

Comparison and Improvement of Different Methods of RNA Isolation from Strawberry (*Fragaria × ananassa*)

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Abstract

Isolation of high-quality RNA from different strawberry tissues is often affected by the presence of high levels of contamination by polysaccharides and phenolic compounds. By the methods of improved CTAB, SDS, and guanidinium thiocyanate, total RNA was isolated from leaves of strawberry. The result indicated that total RNA is of high quality and undegraded by using CTAB. However, it was difficult to isolate total RNA from leaves of strawberry by using guanidinium thiocyanate, and total RNA could be extracted by using SDS with low concentration, impurity and degradation in some degree. After comparing, an improved 3% CTAB₃ was used to isolate RNA from tissues of strawberry, and the isolated RNA was good enough for further applications. After RT-PCR, the 699 bp sequence of glucose-6-phosphate dehydrogenase (*G6PDH*) gene was obtained to identify the quality of total RNA from strawberry. In this experiment, improved 3% CTAB₃ method could be regarded as a simple, rapid, economic and convenient method for the RNA isolated from strawberry.

Keywords: strawberry, RNA extraction, *G6PDH* gene

1. Introduction

Extraction of RNA of high quantity and quality is an important procedure and can be a limiting factor in plant molecular biology experiments such as Northern blot hybridization, mRNA purification, PCR amplification, cDNA synthesis and cDNA library construction and RNAi (Murillo et al., 1995; Hu et al., 2002; Wang et al., 2006). However, to isolate RNA from some plant tissues is manifestly difficult due to the presence of high amounts of secondary metabolites, such as polysaccharides and polyphenolic compounds, which could co-precipitate or bind to RNA and result in poor yields (Gasic et al., 2004; Djami-Tchatchoua et al., 2011).

Strawberry (*Fragaria × ananassa*), a fruit of economic and nutritional importance, is also a model species for soft fruits and genomics in the Rosaceae (Zhang et al., 2011). Its leaves are rich in carbohydrates, pigments and proteins and also contain polyphenols which interfere with RNA isolation in high quantity and quality (Cai et al., 2008). After breaking, strawberry leaf cells release out phenolic compounds leading to the browning effect (Li, 2001), and oxidation of phenolic compounds could irreversibly bind with RNA causing its loss (Scheiderbauer et al., 1991). Furthermore, this fruit is one of the most complex tissues for RNA isolation (Pandit et al., 2007). Therefore, a reliable method is necessary for the isolation of good-quality RNA from strawberry tissues.

Thompson et al., (2003) used the Plant RNeasy Kit produced by QIAGEN to quickly extract high-quality total RNA from strawberry leaves, but the kit is expensive. Cai et al., (2008) and Yang et al., (2006) extracted RNA from strawberry leaves with the improved CTAB method, but it is time-consuming or contaminates the DNA seriously. The aim of this study was to optimize a simple, rapid, economic and convenient RNA isolation method from strawberry tissues for subsequent experiments.

2. Materials and Methods

2.1 Materials

Flushing roots, stems, leaves, flowers and mature fruits of strawberry (*Fragaria × ananassa*) were obtained from in field-grown plants at the Sichuan Agricultural University, Sichuan province of China. Tissues were

immediately frozen in liquid nitrogen and stored at -80°C until needed. Vessels and plastic tubes were dipped in DEPC-treated water overnight and autoclaved. Mortars and pestles were treated with DEPC-treated water, and then baked for 6 h at 180°C .

2.2 RNA Extraction Methods

2.2.1 RNA Extraction Protocol

1. Frozen leaves (0.1 g) were ground with a mortar and a pestle in the presence of liquid nitrogen and PVPP (0.01 g).
2. The resulting powder was transferred to a sterile centrifuge tube containing 700 μl of pre-warmed (65°C) extraction buffer. The sample was vortexed vigorously for 1 min and then incubated at 65°C for 10 min to lyse cells completely.
3. Centrifuging at 15 700 g for 5min and transferring the supernatants into a new sterile tube.
4. Adding an equal volume of chloroform: isoamyl alcohol (24:1) mixture extraction (replace mixture by Tris-Phenol in SDS₂ method), the tube was vortexed vigorously for 1 min and centrifuged at 15 700 g for 10 min at room temperature. After centrifugation, the upper phase was transferred to a new tube.
5. Following the addition of an equal volume of chloroform, the tube was vortexed vigorously for 1 min and centrifuged at 15 700 g for 10 min at room temperature.
6. After centrifugation, the upper phase was transferred to a new tube following the addition of an equal volume of 4 M LiCl.
7. After precipitated at 4°C for 4 h, the tube was centrifuged (15 700 g, 15 min, 4°C) and the pellets were washed gently twice with 75% EtOH.
8. Then the tubes were centrifuge at 5 900 g for 5min, and resultant pellet was air dried and re-suspended in 30 μl DEPC-treated water.

2.2.2 RNA Extraction Methods

Different extraction methods were listed in table 1.

Table 1. Different methods

Method	extraction buffer	extraction protocol	extraction tissue
3% CTAB ₁	3% CTAB, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 5%PVP, 1% β -mercaptoethanol added just before use	1,2,5,6,7,8	leaves
3% CTAB ₂	the same as 3% CTAB ₁	1,2,4,5,6,7,8	leaves
4% CTAB	4% CTAB, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 5%PVP, 1% β -mercaptoethanol added just before use	1,2,5,6,7,8	leaves
3% CTAB ₃	the same as 3% CTAB ₁	1,2,3,5,6,7,8	roots, stems, leaves, flowers, mature fruits
SDS ₁	3% SDS, 100 mM Tris-HCl pH 8.0, 0.2 M NaCl, 20 mM EDTA, 5% PVP β -mercaptoethanol added just before use	1,2,5,6,7,8	leaves
SDS ₂	the same as SDS ₁	1,2,4,5,6,7,8	leaves
Guanidinium thiocyanate ₁	according to Sambrook et al. (2000)	adding the above step 5 before Sambrook's step 8	leaves
Guanidinium thiocyanate ₂	according to Sambrook et al. (2000)	according to Sambrook et al. (2000)	leaves

2.3 Gel Electrophoresis and Analysis of RNA Purity

3ul RNA samples were detected by 1.0% agarose gel electrophoresis, 75mA for 18min. Added 5ul RNA to 45ul DEPC-treated water, and detected ratios A_{260}/A_{230} and A_{260}/A_{280} .

2.4 RT-PCR

RT-PCR was used to detect the RNA of roots, stems, leaves, flowers and fruits extracted by 3%CTAB₃. The first strand cDNA is synthesized from 1μg of the total RNA by reverse transcriptase with Oligo-(dT) primer according to the instructions of the RevertAid™ First Stand cDNA Synthesis Kit (MBI Fermentas).

Based on the conserved regions of the *G6PDH* gene nucleotide sequences from *Fragaria × ananassa* (JQ260862), the gene-specific primers were designed as follows: G6-F: 5'-AGAGGGA GAATTGTGGTG-3', G6-R: 5'-CAGTGTAGGCTGGGTAAG-3'. PCR was performed with a 25 μl reaction mixture containing 2μl of first-strand cDNA, 12.5μl 2 × *Taq* PCR Master Mix (TIANGEN, China), 1μl primer G6-F and 1μl primer G6-R, and 8.5μl ddH₂O.

The PCR procedure started with 94°C for 3min, then 35 cycles of 94°C for 1min, 60°C for 1min, 72°C for 1min, and finally 72°C for 10min. The PCR products were analyzed on 1.0% agarose/EtBr gel and the corresponding DNA bands were recovered. Then the PCR products were sequenced with Big Dye Terminators using ABI-PRISM3730 Sequencer (PE Applied Biosystems, Foster City, California, USA).

3. Results

3.1 Results of Total RNA by CTAB_s, SDS_s, Guanidinium Thiocyanate_s from Leaves

By improved CTAB, SDS, and guanidinium thiocyanate methods, total RNA was isolated from strawberry leaves. Total RNA was compared by gel electrophoresis (Figure 1) and the ratio of A_{260}/A_{280} was measured (Table 2). The result indicated that the RNA was of high quality and undegraded, and the sharpness of 28S bands was twice than 18S of bands using the 3%CTAB₁ compared to the 4% CTAB method. The ratio of A_{260}/A_{280} was from 1.8 to 1.9, and ratio of A_{260}/A_{230} was higher than 2.0 by two methods. It indicates the results of RNA isolation were basically qualified to the next experiments. The brightness of 28S bands was the same to 18S bands by using 3%CTAB₂ method. Some RNA lost by procedure of chloroform: isoamyl alcohol (24:1) mixture extracting.

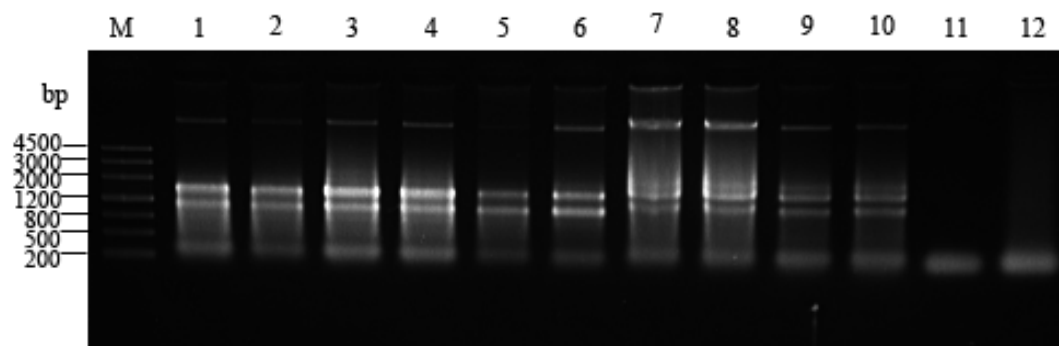


Figure 1. Results of all CTAB_s, SDS_s, guanidinium thiocyanate_s

M = DNA ladder; lane 1, 2 by 3%CTAB₁; lane 3, 4 by 4%CTAB; lane 5, 6 by 3%CTAB₂; lane 7, 8 by SDS₁; lane 9, 10 by SDS₂; lane 11 by Guanidinium thiocyanate₁; lane 12 by Guanidinium thiocyanate₂

Table 2. Purity analysis of total RNA by CTAB_s, SDS_s and guanidinium thiocyanate_s (GT_s) from leaves

Absorbance	3%CTAB ₁		4%CTAB		3%CTAB ₂		SDS ₁		SDS ₂		GT ₁	GT ₂
A ₂₃₀	0.637	0.452	1.106	0.887	0.402	0.283	0.506	0.346	1.809	1.519	1.32	1.127
A ₂₆₀	1.523	0.997	2.421	2.119	0.571	0.504	0.887	0.742	1.393	1.847	0.086	0.828
A ₂₈₀	0.802	0.539	1.343	1.115	0.456	0.258	0.467	0.410	1.330	1.488	0.041	0.714
A ₂₆₀ /A ₂₃₀	2.39	2.21	2.19	2.39	1.42	1.78	1.75	2.14	0.77	1.21	1.16	0.73
A ₂₆₀ /A ₂₈₀	1.90	1.85	1.80	1.90	1.45	1.95	1.90	1.81	1.05	1.24	1.26	1.16

However, the results of extracting RNA by SDS_s and guanidinium thiocyanate_s were not satisfactory. It was difficult to isolate total RNA from leaves of strawberry by using guanidinium thiocyanate_s. Although total RNA could be extracted by using SDS₁ with low concentration, impurity and degradation in some degrees, the

sharpness of 28S bands was no better than 18S, which indicated that the bands of 28S RNA might be degraded. Reasons resulting in these phenomena were some RNase and insoluble substances existing. So some powerful denaturants were required to degrade RNase and get rid of polysaccharides before precipitating.

3.2 Results of Total RNA Extracting from Tissue by 3%CTAB₃ Method

The result of RNA extracting from roots, stems, leaves, flowers and mature fruits of strawberry by 3% CTAB₃ were shown by gel electrophoresis (Figure 2) and the ratio of A_{260}/A_{280} (Table 3). The sharpness of 28S bands was twice than 18S of bands in all extracting RNA. The ratio of A_{260}/A_{280} is above 1.90, and ratio of A_{260}/A_{230} is higher than 2.0. It indicates the results of RNA isolation from roots, stems, leaves, flowers and mature fruits are qualified to the next experiments.

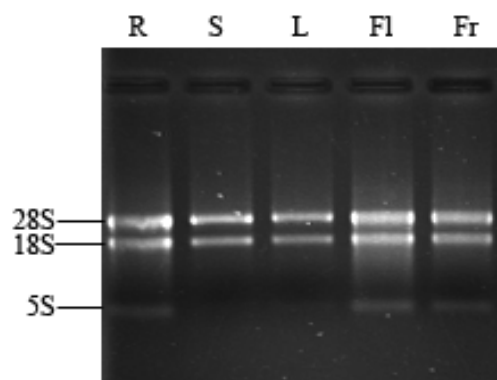


Figure 2. Isolated RNA from different tissue by 3%CTAB₃

M: DNA ladder; R: roots; S: stems; Fl: flowers; L: leaves; Fr: fruits

Table 3. Purity analysis of total RNA from roots, stems, leaves, flowers, fruits by 3%CTAB₃

Absorbance	roots	stems	flowers	leaves	fruits
A_{230}	0.372	0.263	0.292	0.637	0.351
A_{260}	0.870	0.668	0.589	0.889	0.756
A_{280}	0.442	0.326	0.307	0.802	0.395
A_{260}/A_{230}	2.34	2.54	2.02	2.13	2.15
A_{260}/A_{280}	1.97	2.05	1.92	2.17	1.91

3.3 RT-PCR and Sequence Analysis

The RNA isolated from roots, stems, leaves, flowers and mature fruits of strawberry by 3%CTAB₃ are suitable for RT-PCR. By primer G6-F and G6-R and RNA from tissue, PCR amplification generated DNA products with the expected size of about 700 bp (Figure 3). Sequence analysis revealed a PCR product of 699 bp long. The similarity analysis of nucleotide sequence within different plants species revealed that they shared high homology by Blastx (Table 4). The sequence of target gene shared a similarity of the deduced amino acid sequences (91-94%) with *G6PDH* genes of other plants, among which it shared the highest sequence homology with castor strawberry. Therefore, we presumed that it was the target fragment and submitted it to GenBank (JQ323094).

Table 4. Results of sequence similarity analysis

Genbank Accession number	Compared gene	Score(bits)	E value	Identities
EEF47431.1	<i>Ricinus communis G6PDH</i>	415	2e-146	200/215(93%)
XP_003552660.1	<i>Glycine max G6PDH</i>	410	1e-144	202/215(94%)
XP_002266930.1	<i>Vitis vinifera G6PDH</i>	405	5e-143	198/215(92%)
AED91851.1	<i>Arabidopsis thaliana G6PDH</i>	405	1e-142	196/215(91%)
EEE82659.1	<i>Populus trichocarpa G6PDH</i>	403	7e-142	197/215(92%)
CAB52708.1	<i>Solanum tuberosum G6PDH</i>	400	9e-141	195/215 (91%)

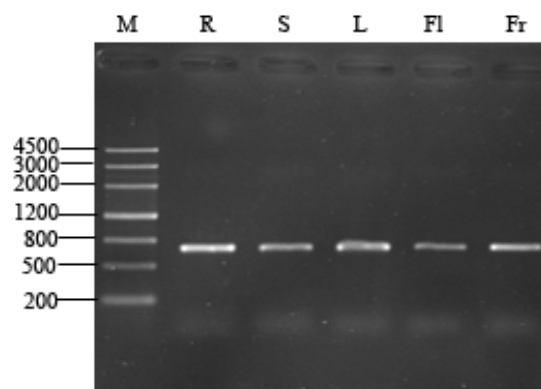


Figure 3. RT-PCR product of tissue

M: DNA ladder; R: PCR product of roots; S: PCR product of stems; L: PCR product of leaves; Fl: PCR product of flowers; Fr: PCR product of fruits

4. Discussion

Plant RNA isolation plays an important role in various experiments. To successfully isolate RNA from strawberry, we applied seven different methods (CTAB_s, SDS_s and guanidinium thiocyanate_s) to extract RNA from leaves. Firstly, CTAB_s were better than SDS_s and guanidinium thiocyanate_s. By 3%CTAB₁ and 4%CTAB₂, no RNA degradation was detected and the result was basically free of protein contamination. Although the 3% CTAB₂ method spend much more time and add chloroform: isoamyl alcohol (24:1) mixture to remove proteins, no better quality was obtained. It might be some important RNA loss by mixture extraction. Secondly, RNA isolated from leaves by SDS_s was not satisfactory. There were lots of insoluble substances existing in RNA. They maybe were some insoluble polysaccharids or proteins. What's more, it was hardly to isolate total RNA from leaves of strawberry when we tried the method of guanidinium thiocyanate by the protocol of Sambrook (Sambrook et al., 2000). Only the 5S RNA band was obtained with some insoluble substances, even though we added chloroform to deal with.

Although a number of different methods have been described for the isolation of plant RNA, in most cases, they involve either the use of detergents SDS or phenol extraction (Logemann et al., 1987; Mazzara and James, 2000; Vasanthaiah et al, 2008). When we tried to apply these methods to the isolation of RNA from strawberry, these methods failed due either to RNA degradation or very low yield. After comparing, we developed a modified 3%CTAB₃ to extract RNA from roots, stems, leaves, flowers and mature fruits of strawberry.

Proved by this experiment, we found that 3%CTAB₃ was appropriate for RNA extracting from tissues of strawberry and total RNA obtained was of sufficient quality to be used for downstream experiments. We successfully obtained *G6PDH* gene fragment by extracted RNA, and shared a similarity of 91%-94% with *G6PDH* genes of other plants. In conclusion, 3%CTAB₃ is efficient to get more RNA and prevent degradation of RNA, and is the best method for RNA purification from strawberry tissues.

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