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Full Length Research Paper

An effective method for RNA extraction from grapevine berry skins

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RNA extraction is the foundation of modern molecular biology. It is difficult to extract high quality RNA from grapevine tissues, especially in cases where polysaccharides and polyphenols are rich. In this paper, the modified cetyltrie thylammnonium bromide (CTAB), sodium dodecyl sulphate (SDS) and guanidine isothiocyanate methods were applied to extract high purity and integrity RNA from grapevine green and mature berry skins. Highly-grade RNA was isolated by using modified CTAB method and it is suitable for the demands of further molecular biological research such as reverse-transcription polymerase chain reaction (PCR) (RT-PCR), cDNA library construction and Northern hybridization.

Key words: Grapevine berry skins, RNA extraction, reverse-transcription polymerase chain reaction (RT-PCR).

INTRODUCTION

Grapevine is one of the most important economic species in the world. The research of grapevine molecular biology is more and more valuable. Consequently, grapevine research requires studies of gene expression and function, which are mainly dependent on RNA quality. However, a rapid, inexpensive and reliable protocol for the extraction of RNA from grapevine berry skins is particularly challenging because of high concentrations of polysaccharides, polyphenols and other secondary metabolites. Polyphenolics are easily oxidized quinone compounds which with irreversible binding to RNA (Graham, 1993), results in RNA inactivity and loss in the later extraction with phenol and chloroform (Schneiderbauer et al., 1991; Lin et al., 2003).

Polysaccharides have similar structure with RNA. In the process of extraction to form a common jelly precipitation with RNA (Logemann et al., 1987), the quality of RNA is affected. This paper compared several RNA extraction methods such as sodium dodecyl sulphate (SDS) method (Xia et al., 2000), cetyltrie thylammnonium bromide (CTAB) method (Chang et al., 1993) and guanidine isothiocyanate method (Zhang et al., 2003) in order to find an effective method for RNA extraction.

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Abbreviations: DEPC, Diethyl pyrocarbonate; **EDTA,** ethylene diamine tetra-acetic acid; **RT-PCR,** reverse transcription-polymerase chain reaction; **SDS,** sodium dodecyl sulphate; **CTAB,** cetyltrie thylammnonium bromide.

MATERIALS AND METHODS

Berries of Amur grape (*Vitis amurensis Rupr.*) were collected at Northeast Forestry University campus. Skins were peeled and frozen in liquid nitrogen in aliquots of 500 – 600 mg. Samples were stored at -80°C until extraction. Disposable plastic supplies and various reagents (except Tris) were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) and autoclaved to inactivate RNases. Glassware were kept overnight at 180°C.

For the SDS procedure, after grinding in liquid nitrogen, 500 mg of the frozen tissue was added to pre-chilled sterile centrifuge tube containing 700 µl SDS extraction buffer (0.1 M Tris-HCl, 0.05 M ethylenediamine tetra-acetic acid (EDTA), 1% SDS, 0.09 M LiCl, 2% of β-mercaptoethanol added just before use), 350 µl chloroform and 350 µl phenol. The tube was shocked vigorously for 15 min and centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was recovered and 350 µl chloroform and 350 µl phenols was added; the tube was shocked vigorously for 5 min and centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was transferred to a new microcentrifuge tube and 700 µl chloroform was added, the tube

Table 1. Results from different RNA (from grapevine berry skins) extraction methods.

Method	RNA yield (µg/µl)	A260:A280	A260:A230
SDS	0.53±0.08	1.72±0.04	1.91±0.1
CTAB	1.11±0.08	1.94±0.02	2.08±0.15
Guanidine isothiocyanate	0.79±0.03	2.19±0.02	1.92±0.01

was centrifuged at 12000 rpm for 10 min at 4°C after vigorous shaking for 5 min. The supernatant was transferred to a new microcentrifuge tube; 350 µl 75% ethanol and 350 µl LiCl (8 M) was then added. The mixture was centrifuged at 12000 rpm for 20 min at 4°C after being incubated at -20°C for 20 min. The pellet was washed with 300 µl ethanol (75%), dried and resuspended in DEPC-water.

For the CTAB procedure, aliquots of 500 mg of frozen skin tissue were grounded by mortar and pestle in the presence of liquid nitrogen. The powder was transferred to a pre-chilled sterile centrifuge tubes containing 1 ml washing buffer (0.1 M Tris-boric acid, 0.35 M sorbitol, 10% PEG 6000 and 2% of β-mercaptoethanol were added just before use); 1 ml pre-chilled washing buffer was then added to sterile centrifuge tubes and fiercely shaked for 2 min at room temperature. The tubes were placed on ice for 10 min, then centrifuged at 8000 rpm for 8 min at 4°C. The supernatant was discarded following the addition of 900 µl CTAB buffer (0.1 M Tris-pengsuan, 1.4 M NaCl, 0.02 M EDTA and 2% CTAB), the samples were placed in the water at 55°C for 20 min, and 100 µl KAc, 100 µl ethanol and 1000 µl chloroform were added. It was stirred rapidly for 20 min at room temperature, and then centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was transferred to a new microcentrifuge tube and 1/3 (v/v) LiCl (10 M) was added. The mixture was incubated at -20°C for 1 h and RNA was selectively pelleted after centrifugation at 13000 rpm for 20 min at 4°C. The pellet was resuspended in 500 µl of DEPC, an equal volume of phenol was added and the mixture was centrifuged at 13000 rpm for 10 min at 4°C. The supernatant was transferred to a new microcentrifuge tube and the RNA was precipitated with 500 µl of cold chloroform and centrifuged at 13000 rpm for 10 min at 4°C. The supernatant was transferred to a new microcentrifuge tube and 1/3 (v/v) LiCl (10M) was added. The mixture was incubated at -20°C for 1 h, and then centrifuged at 13000 rpm for 20 min at 4°C. The pellet was washed with ethanol (70%), dried and resuspended in DEPC–water.

Procedure of the guanidine isothiocyanate method

After grinding in liquid nitrogen, the frozen tissue was added to 800 µl extraction buffer (4 M guanidinium isothiocyanate, 0.025 M sodium citrate (pH7.0), 5% sodium lauryl sarcosine and 1% of β-mercaptoethanol were added just before use). 80 µl 2 M NaAc (pH4.8) and an equal volume of chloroform and isoamyl alcohol (24:1) were added after shaking. The supernatant was transfered to a new centrifuge tube after being centrifuged at 12000 rpm for 15 min at 4°C. An equal volume of isopropanol and the mixture was incubated for 2 h at -20°C. RNA was selectively pelleted after centrifugation at 12000 rpm for 20 min at 4°C. The pellet was resuspended in 500 µl of extraction buffer, an equal volume of isopropanol and 1 / 10 volume of 3 M NaAc was added and the mixture was incubated at -20°C for more than 2 h. RNA was selectively pelleted after centrifugation at 12000 rpm for 15 min at 4°C. The pellet was then dried at room temperature after washing with 75% alcohol precipitate twice and resuspended in DEPC- water.

RNA purity and concentration were assessed by UV spectrophotometer. RNA integrity was analysed by 1.2% agarose gel electrophoresis, stained with ethidium bromide and visualisation with UV light. The total RNA was digested by RQ1 RNase Free-DNase, aiming to eliminate the DNA pollution. First-strand cDNA synthesis was performed as described by Creator™ SMART™ cDNA Library Construction Kit User Manual. Primer sequence was as follows:

SMART IVTM Oligonucleotide (10 mM): 5'-AAGCAGTGGTAT-CAACGCAGAGTGGCCATTACGGCCGGG-3': CDS III/3' PCR primer (10 mM): 5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30N–1N-3' (N = A, G, C, or T; N–1 = A, G, or C); 5' PCR Primer (10 mM): 5'-AAGCAGTGGTATCAACGCAGAGT-3'.

Long distance polymerase chain reaction (LD-PCR) amplification was performed and it generated double stranded (ds) cDNA. The procedure used is as follows: Denaturation at 95°C for 1 min and amplification at 95°C for 15 s, 68°C for 6 min for 22 cycles. When the cycling was completed, 5 µl sample of the PCR product was analyzed alongside 0.1 µg of 2-kb DNA size markers on a 1.1% agarose/EtBr gel.

RESULTS

The result of RNA purity and concentration are shown in Table 1. The A260:A280 ratio of high-quality RNA is 1.7 to 2.0, and the A260:A230 ratio is 2.0 to 2.3. From Table 1, we can see that the total RNA extracted by the three methods was high in purity especially by the CTAB method. However, if there were polysaccharide in sam-ples, it will form a gel complex with the RNA and will have strong spectrophotometric absorbance at the 230 and 280 nm, thus affecting the results (Haruta et al., 1999). Therefore, samples were tested using electrphoresis to check whether there were other impurities, in particular, polysaccharides (Figure 1).

Electrophoresis results (Figure 1) showed that high molecular weight RNA is practically absent in samples extracted with the guanidine isothiocyanate method. Obvious bright band in the lanes showed that the total RNA incomplete removal of impurities have polysaccharides contamination. The smearing observed in the 28 and 18 S bands of the RNA extracted by SDS method, from the Table 1 with low ratio of A_{260}:A_{230} indicated that RNA has been heavily contaminated by phenol and other secondary compounds that impair migration. Only the RNA extracted by CTAB method has two major ribosomal bands and 28:18 S brightness close to 2:1. The RNA extracted by the CTAB method was relatively free of protein, polysaccharide and phenolics compounds. The CTAB method set up for RNA extraction from skins of green and mature grapevine berries was compared with two other RNA extraction methods that have been certified to be successfully.

To further prove that the RNA extracted by the CTAB

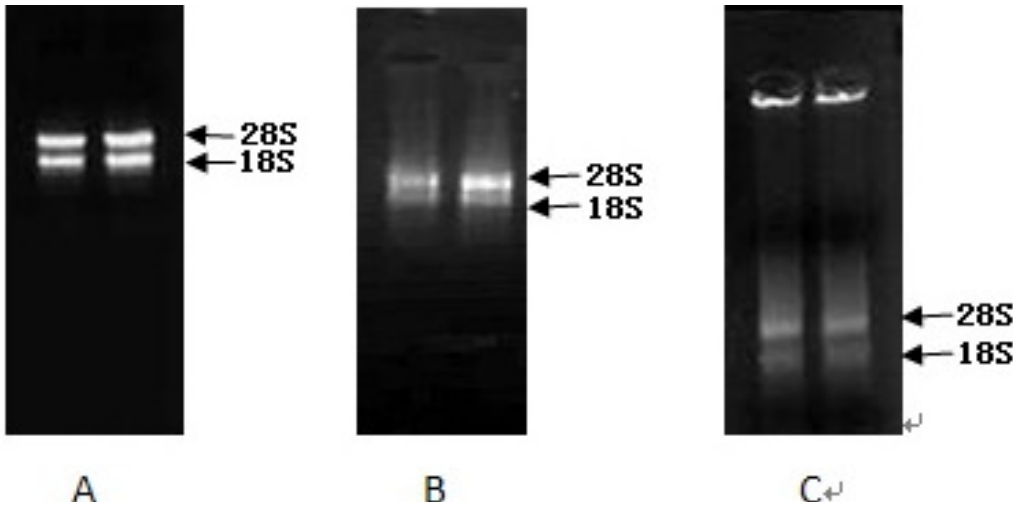

Figure 1. Agarose gel electrophoresis of total RNA from grapevine berry skins. A, CTAB method; B, SDS method; C. guanidine isothiocyanate method.

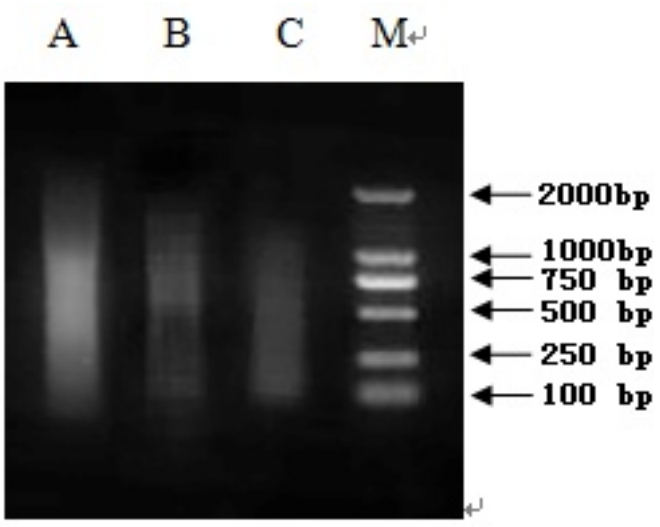

Figure 2. 1.1% agarose gel electrophoresis of dscDNA from grapevine berry skins. A, CTAB method; B, guanidine isothiocyanate method; C, SDS method; M, DL2000 marker.

method can be used for cDNA library construction and other biological experiments, the total RNA was extracted from ripened grapevine berry skins as a template for first-strand synthesis and RT-PCR amplification. Figure 2 shows that the fragment was located in 100 to 3000 bp which was amplified with the RNA extracted by the CTAB method; this complies with the requirement of the experiment such as cDNA library construction. The fragment was respectively located at the 100 to 2000 and 100 to 1000 bp which was amplified with the RNA extracted by the SDS method and guanidine isothiocyanate method. The short fragment and low integrity of the dscDNA indicated that the RNA obtained by these two methods was not suitable for cDNA library construction.

The PCR product obtained by CTAB method was used to construct cDNA library. The titer of primary cDNA library was 1.008×10^6 pfu/ml, the titer of amplified library was 1.97×10^9 pfu/ml^{-1}, the percentage of recombination was about 100% and the fragment size inserted was 0.3 ~ 2 kb. The average insert size was 0.84 kb. We obtained high quality cDNA library. The results further demonstrated that the purity and integrality of total RNA isolated by using CTAB method were significantly satisfactory for the demands of molecular biological research.

DISCUSSION

Polyphenols and polysaccharides are two difficult problems in plant total RNA extraction. In the complete plant cells, nucleic acid and other components of the plant cells are separated when the cells are broken. These when released interacts with the RNA, affecting RNA extraction and reverse transcription reaction (Li et al., 1999).

The time of degradation is greater than for synthesis; following berry skins maturity, a great quantity of various enzymes accumulates including RNase (Chang et al., 1993). Mature grape berries are rich in secondary metabolites such as phenols, pigments and polysaccharides; the general method of RNA extraction is difficult to use in the extraction of full RNA, while the CTAB method adopted by this article extracted high-quality total RNA from grapes berry skins successfully. In the extraction process, addition of β-mercaptoethanol and Tris- boric acid to prevent oxidation of polyphenols and the disulfide bonds can be interrupted so that there would be inactivation of polyphenol oxidase (Chang et al., 1993). CTAB cell lysate with RNA and DNA form insoluble complexes and helps to remove polysaccharides. LiCl precipitation

improves the CTAB method more effectively by the removal of polysaccharide. For protein denatu-ration and removal of DNA, phenol and chloroform extraction could be applied. The results showed that impurities such as polyphenolics and pigments were completely removed by CTAB method.

RNA extraction is the most basic step of molecular biology. In order to obtain the full and wealth full-length cDNA, the key is to extract complete and not degraded RNA. RNA extraction methods have long been reported, but each method is not suitable for all materials (Ainsworth et al., 1994). CTAB method used in this study can effectively isolate high-quality RNA from grapes berry skins which are rich in polysaccharide and polyphenols, and can be widely applied to the RT-PCR and cDNA library construction and other molecular biology research.

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