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Methodology

An alternative cetyltrimethylammonium bromide-based protocol for RNA isolation from blackberry (*Rubus* L.)

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ABSTRACT. Isolation of high-quality RNA free of contaminants, such as polyphenols, proteins, plant secondary metabolites, and genomic DNA from plant tissues, is usually a challenging but crucial step for molecular analysis. We developed a novel protocol based on the cetyltrimethylammonium bromide method to isolate high-quality RNA from blackberry plant tissues, especially fruits. Most DNA was removed when acetic acid was utilized, before RNA precipitation. Thus, lithium chloride, a reagent widely used for RNA purification, was not needed. The isolation time was shortened to less than 3 h. The RNA was quite pure, with little DNA contamination. The quality of the RNA was assessed by spectrophotometric readings and electrophoresis on agarose gels. It was good enough for downstream enzymatic reactions, such as reverse transcription-PCR, cloning and real-time PCR assay.

The method yielded an amount of total RNA comparable to previously described protocols.

Key words: Blackberry fruits; RNA isolation; CTAB; Acetic acid; DNA removal; Polysaccharide

INTRODUCTION

Isolation of intact and high-quality RNA is often a prerequisite and critical step for most molecular biology studies. To date, several standard protocols as well as extensive modifications have been reported and widely used for RNA isolation (Abe et al., 1972; Chomczynski and Sacchi, 1987, 2006). Probably, the most prevalent one is the single-step RNA isolation method based on acid guanidinium thiocyanate-phenol-chloroform extraction (GITC) developed by Chomczynski and Sacchi (1987). Thereafter, commercial kits have been developed through this method under different names such as 'TRIzol' (Invitrogen, USA), and 'RNeasy' (Qiagen, Germany). Unfortunately, most of these kits are not suitable for some plant species, especially those tissues rich in phenolic compounds, polysaccharides and other secondary metabolites, neither because of their inability to obtain RNA nor the negligible quality of RNA for downstream manipulations (Liu et al., 1998; Wang and Stegemann, 2010; Ghawana et al., 2011; Wang et al., 2011). Other conventional methods employing the hash lysate buffer such as sodium dodecyl sulfate (SDS), cetyltrimethyl ammonium bromide (CTAB), N-lauroyl sarcosine, and urea have also been widely used for RNA isolation in plant species (Liao et al., 2004; Almarza et al., 2006; Fort et al., 2008; Rio et al., 2010; Wu et al., 2011). Special measures could be adopted to address the problems caused by inferences of polysaccharides, phenolic compounds, starches, and pigments (Rodrigues et al., 2007; Kansal et al., 2008; Smart and Roden, 2010).

Our main concern was involving cloning of relevant genes from the important horticultural blackberry (*Rubus* spp) tissues, especially in fruits. They were proved to be rich in polyphenolic compounds like anthocyanins and proanthocyanidins (Fan-Chiang and Wrolstad, 2005; Cuevas-Rodriguez et al., 2010). The interference of these compounds brought severely negative effects in RNA extraction. Particularly when fruits were used as the starting material, polysaccharides and the oxidized-prone polyphenols led to brown pellets or even no products after extraction.

In the previous study of Jones et al. (1997), SDS was used to disrupt the raspberry (*Rubus ideaus*) fruit cells and denature the proteins. RNA was then selectively precipitated by lithium chloride (LiCl) from the released total nucleic acids. However, this method took almost two days long and involved laborious extraction and precipitation steps, which put RNA at more risks of being degraded by the stubborn but ubiquitous existing RNase. We also tried other methods like GITC, commercial kits Tri-blue (Shenergy, Shanghai, China) and RNAiso for polysaccharide-rich plant tissues (Takara, Dalian, China) but did not obtain any satisfactory results.

The current study was intended to develop a simpler and more efficient method for RNA extraction from blackberry tissues. In the presence of acetic acid, DNA was co-precipitated with proteins and other metabolites, while RNA remained in the aqueous phase. Relative intact RNA with little DNA contamination could be obtained in less than 3 h. The RNA was of high quality as accessed by gel-electrophoresis, spectrophotometric readings and real-time PCR test.

MATERIAL AND METHODS

Plant materials

The tetraploid blackberry cultivar 'Arapaho' (ARK631 x ARK883) was selected for RNA isolation (Stafne, 2003; Wang et al., 2008). Fruit tissues at the stage of green, red and black (Perkins-Veazie et al., 2000), leaves (<30 days old), and flowers (full blooming) were harvested from plants growing in an orchard located in southern part of Sichuan Province, China. All plant tissues were wrapped in aluminum foil, immediately frozen in liquid nitrogen for transportation to the laboratory and stored at -80°C until used.

Solution and reagents

Diethyl pyrocarbonate (DEPC)-treated water was used for all solutions except Tris-HCl. The extraction buffer was according to Chang et al.(1993) with little modification: 30 g/L CTAB, 20 g/L polyvinylpyrrolidone (PVP) K-30 (soluble), 200 mM Tris-HCl, pH 8.0, 50 mM ethylene diamine tetraacetic acid disodium (EDTA-Na_2), pH 8.0, 1.4 M sodium chloride (NaCl), and 0.5% (v/v) 2-mer-captoethanol (added just before use). Insoluble polyvinylpolypyrrolidone (PVPP), water saturated phenol, pH 4.7-5.2, chloroform, and isopropanol were analytical pure and stored at room temperature.

RNase removal

Pestle and mortars, and glassware were baked for at least 2 h at 180°C. Gel electrophoresis apparatuses were rinsed well in DEPC-treated water and subsequently wiped with absolute ethanol. Eppendorf® tubes and tips (1.5 mL) were treated with a 0.1% solution of DEPC overnight at 37°C and then autoclaved at 121°C for 25 min.

RNA isolation

Frozen plant tissue (0.1 g) was used for each extraction. Plant tissue was first ground to a fine powder in liquid nitrogen with the help of a pre-cooled mortar and pestle. Secondly, aliquots of approximately 0.05 g powder were transferred to a 0.6 mL extraction buffer, which was preheated to 65°C in a 1.5-mL Eppendorf® tube containing 8.5% (w/v) insoluble PVPP and then vortexed vigorously for 30 s to suspend the tissue. Thirdly, all samples were kept at 65°C in a water bath for 35 min with interval vortexing. Afterwards, 0.6 mL water-saturated phenol was slowly added to the crude mixture, with certain amounts of acetic acid (set at 0, 2, 4, 6, 8, or 10 µL per 600 µL), and 0.2 mL chloroform. All samples were vortexed vigorously for 30 s and centrifuged at 16,000 *g* for 10 min, 4°C on a desktop rotor. The upper aqueous crude RNA mixture was transferred to a new 1.5-mL tube with a fine pipet to subject a chloroform re-extraction. The resulting RNA mixture (upper phase) was pipetted again and then mixed with an equal volume of isopropanol and incubated at -20°C for at least 30 min. Subsequently, RNA was recovered by centrifugation, the same as above. The supernatant was discarded and the pellet was washed twice in 1.0 mL 75% ethanol. Finally, the pellets were air-dried and dissolved in 30-50 µL RNase-free water. The RNA was ready for quality test or stored at -80°C until further used.

Estimation of RNA purity, integrity and yield

The purity of the total RNA was accessed with a spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany) at wavelengths of 230, 260, 280, and 320 nm. Yield was determined accordingly as described by Chomczynski and Sacchi (2006). To determine the RNA integrity, total RNA was also subjected to gel electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualized using a UV transilluminator and a gel doc system (Syngene, Cambridge, UK). The bands of 28S rRNA, 18S rRNA and 5.8S rRNA were used as an indicator for RNA integrity.

Reverse transcription and subsequent application

DNAse I (Takara, Japan) was used to remove the last trace of DNA contamination prior to reverse transcription. First-strand cDNA was synthesized using the RevertAid™ First-Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer protocol. Degenerate primers for chalcone synthase (CHS) RuCF along with RuCR and specific primers for the house keeping gene *β-actin* RuACTF and RuACTR (Table 1) were used in a Bio-Rad thermal cycle system. The following protocol was applied: 94°C for 3 min, 35 cycles of 30 s at 94°C, annealing for 30 s, 30 s at 72°C, followed by a final extension at 72°C for 10 min. The PCR product was electrophoresed on a 2% agarose gel as described above.

Table 1. Primers used in this study.

Primer name	Primer sequences (5'-3')	Melting temperature (°C)	Amplification length (bp)
RuCF	CCK TCH YTG GAY GCN MGR CAR GAC	55.0	861
RuCR	GGB CCR AAN CCR AAN ARM ACA CC		
RuACTF	GAG GAT ATT CAG CCA CTC GTT	58.0	294
RuACTR	CTC TTC AGG AGC AAC ACG AA		
qMYB10F	CCA TCA GCA TCA CCA CCT A	64.0	115
qMYB10R	CAC CTG TTC CTG TCA ATG AG		
qACTF	TGA CAA TGG GAC TGG AAT GGT	58.5	57
qACTR	GCC CTG GGA GCA TCA TCA		

Real-time PCR test

PCRs were performed on a 96-well plate with a CFX96 real-time PCR detection system (Bio-Rad) using SYBR Green to detect amplification of our newly isolated transcription factor *RuMYB10* gene (accession No. JQ359611). A 20-µL reaction volume consisted of 10 µL SYBR ExTaq Mix (Takara), 200 nM of each primer (qMYB10F and qMYB10R), and 2X 0.8 µL diluted cDNA template derived from blackberry fruits at 3 typical developmental stages. Reactions followed the manufacturer recommended cycling parameters: 95°C for 30 s, 40 cycles of 95°C for 5 s and 64.5°C for 30 s. The PCR results were analyzed using the CFX Manager software (Bio-Rad), and a threshold cycle of ≤40 was considered to be positive. An *actin* gene, amplified by qACTF and qACTR, was used to normalize raw data and calculate relative transcript levels.

RESULTS

Under the conditions described in this protocol, acetic acid was added to adjust the pH

of the lysate mixture. It was encouraging to find that genomic DNA could be gradually removed along with proteins and other cell debris. However, RNA was not affected. As indicated by the increasing of optical density of 28S and 18S rRNA shown in Figure 1, genomic DNA was gradually removed from the solution and could be pre-precipitated via organic solution extraction. On the contrary, total RNA was enriched at first and then reached a maximum plateau. When approximately 1/50 volume upper phase of acetic acid was added (10 μL), DNA could not be detected upon analyzing the RNA on an agarose gel stained with ethidium bromide.

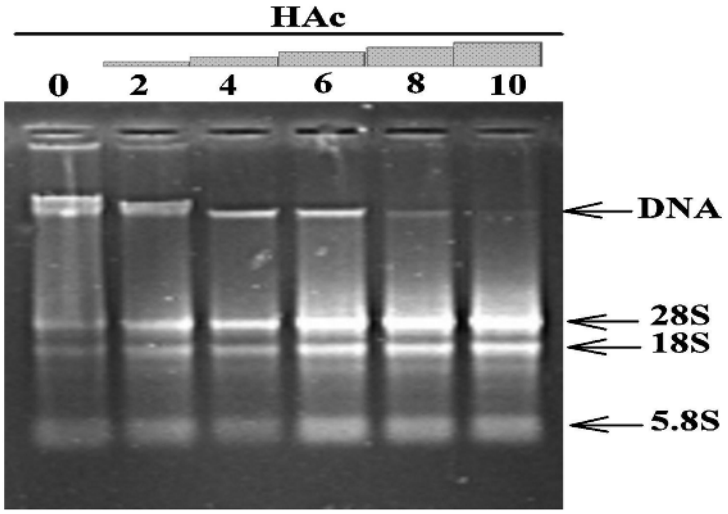

Figure 1. Effects of different volumes of acetic acid (HAc) on genomic DNA removal in the reaction. Six different volumes were set at 0, 2, 4, 6, 8, 10 μL/600 μL mixture, 3 μL total RNA was loaded each lane.

Based on these findings, fruit tissues of blackberry as well as leaves and flowers were used to test such a method. Electrophoresis of the extracted RNA on 1.2% agarose gels showed intense and well-resolved 28S and 18S rRNA bands (Figure 2). Interestingly, the low molecular weight RNAs were still present, indicating good integrity of total RNA (Figure 2). When accessed by spectrophotometric readings, the ratio of OD_{260}/OD_{280} from all samples was above 1.8 and OD_{260}/OD_{230} was greater than 1.9 (Table 2). The results indicated that the RNA isolated by CTAB reagent was scarcely contaminated by proteins and other impurities. At the same time, RNA yield was calculated accordingly. Average yields of total RNA isolated from fruits, leaves and flowers were 101.2, 190.5 and 70 μg/g fresh weight (FW), respectively. This is higher than that obtained by Jones et al. (1997) in their SDS protocol's 50-100 μg/g FW. Furthermore, RNA samples were successfully reverse transcribed and clear bands for CHS (accession No. JN602374) and *actin* gene were amplified at the expected length (Figure 3).

Our recent isolated *RuMYB10* gene, which was a candidate anthocyanin-promoted transcription factor was determined by real-time PCR in three stages according to the developing characters of color appearance. The transcript level of *RuMYB10* gene throughout the fruit growth dramatically increased along with the maturation of the fruits (data not shown). Through the standard and melting curve of the amplification (Figure 4), we could ascertain that the RNA isolated by the depicted method was suitable for gene expression analysis.

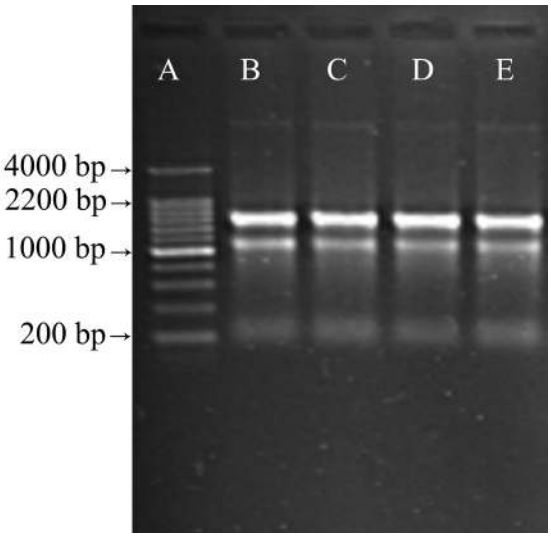

Figure 2. Non-denaturing agarose gel showing the relative concentrations of 28S, 18S, and 5.8S ribosomal RNA. *Lane A* = 200-bp DNA ladder, *lane B-E* = RNA extracted from full ripening fruits.

Table 2. Purity and yield of RNA from different tissues by this protocol.

Tissue	RNA yield[d] (μg/g tissue)	Absorbance ratio (A_{260}/A_{280})	Absorbance ratio (A_{260}/A_{230})
Fruit[a]	101.2 ± 15.2	1.89 ± 0.04	1.91 ± 0.01
Leaf[b]	190.5 ± 13.9	1.97 ± 0.02	1.92 ± 0.04
Flower[c]	70 ± 10.4	2.03 ± 0.01	1.98 ± 0.03

Absorbance at 230, 260, and 280 nm was determined for RNA samples isolated and used in this method. [a]RNA isolated from fruit (black, full ripening); [b]RNA isolated from the first two unfolding leaves; [c]flowers of full blooming was chosen as start material; [d]yield was determined spectrophotometrically as described by Chomczynski and Sacchi (2006).

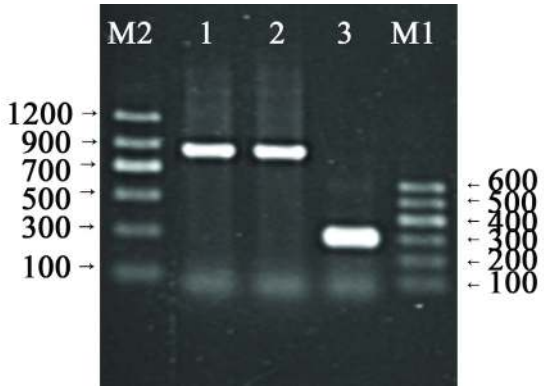

Figure 3. PCR products with degenerate primers (*lanes 1* and *2*) for chalcone synthase and specific primers for *β-actin* (*lane 3*) using cDNA generated from total RNA isolated from blackberry fruit. *Lane M2* = DNA marker II. *Lane M1* = DNA marker I.

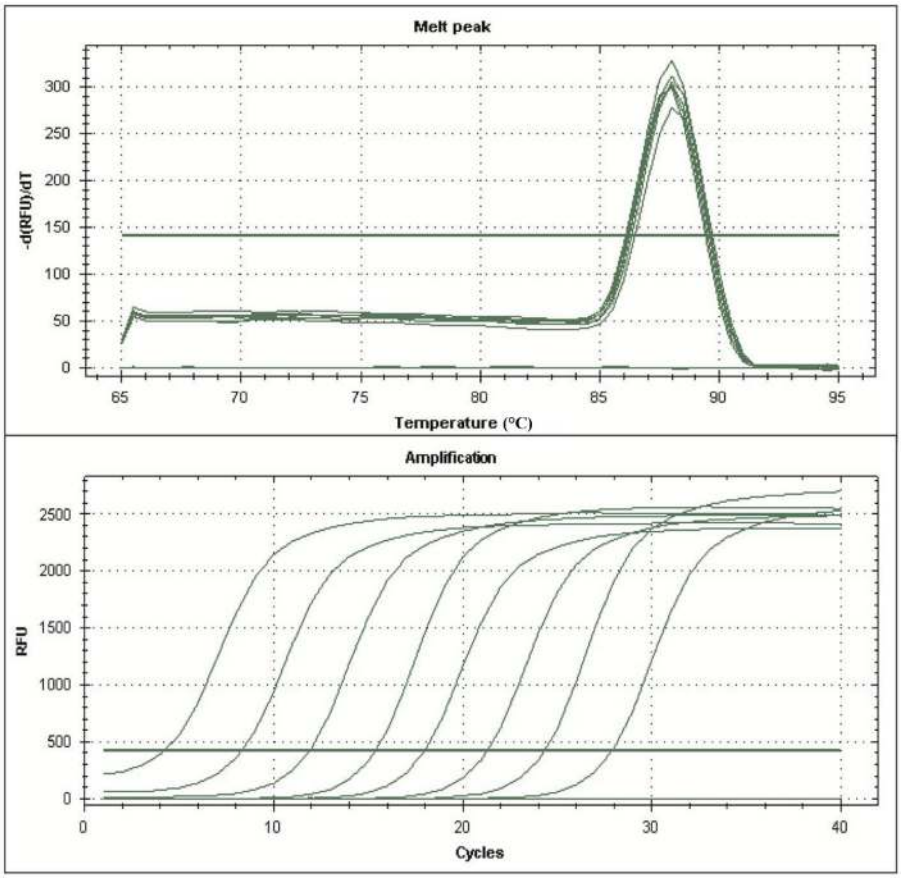

Figure 4. Standard curve and melting curve for amplifying *RuMYB10*.

DISCUSSION

To isolate RNA with high quality and free of contaminations like polyphenols, proteins and genomic DNA from plant tissues is usually a challenging but crucial step for molecular analysis. The two most important factors to address are RNase inhibition and impurities removal (Robert Jr., 2010). Perhaps the most effective reagents for RNase inactivation are guanidinium salts, such as guanidinium thiocyanate and guanidine hydrochloride (Heath and Minnetonka, 1999; Robertson and Leek, 2006). The other detergents commonly used in lysing reagent formulation are SDS, urea and N-lauroyl sarcosine (Liao et al., 2004; Almarza et al., 2006; Rio et al., 2010; Wu et al., 2011). Methods involving CTAB, originally developed for pine tree tissue (Chang et al., 1993), is now prevalently chosen to extract nucleic acid from plant tissues, which are high in polysaccharides, polyphenols and other metabolites (Fort et al., 2008; Ghangal et al., 2009; Wang et al., 2011).

Before we switched to take CTAB as our main detergent for RNA isolation, the lysate formulation described by Jones et al. (1997) was tested. By involving two rounds of incubation

with LiCl and long-time precipitation (90 min, 20,000 *g*), the total procedure could be finished in nearly 2 days. This prompted a higher demand for stringent manipulation environment and experienced operators so as to keep RNA from being degraded by RNase. Meanwhile, reducing the buffer and tissue volumes to perform the procedures in 1.5- to 2.0-mL tubes to increase the throughput number was also needed. Thus, GITC (Chomczynski and Sacchi, 1987) and commercial kits Tri-blue (Shenergy), RNAiso (Takara) were also tried. All these protocols and kits produced either a brown pellet, insoluble RNA or even no RNA.

To minimize the time when the method of Jones et al. (1997) was used, isopropanol was chosen to precipitate RNA instead of LiCl. The subsequent results were severe genomic DNA contamination (data not shown). This can be expected because of the utilization of LiCl in the original flowchart to selectively recover RNA but leaving DNA and some other cell compounds in the solution. That is why LiCl was primarily used in published protocols for RNA isolation. Some other common practices for removing contaminating DNA were either to treat an RNA-containing sample with deoxyribonuclease (DNAse I), followed by phenol and chloroform extraction and additional RNA precipitation step (Fort et al., 2008) or to extract RNA with a relative lower pH reagent as described by Heath and Minnetonka, (1999) in the patent No. 5973137, or selectively bind RNA to a solid matrix and wash DNA away (as most kits do). They were not suitable because cost-effectiveness or copy-right protection.

Considering that high salt lysis buffer could reduce the impact of polysaccharides, starches and other metabolites on nucleic acids (Fang et al., 1992; Wang et al., 2012), CTAB was used as the main cell disruption and RNase inactivation reagent instead of SDS. Significant modifications of the method presented here included more CTAB (3%), and higher Tris-buffer concentration (200 mM) to increase the buffer capacity. It has been added to our RNA isolation procedure that lower concentration of CTAB (2%) could also work for flowers but not so satisfactorily for fruits. Factors/reagents, which facilitated the removal of polyphenolic compounds (PVP and PVPP), and which inhibited ribonucleases (2-mer-captoethanol), were also included. After the formation of CTAB-nucleic complex, we tried to adjust pH of the mixture by directly adding acetic acid. Then, routinely extracted by organic solution such as phenol and chloroform, we were pleased to receive the results speculated in the Results section.

The first report of utilizing glacial acetic acid in RNA isolation with CTAB was based on such a nature of it that intends to be more sensitive to strand cleavage than DNA in an alkali system (Ghangal et al., 2009). In the protocol, use of glacial acetic acid (1 M) along with ethanol for precipitation after phenol and chloroform extraction enhanced the RNA yield. Afterwards, high salt buffer (5 M NaCl) was used to remove the precipitated polysaccharides. When the same procedure was tried on blackberry tissues, viscous pellets were obtained due to the large quantities of polysaccharides.

Actually, the lysing reagents' pH had previously been taken into consideration before writing the manuscript. Early in the method that at lower pH DNA was selectively precipitated by centrifugation into the organic-aqueous interface (Chomczynski and Sacchi, 1987), the single-step GITC protocol might be the first one making use of such a nature. Afterward, nearly all GITC-based kits/protocols containing acid lysing reagent, the pH of which can be lower than 4 (Chomczynski, 2004) and can be as high as 9 (Bugos et al., 1995), perhaps the main differences occur in the art of adjusting lysate pH so as to minimize DNA contaminations.

In the present method, we successfully removed most part of DNA contaminations by adjusting the mixture's pH, and also removed polysaccharides before RNA recovery. When

combined with other strategies to prevent interference by polyphenols, this could be a useful, economic and simple method for plant RNA isolation, especially for those tissues rich in polysaccharides.

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