with occasional shaking to mix the samples, an equal volume of chloroform/isoamyl alcohol (24:1, V/V) was added to the mixture, shaken vigorously for 30 s and placed on the ice for 15 min. The extract was then centrifuged at 13,000 g for 15 min at 4°C. The supernatant was transferred to a new tube, 1/4 volume of 10 M LiCl was added, mixed thoroughly, and stored at −70°C for 1 h. The extract was centrifuged at 13,000 g at 4°C for 15 min to obtain an RNA pellet, which was air-dried at room temperature for 10 min. The RNA pellet was dissolved in 1 mL DEPC-treated water and suspended with one equal volume of chloroform/isoamyl alcohol (24:1, V/V). The extract was mixed well and placed on the ice for 15 min. It was centrifuged at 13,000 g at 4°C for 15 min. The chloroform/isoamyl alcohol extraction and centrifugation procedure were repeated for two or three times until the interface of the extract was clear. The supernatant was then transferred to a new tube, to which 2 volumes of absolute ethanol (pre-cooled at −20°C) and 3 M NaAc (pH 5.6) (pre-cooled at −20°C) were added and mixed gently. The mixture was then stored at −70°C for at least 1 h. After centrifugation at 13,000 g at 4°C for 15 min, the RNA pellet was washed with 70% cold ethanol (V/V) for at least twice to eliminate residuum and then air-dried. The RNA was suspended in 50 μL DEPC-treated water and stored at −70°C until use.

Reverse-Transcription (RT)-PCR and cDNA Synthesis for PCR Amplification

First strand cDNA synthesis was performed with 1 μg of RNA in a final volume of 10 μL by using the SMART PCR cDNA Synthesis Kit (BD
Clontech, Mountain View, CA, USA) according to the manufacturer’s instructions. One to three microliters of cDNA (equivalent to 0.1 to 0.3 μg of RNA as indicated in the results) was used in a PCR reaction. Amplification was performed in a total volume of 25 μL under the following conditions: 50°C for 30 min, 94°C for 2 min followed by 25 cycles of 94°C for 50 s, 50°C for 50 s, 72°C for 90 s. The RT-PCR reaction for cDNA of the metallothionein-like protein gene (GI4 gene), which was used as internal control, was performed by using specific primers FG14 (5'-ATGTCCGACAAGTGCGGAACTGC-3') and RG14 (5'-TTAGTG GarcAGGTCGTCGTA-3'). The opening reading fragments (ORF) of cDNAs of the two genes (F3'H and DFR) were amplified using the first-strand 3'-RACE-ready cDNA as templates which were synthesized from the total RNA extraction. PCR products (10 μL) were separated on 1% agarose gels, which were then stained with ethidium bromide after electrophoresis.

RESULTS

RNA Purity and Yield

Using the procedure described above, more than 0.2 mg total RNA could be obtained from 1 g (FW) samples from different tissues of the purple-fleshed sweet potato. As shown in Table 1, purities and yields of RNA were varied depending on the tissues used. The yields ranged from 201.8 to 245.2 μg/g(FW). The total RNA yield of leaf blade was higher than that of other tissues, while fibrous root generated the lowest yield. A₂₆₀/A₂₃₀ ratios were greater than 2 in all the samples, ranged from 2.59 to 3.60, indicating that the RNA was only slightly contaminated by polysaccharides or polyphenols. A₂₆₀/A₂₈₀ ratios ranged from 1.90

<table>
<thead>
<tr>
<th>Tissue</th>
<th>A₂₆₀/A₂₃₀</th>
<th>A₂₆₀/A₂₈₀</th>
<th>Yield (μg/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf blade</td>
<td>3.25</td>
<td>1.92</td>
<td>245.2</td>
</tr>
<tr>
<td>Petiole</td>
<td>3.57</td>
<td>1.96</td>
<td>237.1</td>
</tr>
<tr>
<td>Stem</td>
<td>2.59</td>
<td>1.90</td>
<td>212.6</td>
</tr>
<tr>
<td>Fibrous root</td>
<td>3.43</td>
<td>1.97</td>
<td>201.8</td>
</tr>
<tr>
<td>Thick root</td>
<td>3.60</td>
<td>2.12</td>
<td>232.7</td>
</tr>
<tr>
<td>Storage root</td>
<td>2.87</td>
<td>2.07</td>
<td>219.3</td>
</tr>
</tbody>
</table>

Table 1. Analysis of RNA quality and yield by spectrophotometric analyses
to 2.12, indicating that little or no protein contamination was present in the RNA samples.

**Integrity of the Total RNA**

When 1% agarose gel electrophoresis was applied to check the integrity of all extracted total RNA from different tissues, distinct 28S, 18S, and 5.8S ribosomal RNA bands (Figure 1) were found in ethidium bromide staining and the brightness of 28S was nearly twice as much as that of 18S.

**RT-PCR Analysis and PCR Amplification**

To further evaluate the integrity of extracted RNA, total RNA was examined by RT-PCR and gene cloning analysis. A cDNA fragment (195 bp) of *G14* gene that encodes a metallothionein-like protein was amplified (Fig. 2). Two full-length cDNAs of the key genes (*F3’H* and *DFR*) in the pathway of anthocyanidin synthesis were amplified successfully by RT-PCR and then 5’- and 3’-RACE. Besides, the opening reading fragments (ORF) of cDNAs (1554bp and 1185bp) of the two genes (*F3’H* and *DFR*) were successfully amplified.
and $DFR$) were successfully amplified using the first-strand $3'$-RACE-ready cDNA as templates which were synthesized from the total RNA extraction (Figure 3). The amplification products were confirmed by sequencing and homology alignment (Table 2).

**Figure 2.** RT-PCR amplification products of $Gl4$ gene using the various total RNA samples (M, DNA marker; SR, storage root; TR, thick root; FR, fibrous root; ST, stem; PT, petiole; LB, leaf blade).

**Figure 3.** Electrophoresis of PCR product used first-strand $3'$-RACE-ready cDNA as templates which were synthesized from the total RNA extraction.
DISCUSSION AND CONCLUSION

As we know, purple-fleshed sweet potato cultivars are rich in starch, polysaccharides, and many other pigment compounds. To isolate high quality and yield of total RNA, it is necessary to efficiently decrease the interference of these components in the extracts. In our preliminary experiment, we found that the commonly used protocols, including TriZOL reagent (Invitrogen), SDS phenol precipitation, and CTAB precipitation were not optimal to isolate high quality total RNA from the purple-fleshed sweet potato. These methods usually result in low yield and poor quality of total RNA (Figure 4 and Table 3) and are only suitable for isolation of total RNA from common sweet potato cultivars which contain low levels of polysaccharides and pigmented compounds. Although there is a report that total RNA was isolated from sweet potato using modified CTAB method,[7] we found that this method was not optimal for the purple-fleshed sweet potato that contains high level of pigments, because RNA degradation was apparently found.[7]

In this study, we made some further modification to the CTAB. A high-strength CTAB (2%) solution was used as a lysis buffer. In addition, high concentrations of PVP (5%) and β-mercaptoethanol (4%) were added to the CTAB buffer, which ensured rapid removal of phenolic compounds, which otherwise oxidize and bind to RNA molecules. Similar to Sun’s method,[7] the first step in the purification procedure in our method also used LiCl precipitation. Although a high concentration of Na+ in the lysis buffer would increase the solubility of polysaccharides and reduce co-precipitation of polysaccharides with RNA in LiCl precipitation step,[9] a small amount of polysaccharide might still

Table 2. Representative cDNA cloned from total RNA isolated from purple-fleshed sweet potato using RT-PCR and RACE

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Best match in databasea</th>
<th>E valueb</th>
<th>Max identityc</th>
</tr>
</thead>
<tbody>
<tr>
<td>EU402465</td>
<td>Flavonoid 3’-hydroxylase [Ipomoea purpurea] (gb</td>
<td>AAR00229.1))</td>
<td>0.0</td>
</tr>
<tr>
<td>EU402466</td>
<td>Dihydroflavonol4-reductase [Ipomoea batatas] (dbj</td>
<td>BAA34637.1))</td>
<td>0.0</td>
</tr>
</tbody>
</table>

aBest match in different databases (Gen Bank or DDBJ or EMBL). The accession number is noted in the parentheses.

bThe expected (E) value refers to the number of matches expected by chance alone. The lower the E value, the more strongly supported the match.

cThe amino acid identity. The coding amino acid residue number of cloned cDNA is shown in parentheses.
coexist with total RNA. To solve this problem, we used 2 volumes of absolute ethanol and 3 M NaAc (pH 5.6) (both pre-cooled at −20°C) to precipitate RNA. The treatment with 3 M sodium acetate and pre-cooled ethanol at −20°C could improve the output of RNA \cite{10}. Precipitation with 3 M sodium acetate at pH 5.6 could also facilitate removal of residual polysaccharides \cite{11} and remove the residuary genomic DNA from RNA \cite{12}. Additionally, this protocol was more time-saving and more easily carried on than the previous methods. The whole extraction procedure could be accomplished within 4 hours.

Effectiveness of this RNA isolation protocol was confirmed by several ways, including spectrophotometric analysis, agarose gel electrophoresis, and RT-PCR. Firstly, the $A_{260}/A_{280}$ absorbance ratios

\begin{table}
\centering
\begin{tabular}{lccc}
\hline
 & $A_{260}/A_{230}$ & $A_{260}/A_{280}$ & Yields ($\mu g/g$ FW) \\
\hline
TriZOL reagent & 3.02 & 1.72 & 141.4 \\
SDS/Phenol precipitation & 2.45 & 1.63 & 102.8 \\
CTAB precipitation & 2.35 & 2.27 & 158.7 \\
\hline
\end{tabular}
\caption{Analysis of RNA quality and yield by spectrophotometric analyses}
\end{table}

*Figure 4.* Electrophoresis of total RNA from sweet potato tuberous root with different methods (a) TriZOL reagent; (b) SDS/Phenol precipitation; (c) CTAB precipitation.
ranged from 1.9 to 2.1 and \( A_{260}/A_{230} \) absorbance ratios ranged from 2.59 to 3.60, indicating that the total RNA was free or had little protein and polysaccharide contamination. Secondly, through 1.2% denaturing agarose gel electrophoresis, three discrete ribosomal RNA bands with no apparent RNA degradation were observed, suggesting that the total RNA might be free of RNases. Thirdly, the maximum yield of total RNA reached to 245.2 \( \mu \text{g/g (FW)} \), which could meet with use for Northern-blot analysis. Reverse transcription PCR usually is sensitive to any interference. Especially the efficiency of amplifying the 3'-end and 5'-end of any cDNA using the SMARTTM RACE cDNA Amplification Kit (Clontech, USA) is dependant on the quality of RNA. Successful amplification of the \( G14 \) gene fragment and the three total length of cDNA (\( F3'H \) and \( DFR \)) successfully indicates that the total RNA extracted using this method was of excellent integrity.

In conclusion, a quick and reliable method for isolating total RNA from the purple-fleshed sweet potato was developed. The extracted total RNA was good enough to be used for gene cloning.

**ABBREVIATIONS**

RNA, ribonucleic acid; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; CTAB, cetyl trimethyl ammonium bromide; cDNA, copy DNA; bp, basepairs; mRNA, messenger RNA; RACE, rapid amplification of cDNA ends; \( F3'H \), flavonoid 3'-hydroxylase; \( DFR \), dihydroflavonol 4-reductase; \( G14 \), metallothionein-like protein gene; ORF, opening reading fragment.

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