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RNA Extraction From Different Apple Tissues Rich in Polyphenols and Polysaccharides for cDNA Library Construction

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Abstract. Recovering RNA of high quality and quantity is a prerequisite for ensuring representation of all expressed genes in a cDNA library. An efficient procedure for isolating RNA from bud, internodal shoot, flower, and fruit tissues of apple has been developed. This protocol does not involve the use of phenol, lyophilization, or ultracentrifugation. In addition, this protocol overcomes problems of both RNA degradation and low yield attributed to oxidation by polyphenolic compounds and coprecipitation with polysaccharides, both abundant components in apple fruit and flower tissues. Isolated RNA is of high quality and is undegraded as assessed by spectrophotometric readings and electrophoresis in denaturing agarose gels. RNA quality is further assessed following its use in reverse transcription and cDNA library construction, and it can be used for a number of downstream analyses, including Northern blot hybridization and reverse transcription–polymerase chain reaction (RT-PCR). With this modified protocol, 25–900 µg of total RNA is routinely obtained from 1 g of fresh material. This method is of low cost and easy to perform.

Full text[†]: This article, in detail, is available only in the electronic version of the *Plant Molecular Biology Reporter*.

Key words: apple, bud, cDNA libraries, flower, fruit, RNA extraction, shoot

Abbreviations: Chl:Iaa, chloroform-isoamylalcohol, CTAB, cetyltrimethylammonium bromide; DAP, days after pollination; DEPC, diethyl pyrocarbonate; EST, expressed sequence tag; FW, fresh weight; PVP, polyvinylpyrrolidone; RT-PCR, reverse transcription–polymerase chain reaction.

Introduction

Obtaining RNA of high quality and quantity is a prerequisite for constructing good-quality cDNA libraries with adequate representation of all expressed genes. However, extracting RNA from plant tissues can be difficult and often requires the

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modification of existing protocols or the development of new procedures. This is particularly true for woody species such as apple and pear because they contain high levels of extractable phenols and polysaccharides (Wilson and Blunden, 1983; Mitra and Kootstra, 1993; Salzman et al., 1999) and for mature pre-climacteric and postclimacteric fruit tissues in which biosynthesis of potential interfering substances is known to increase during maturation and ripening (Shellie et al., 1997). Several cetyltrimethylammonium bromide (CTAB)-based methods have been developed for RNA extraction from tissues containing high levels of polysaccharides and phenols (Chang et al., 1993; Malnoy et al., 2001; Jaakola et al., 2001; Zeng and Yang, 2002; Hu et al., 2002). Moreover, 2 extraction reagents, TRIzol (Sigma) and Concert Plant RNA reagent (Invitrogen), are commercially available and can be used for extracting total RNA from plant tissues. However, attempts to use these reagents to extract total RNA from apple fruit and flower tissues have failed to provide high enough quality and quantity of total RNA necessary for cDNA library construction. In this report, we describe a simple and low-cost extraction protocol that provides high-quality and high-quantity RNA suitable for constructing good-quality cDNA libraries from various apple tissues (bud, shoot, flower, and fruit). This protocol is a modification of the pine tree RNA extraction method described by Chang et al. (1993).

Materials and Methods

Plant material

Bud, internodal shoot, flower, and fruit tissues of apple (*Malus x domestica*) cv. GoldRush were collected from mature trees growing in an orchard at the University of Illinois, immediately frozen in liquid nitrogen, and stored at -80°C until needed. All tissues were collected at different stages of development, as follows:

- Dormant and actively growing lateral buds as well as dormant terminal buds
- Dormant and actively growing internodal shoot segments from previous season growth as well as actively growing internodal shoot segments from current season growth
- Flower buds at the pink stage (first pink), flower buds at the balloon stage (full pink), flowers at full bloom (open flowers), and flowers at petal fall (after pollination)
- Fruits collected at 6 stages of development, including young fruitlet I (9 days after pollination [DAP]), young fruitlet II (16 DAP), young fruitlet III (44 DAP), maturing fruit I (104 DAP), maturing fruit II (145 DAP), and ripe fruit (166 DAP)

Solutions and reagents

Diethyl pyrocarbonate (DEPC)-treated water was used for all solutions. The extraction buffer, without β -mercaptoethanol, was autoclaved and stored at room temperature.

- Extraction buffer - 2% CTAB, 2% polyvinylpyrrolidone (PVP) K-30 (soluble), 100 mM Tris HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 0.5 g/L spermidine (free acid) (HRS), 2% β -mercaptoethanol (added just before use)

- Chloroform-isoamylalcohol (Chl:Iaa) (24:1 [v/v])
- 7.5 M LiCl
- 3 M sodium acetate (pH 5.5)
- DEPC-treated water
- 70% (v/v) ethyl alcohol
- RNase out

Protocol

1. Prewarm 10 mL extraction buffer per sample at 60°C in a water bath.
2. Grind 1 g of frozen apple tissue in liquid nitrogen by using a baked mortar and pestle. Transfer powder to 50-mL polypropylene tubes with a chilled autoclaved spatula (1 g frozen tissue = ~5 mL vol). Keep samples in liquid nitrogen until ready.

The following steps must be conducted in a flow hood.

3. Add 10 mL extraction buffer to each tube and keep at 60°C for 15 min. Vortex briefly and keep at 60°C to allow thawing. Then, homogenize with a Tissue Tearer (Biospec Products, Inc.) at a maximum of 30,000 rpm (2,500g). Initially clean Tissue Tearer with RNase out (RNaseZap, Ambion) and then with distilled water between samples. Vortex for 2 min. Keep samples at 60°C for 15 min and vortex several times. Use RNase out to spray gloves occasionally.
4. Add an equal volume of Chl:Iaa (24:1) and immediately vortex for 2 min. Transfer samples to prelabeled, clean, and autoclaved 30-mL Oakridge tubes and vortex.
5. Centrifuge at 10,000g for 10 min at 4°C.
6. Transfer supernatant to a clean Oakridge tube. Carefully pipette aqueous phase and do not collect the white phase. Re-extract with an equal volume of Chl:Iaa. Centrifuge at 10,000g for 15 min at 4°C.
7. Transfer 1.5 mL of supernatant to 2 mL (RNase-free nonstick) Microfuge tubes (~5 tubes for each stage of fruit). Carefully pipette all supernatant but do not collect the white layer. If you notice fibers, add Chl:Iaa and spin again.
8. Add 1/3 vol of 7.5 M LiCl to each tube. Mix by inversion and store at 4°C overnight.
9. Centrifuge tubes at 12,000g for 30 min at 4°C. Discard supernatant by pipetting and quickly dry tubes on a Kimwipe tissue. Wash pellet in 70% ethanol and air-dry it for 5 min.
10. Dissolve RNA in 80 µL DEPC-treated water and pool RNA from the same samples into new 1.5-mL nonstick RNase-free Microfuge tubes.
11. Quantify total RNA by using a spectrophotometer and precipitate in 1/10 vol of 3 M sodium acetate (pH 5.5) and 2.5 vol of 70% ethyl alcohol for 1-3 h at -80°C.
12. Centrifuge at 12,000g for 30 min. Wash pellet with 70% ethyl alcohol, air-dry for 10 min, and dissolve in 200 µL DEPC-treated water. Store RNA at -80°C.

Table 1. Modified oligo₁₈(dT) primers with identifying tag sequences used for fruit tissues.

Stage	Tag Identification When Sequencing From 5' End	Tag Identification When Sequencing From 3' End
Young fruitlet, 9 DAP	Insert ₁₈ (A) TCGTG	cagca ₁₈ (T) insert
Young fruitlet, 16 DAP	Insert ₁₈ (A) TGCTG	cagca ₁₈ (T) insert
Young fruitlet, 44 DAP	Insert ₁₈ (A) TCGGT	ACCGA ₁₈ (T) insert
Maturing fruit I, 104 DAP	Insert ₁₈ (A) TGCCA	TCGCA ₁₈ (T) insert
Maturing fruit II, 145 DAP	Insert ₁₈ (A) TCGGA	TCCGA ₁₈ (T) insert
Mature fruit, 166 DAP	Insert ₁₈ (A) TGCGT	ACGCA ₁₈ (T) insert

RNA analysis

The purified total RNA was quantified with a spectrophotometer (NanoDrop, Technologies Inc.) at wavelengths of 230, 260, and 280 nm. The integrity of total RNA was verified by running samples on 1.2% denaturing agarose gels (Qiagen, RNeasy Mini Handbook).

mRNA extraction and cDNA synthesis

Poly(A)⁺mRNA was isolated twice from total RNA from each tissue by using the Oligotex Direct mRNA kit (Qiagen). mRNA was reverse transcribed into double-stranded cDNA by using a modified oligo₁₈(dT) primer with an identifying tag sequence (Table 1). cDNA from different stages of development from each tissue type were pooled in equal amounts prior to adaptor ligation.

cDNA library construction

cDNA libraries were constructed following procedures described by Bonaldo et al. (1996) and Soares et al. (1994). Double-stranded cDNAs were size-selected (>450 bp), adapted with *EcoRI* adapters at both ends, and then digested with *NotI*. The cDNAs were then directionally cloned into *EcoRI-NotI*-digested pBS II SK(+) phagemid vector (Stratagene). Purified plasmid DNA from the primary library was converted to single-stranded circles and used as a template for PCR amplification utilizing the T7 and T3 priming sites flanking the cloned cDNA inserts. The purified PCR products, corresponding to the entire cloned cDNA population, were used as drivers for normalization. Hybridization between the single-stranded library and the PCR products was carried out for 44 h at 30°C. Unhybridized single-stranded DNA circles were separated from hybridized DNA, rendered partially double-stranded, and electroporated into DH10B cells to generate the normalized library.

RT-PCR

Reverse transcription–polymerase chain reaction (RT-PCR) amplification was performed to demonstrate that the RNA could also be used for other analyses. Primers were constructed on the basis of the conserved sequence for an apple actin gene (Apple_0902.385.C2.Contig633). Primers synthesized were as follows:

Actin - forward (5'-CTACAAAGTCATCGTCCAGACAT-3')

Table 2. RNA yield and quality using spectrophotometric determinations.

Apple Tissue		Absorbancy Ratios		
		OD 260/230	OD 260/280	Yield, $\mu\text{g/g}$ FW
Bud	Dormant terminal	2.26	1.97	648
	Dormant lateral	2.24	1.96	681
	Active lateral	2.16	1.91	777
Shoot	Dormant, previous season	2.25	1.96	691
	Actively-growing, previous season	2.36	1.99	364
	Actively-growing, current season	2.26	1.95	504
Flower	Bud (first-pink stage)	2.26	1.95	680
	Balloon (full-pink stage)	2.29	1.97	640
	Full bloom (open flowers)	2.34	2.01	600
	Petal fall (after pollination)	2.23	1.95	680
Fruit	Young fruitlet, 9 DAP	2.48	1.96	898
	Young fruitlet, 16 DAP	2.19	1.91	692
	Young fruitlet, 44 DAP	2.46	2.02	786
	Maturing fruit I, 104 DAP	2.28	2.02	743
	Maturing fruit II, 145 DAP	2.33	1.95	48
	Mature fruit, 166 DAP	2.28	1.98	18.9

Actin - reverse (5'-TGGGATGACATGGAGAAGATT-3')

Following initial denaturation at 94°C for 3 min, the PCR reaction was carried out for 35 cycles under the following conditions: denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 80 s. The final extension was carried out at 72°C for 6 min.

Results and Discussion

High-quality total RNA was obtained by using the modified pine tree RNA isolation method (Chang et al., 1993). The yields of total RNA ($\mu\text{g/g}$ fresh weight [FW]) were as follows: 648-777 for bud, 364-691 for internodal shoot, 600-680 for flower, and 25-898 for fruit tissues (Table 2). The extraction protocol described herein was efficient in yielding a high quality and quantity of total RNA from all apple tissues and stages tested. However, mature fruit tissue (consisting of both flesh and skin) produced high-quality but low-yield total RNA, which was insufficient for cDNA library construction. To accumulate enough total RNA, it was necessary to extract RNA from 24 and 36 g FW of mature fruit flesh, stages V and VI, respectively. For all samples, the $A_{260/230}$ ratio was higher than 2.0. This indicated that the RNA was of high purity and without polyphenol and polysaccharide contamination (Table 2). The $A_{260/280}$ ratios ranged from 1.91-2.02, indicating a lack of protein contamination (Table 2). The RNA integrity was assessed by the sharpness of ribosomal RNA bands visualized on a denaturing 1.2% agarose gel. For all RNA samples tested, distinct 28S and 18S rRNA bands without degradation were observed (Figure 1A).

The extracted RNA could be used for further analyses as demonstrated by cDNA library construction and RT-PCR. After synthesizing cDNA, we successfully constructed normalized cDNA libraries from bud, shoot, flower, and fruit

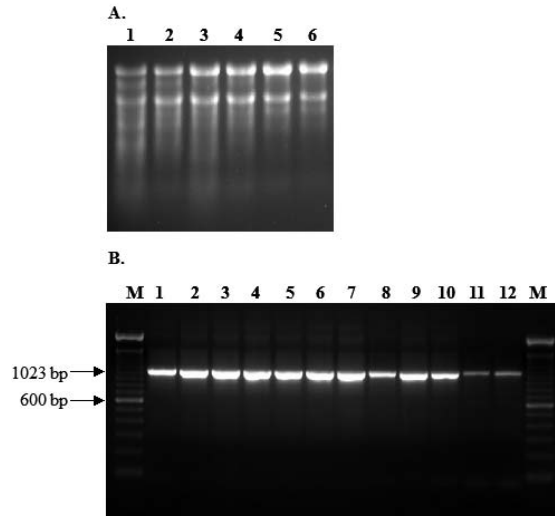


Figure 1. (A) Agarose gel electrophoresis of total RNA from fruit at different developmental stages, including young fruitlets at 9 (lane 1), 16 (lane 2), and 44 (lane 3) days after pollination (DAP) and maturing fruit at 104 (lane 4), 145 (lane 5), and 166 (lane 6) DAP. (B) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of various apple tissues with actin. Lanes 1-4, apple flower stages 1-4; lanes 5-10, apple fruit stages 1-6; and lanes 11-12, dormant and actively growing lateral buds.

Table 3. Sequencing success and quality read-lengths for normalized cDNA libraries from different apple tissues.

Library	Tissue	Successful Sequences, %	Average Insert Size, bp
Mddb	Bud	99.0	760
Mdst	Shoot	96.9	805
Mdfw	Flower	99.0	712
Mdft	Fruit	94.8	706

tissues; expressed sequence tags (ESTs) have been deposited in NCBI. Inserts ranged from 0.5-4 kb, as determined by means of PCR. A total of 192 clones per library were picked and sequenced using an automated sequencer (Model 3730xl Applied Biosystems) to assess quality of the cDNA libraries. Sequencing success was high at 94.8-99%, and the average read-length ranged from 706-805 bp (Table 3).

Furthermore, we successfully amplified actin fragments from flower, fruit, and bud tissues (Figure 1B). End-point PCR analysis showed a differentially expressed actin gene in apple tissues, with the lowest level of expression detected in bud tissue (Figure 1B).

The extraction protocol was completed in 2 d, and the total average extraction time was approximately 4-6 h, including LiCl precipitation. Similar protocols were previously described for leaf and fruit tissues of apple, peach, and kiwifruit by Hu et al. (2002) and for pear leaf tissue by Malnoy et al. (2001). However,

unlike these protocols, our proposed protocol involves the following modifications: grinding of tissue is done by using mortar and pestle; all tissues, including fruit, were frozen in liquid nitrogen prior to extraction, thus eliminating the need for lyophilization; the extraction step was simplified by using only ChI:Iaa (24:1) and eliminating phenol; the frequency of centrifugation was reduced; and ultracentrifugation was completely eliminated.

Acknowledgments

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