A Quick Method for Isolating RNA from Raw and Ripe Fleshy Fruits as Well as for Co-Isolating DNA and RNA from Polysaccharide- and Polyphenol-Rich Leaf Tissues

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Qualitative and quantitative changes in its chemical composition make it difficult to use any single procedure for isolating good-quality RNA from fruits at various ripening stages. Although the CTAB method has eliminated some specific problems, e.g., low pH in raw fruit or high levels of polysaccharides, oligosaccharides and phenolics in raw and ripe fruits, the total time required is long and unsuitable for high throughput. Here, we successfully modified this CTAB protocol to isolate good-quality RNA from (i) fleshy fruits, especially raw and ripe mangos; (ii) the leaves of a succulent air plant; and (iii) an oligosaccharide-rich onion epidermis. This RNA proved useful for downstream transcriptomic applications, where RT-PCR followed by RACE yielded the complete open reading frame of the (mango) terpene synthase gene. We also extended the utility of this protocol to co-isolate good-quality genomic DNA from the supernatant that remained after RNA precipitation. This preparation was useful for the arbitrary primer multilocus amplification of genomic DNA as well as for single locus diversity marker amplifications of the ctDNA and mtDNA.

Keywords: DNA-RNA co-isolation, fleshy fruit, mango, oligosaccharide-rich, ripening, transcriptomic studies

Oligosaccharides, polysaccharides, polyphenols, and other chemical constituents can be the limiting factors when trying to isolate good-quality RNA from plant tissues. Various procedures often fail when used with tissues rich in polysaccharides and secondary metabolites (Logemann et al., 1987; Levi et al., 1992; Lopez-Gomez and Gomez-Lim, 1992). Different chaotropic substances, e.g., guanidine hydrochloride, guanidine thiocyanate, phenol and some detergents, are often incorporated to avoid the co-extraction of polysaccharides. To eliminate phenolic substances, one can use either antioxidants, such as βME, dithiothreitol and PVP or PVPP adsorbents. The selection and optimization of these components in the extraction buffer is a hectic 'trial and error' job when tested with complex plant tissues. Therefore, species-specific or tissue-specific protocols are often developed.

Fruit is one of the most complex tissues to isolate the RNA from. For example, the chemical composition of mango [*Mangifera indica* L. (Anacardiaceae)] changes remarkably during development and especially, during ripening. This may include alterations in fatty acid, lipid and protein contents (Bandopadhyay and Gholap, 1973), a dramatic shift in pH (Germain and Linden, 1981; Kansci et al., 2003), the conversion of starch to sugars, protopectins to pectin (Kansci et al., 2003) and changes in the pulp, from terpenoid-rich to one that is rich in alcohol-aldehyde-esters (Saby John et al., 1999). We have utilized different RNA isolation protocols at various stages to track the transcriptome dynamics of the ripening mango. However, such methodology is unacceptable when trying to illustrate the dynamic transcriptome. Therefore, a comprehensive method is necessary for the isolation of good-quality RNA at all ripening stages. A protocol by Asif et al. (2000), though most effective, requires 2 d for its completion, making it unsuitable for high-throughput applications. Because most published protocols have restricted specificity for tissue types, our objective in this study was to modify this protocol of Asif et al. (2000) in order to develop one that would extend across various complex tissues, such as succulent or oligosaccharide-rich tissues. For RNA isolations, we selected two plant types: 1) a recalcitrant species (Gehrig et al., 2000), the air plant [*Kalanchoe pinnata* (Lam.) Pers. (Crassulaceae)] – which exhibits crassulacean acid metabolism (CAM); and 2) the epidermal tissues of onion [*Allium cepa* L.(Liliaceae)], which are rich in oligosaccharides (Zhou et al., 1999). To confirm the comprehensiveness of our new method, we also attempted to co-isolate DNA and RNA from leaf tissues of mango and air plant.

MATERIALS AND METHODS

Plant Material

The flushing leaves, flowers, and mature fruits of mango (*M. indica* L. cv. Alphonso) were collected from local orchards. Fruits were allowed to ripen at 25°C to 28°C, and were tested at Day 0 (the day of harvesting), and at 2, 5, 10, 15 and 20 d post-harvest. Leaves of the succulent air plant

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Abbreviations: βME, 2-mercaptoethanol; DEPC, diethyl pyrocarbonate; EtBr, ethidium bromide; EtOH, ethanol; PVPP, polyvinylpolypyrrolidone; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; TE, Tris (10 mM) with EDTA (1 mM).

(*K. pinnata*) were collected from a local garden. Bulbs of the red onion (*A. cepa*, locally grown variety) were purchased from a market and the inner epidermis of the scales was peeled. All tissues were frozen in liquid nitrogen and stored at -80°C before processing.

Solutions and Reagents

The extraction buffer contained 100 mM Tris-Cl (pH 8.2), 1.5 M NaCl, 30 mM EDTA (pH 8.0), 2% CTAB, 10 M LiCl, 3 M sodium acetate (pH 5.4), βME, 70% EtOH, chloroform: iso-amyl alcohol (24:2), DEPC-treated and autoclaved MilliQ-grade water, Isopropanol (prechilled), RNase A [prepared and used as directed by Sambrook et al. (1989)], Sterile MilliQ-grade water, TE (pH 8.0), Tris saturated phenol (pH 7.8 to 8.0), and water-saturated phenol (pH 5.2 to 5.5).

RNA Extraction Protocol

For our sampling, 10 mL extraction buffer/gram of tissue was used, to which 20 µL of 2-mercaptoethanol per mL of buffer (~0.2%) was then added. βME could be optimized for different fruits, within a range of 0.1 to 5.0%. Frozen tissue was crushed to a fine powder using mortar and pestle in liquid nitrogen. This powder was then homogenized to slurry with preheated (65°C) extraction buffer. The homogenate was incubated at 65°C for 20 min, with intermittent, thorough vortexing. After the slurry cooled to room temperature (RT), an equal volume of chloroform: iso-amyl alcohol (24:2) was added, followed by vigorous shaking to form an emulsion, with intermittent venting of the tube. Samples were centrifuged at 12000*g* for 10 min at RT. The aqueous phase was collected, to which was added prechilled 10 M LiCl, to a final concentration of 3 M. The RNA was allowed to precipitate at -20°C for 30 min (although incubation between -20°C and -80°C would be equally efficient). The pellet was recovered by centrifugation at 12000*g* at 4°C for 10 min. This RNA yield could be marginally improved by extending the centrifugation period by up to 30 min. The supernatant was preserved at 4°C for DNA isolations. The RNA pellet was dissolved in 10 mL DEPC-treated water, then extracted once with water-saturated phenol, followed by extraction with chloroform: iso-amyl alcohol (24:2). To the aqueous phase, 3 M Na acetate (pH 5.4) was added to a final concentration of 0.3 M, followed by a range of 0.6 to an equal volume of prechilled isopropanol. RNA was allowed to precipitate at -40 to -70°C for 20 to 120 min. Longer incubations could marginally improve this yield. The RNA was recovered by centrifugation at 12000*g* at 4°C for 10 min. Afterward, the pellet was suspended in 1 mL of 70% EtOH, then centrifuged at 12000*g* at RT for 10 min. The vacuum-dried pellet was dissolved in an appropriate volume of DEPC-treated water (100 µL g^{-1} starting material). RNA was quantified spectrophotometrically at 260 nm, and the purity was determined by ratios of A_{260}/A_{230} and A_{260}/A_{280}. RNA quality was assessed by electrophoresing on 1% non-denaturing EtBr-stained agarose gel, using a 1-kB ladder (Promega, USA) as the size marker. Here, we recommend that all reagents be DEPC-treated and autoclaved. The exception is Tris-Cl, which must be prepared in DEPC-treated water and autoclaved. The entire procedure should be conducted under RNase-free conditions.

DNA Extraction Protocol

To the preserved supernatant from the RNA isolation procedure, we added 0.1 volume of 3 M sodium acetate (pH 5.4) and an equal volume of prechilled isopropanol. DNA was allowed to precipitate for 1 h at -20°C, before being recovered by centrifugation at 12000*g* at 4°C for 10 min. The pellet was suspended in 1 mL of 70% EtOH, and then centrifuged at 12000*g* at RT for 10 min. The vacuum-dried pellet was dissolved in an appropriate volume of sterile MilliQ-grade water, i.e., 1 mL g^{-1} starting material. DNA was treated with RNase A for 30 min at 37°C (15 µg mL^{-1} DNA solution). RNase A was removed via extraction with an equal volume of Tris-saturated phenol (pH 7.8 to 8.0) and centrifugation at 12000*g* at RT for 10 min. The aqueous phase was re-extracted with equal volumes of chloroform: iso-amyl alcohol (24:2) to remove the traces of phenol by the centrifuging at 12000*g* at RT for 10 min. To the aqueous phase, 0.1 volume of 3 M sodium acetate (pH 5.4) was added, followed by 0.6 to an equal volume of prechilled isopropanol. DNA was allowed to precipitate for 1 h at -20°C and then recovered by centrifugation at 12000*g* at 4°C for 10 min. The pellet was suspended in 1 ml of 70% EtOH (RT), then centrifuged at 12000*g* at RT for 10 min. After vacuum-drying, the pellet was dissolved in an appropriate volume (100 µL g^{-1} starting material) of TE (pH 8.0). DNA was quantified spectrophotometrically at 260 nm, and its purity was determined by ratios of A_{260}/A_{230} and A_{260}/A_{280}. The quality of this DNA was assessed by electrophoresing in 0.8% EtBr-stained agarose gel.

Testing the Quality of RNA

To assess the quality of total RNA from mango fruit, we isolated the poly(A)$^+$ mRNA and performed RT-PCR, followed by RACE, to obtain a complete reading frame of the gene of interest.

Poly(A)$^+$ mRNA Isolation

Poly(A)$^+$ mRNA was isolated from the total RNA of mango flowers, fruits, and leaves by using Oligo dT cellulose (Sigma, USA), as described by Breeden (2006). The Poly(A)$^+$ mRNA was then quantified spectrophotometrically at 260 nm, and the purity was determined by A_{260}/A_{280} ratio. We assessed its quality by electrophoresing in 1% non-denaturing EtBr-stained agarose gel, using a 1-kb ladder as the size marker.

RT-PCR Analysis

To synthesize the single-stranded cDNA, we performed RT-PCR according to the protocol for the Clontech Advantage™ (Clontech, USA) RT-for-PCR kit. Reverse transcription was carried out at 42°C for 1 h, followed by heat-inactivation of the MMLV reverse transcriptase at 94°C for 5 min, using oligo-$(dT)_{18}$ primers. This first-strand cDNA served as template for further PCR analysis, as directed by the manufacturer. A terpene synthase gene-specific primer pair was used to amplify the cDNA fragment of interest. PCR was

carried out for 35 cycles, with conditions including denaturation at 94°C for 45 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min 30 s. A final extension was carried out at 72°C for 20 min. Amplicons of the expected size were obtained for the desired genes and were inserted into the pGEM®-T Easy vector system (Promega) for transformation into an *Escherichia coli* strain TOP10. The transformed clones were sequenced with a Megabase 1000 DNA sequencer (Amersham Biosciences, USA), then annotated with help from a Basic Local Alignment Search Tool (BLAST) homology search in the National Center for Biotechnology Information (NCBI) sequence database (http://www.ncbi.nlm. nih.gov).

RACE Reaction

From the annotated cDNA sequence, gene-specific primers for the RACE reaction were synthesized. We used the Clontech SMART™ RACE cDNA Amplification Kit to obtain 5′- and 3′- end fragments that overlapped each other in the middle. Their identities were confirmed by sequencing. A complete reading frame was obtained using the terminal primers, and was confirmed by sequencing.

Polymerase Chain Reaction

The utility of this isolated DNA was checked with polymerase chain reactions (PCRs). Inter simple sequence repeat (ISSR) primers - UBC 808, 834 and 845, procured from the University of British Columbia (UBC, Canada) were used to test the nuclear DNA. Viability of the ctDNA in the preparation was tested by amplifying the gene for the chloroplastic RuBisCO large subunit (RbcL). Similarly, the viability of mtDNA was tested by amplifying the intron between exons A and B of the *nad*1 gene. All PCR products were electrophoresed in 2% agarose gel with a 1-kb ladder as the size marker.

RESULTS AND DISCUSSION

Using the Phenol-SDS protocol, as well as those of Chomczynski and Sacchi (1987) and Lopez-Gomez and Gomez-Lim (1992), we obtained about 100 μg g^{-1} of high-quality RNA from ripe mango fruits. However, direct treatment of raw fruits with strong chaotropic chemicals, e.g., guanidine thiocyanate, guanidine hydrochloride, and phenol-SDS (Sambrook et al., 1989), yielded very little (10 to 20 μg g^{-1}) or no RNA. This failure was probably due to high levels of oligosaccharides, polysaccharides and polyphenols. Furthermore, use of the protocol by Liu et al. (1998), which includes ice-cold potassium acetate to precipitate genomic DNA and secondary metabolites, produced very low amounts of RNA. Incorporating PVP and PVPP did not increase this yield either. In contrast, the modified protocol described here could be successfully used to isolate large amounts (consistently 100 to 250 μg g^{-1}) of good-quality RNA from mango fruits at all ripening stages, as well as from mango flowers and leaves and complex tissues from other species (Fig. 1). Supplementing CTAB in the extraction buffer successfully eliminated contamination from oligosaccharides and polysaccharides. This was especially true for the onion epidermis, where no gel formed in the preheated extraction buffer containing CTAB. Furthermore, βME inhibited the oxidation of phenolic compounds, thereby limiting damage from free radicals. Lithium chloride selectively precipitated RNA and the following treatment by acidic phenol, completely removed the polysaccharide and protein impurities. Phenol traces were cleared by treatment with chloroform: iso-amyl alcohol. We were unable to use PVP in the extraction buffer because it was incompatible with the phenol extraction. Moreover, when PVPP was used, the protocol had to be extended to accommodate the additional steps required to remove it later.

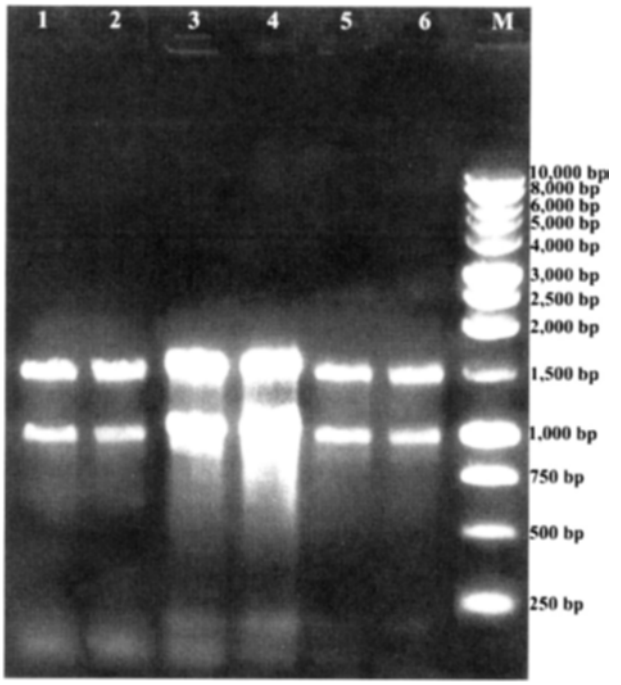

Figure 1. Total RNA separated on 1% non-denaturing EtBr-stained agarose gel isolated from: Lane 1, raw mango fruit; Lane 2, ripe mango fruit; Lane 3, mango flowers; Lane 4, mango leaves; Lane 5, air plant leaves; Lane 6, onion bulb; Lane M, 1-kb DNA size ladder.

During the initial trials, the extraction buffer suggested by Asif et al. (2000) did not work efficiently with the mango pulp, such that a high proportion of degraded RNA was frequently found in the preparation. However, increasing the concentrations of EDTA and βME solved that problem. This protocol could be completed within five hours therefore, was advantageous over the Asif et al. (2000) protocol, which extended up to two days. We eliminated the polysaccharide removal step from Asif et al. (2000) protocol as the preheated CTAB buffer and later treatment of phenol sufficiently removed the carbohydrate contamination. Thus two centrifugation steps were reduced. Furthermore, reducing the initial incubation at 65°C and shortening the overnight precipitation steps yielded equally good-quality RNA.

When analyzing the RNA from all plant tissues, including the succulent leaves and the onion bulb epidermal scale, we found that the spectrophotometric A_{260}/A_{280} ratio to detect protein contamination (Logemann et al., 1987; Manning, 1990) was consistently between 1.8 and 2.0, while the A_{260}/A_{240} ratio, for detecting polysaccharide or polyphenolic con-

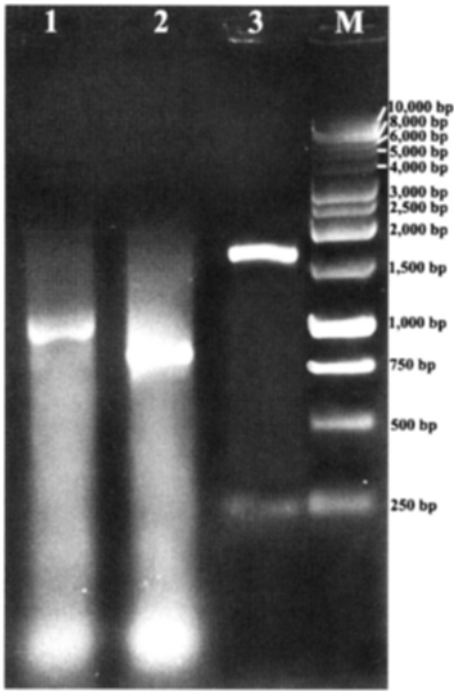

Figure 2. Electrophoresis of RACE fragments. Lane 1, 5′ RACE fragment; Lane 2, 3′ RACE fragment obtained with Clontech SMART™ RACE cDNA Amplification Kit; Lane 3, complete cDNA reading frame annotated as sesquiterpene synthase, obtained by end-to-end PCR; Lane M, 1-kb DNA size ladder.

tamination, always exceeded 1.0. Visualization on an EtBr-stained agarose gel revealed equally intense bands of 25S and 18S rRNA, without any degradation (Fig. 1).

The Poly(A)$^+$ mRNA from our mango tissues, air plant leaves, and onion bulbs appeared as smears ranging from 6000 to 200 b on the agarose gel. A_{260}/A_{280} ratios for each poly(A)$^+$ mRNA preparation always remained between 1.6 and 2.0, indicating that our total-RNA preparations were of good quality. Furthermore, we were able to obtain a 600-bp fragment via PCR with gene-specific primers. This fragment exhibited partial sequence similarity with the sesquiterpene synthase genes from various plants. Based on the sequence of this cDNA fragment, RNA was subjected to RACE, from which we obtained a 900-bp 5′ RACE fragment and a 750-bp 3′ RACE fragment (Fig. 2). Start and Stop codons were easily identified in fragment sequences, and those fragments also showed high similarity with known orthologous genes. The complete reading frame of the gene (1650 bp) was obtained by using terminal primers (Fig. 2); its fragment sequence exhibited ~60% similarity (for nucleotide as well as deduced amino acid sequences) with the conserved domains of known genes for sesquiterpene synthase. Successful isolation of this 1650-bp cDNA fragment demonstrated the utility of this RNA isolation method for the functional genomics experimentation.

DNA isolated from the leaves of mango and air plants showed A_{260}/A_{280} ratios of 1.6 and 2.0, and an A_{260}/A_{240} ratio of >1.0. On an agarose gel, this DNA was observed as an intact, single band. Although isolation of DNA from polyphenol- and polysaccharide-containing tissues is as tedious as that of RNA from such tissues, the use of CTAB eliminates these problems (Aljanabi et al., 1999; Tel-Zur et al., 1999). Previous co-isolation protocols using SDS (La Claire and Herrin, 1997; Alvarez et al., 2004) are of little value with complex tissues, whereas extraction buffer containing CTAB is extremely successful. This enhances the effectiveness of co-isolation procedures, even when considering tissue limitations. Here, amplifications with all three ISSR primers yielded polymorphic band patterns with our mango and air plant DNAs, demonstrating the quality of nuclear DNA in preparations for multilocus DNA marker applications. The chloroplastic RbcL gene and mitochondrial *nad*1 intron were also successfully amplified (Fig. 3). Both the conserved length of RbcL and the size variation in the *nad*1 intron prove the viability of chloroplastic and mitochondrial DNA in such preparations.

In summary, our modified protocol enabled us to shorten the time required to isolate good-quality RNA and DNA from various ripening stages of fruit and from other complex tissues. These isolated RNA and DNA could be successfully

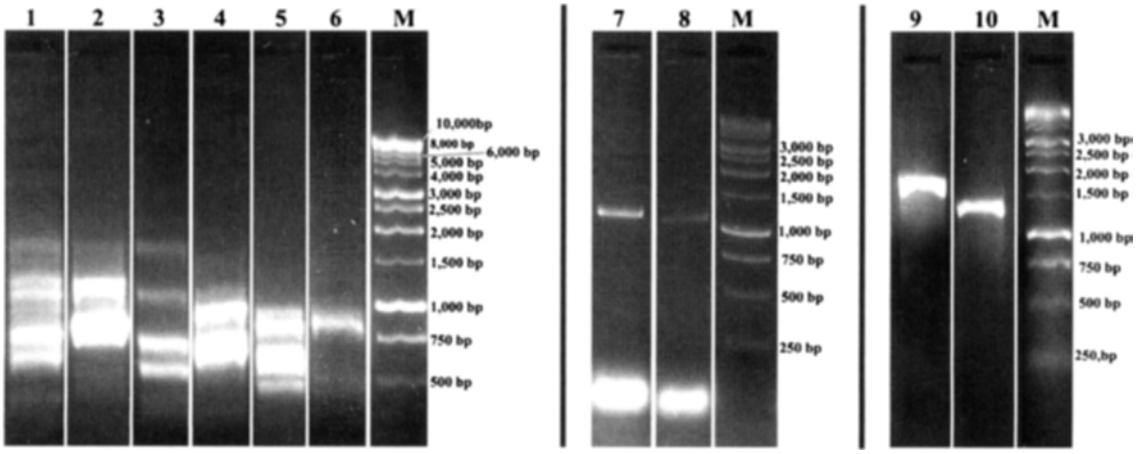

Figure 3. DNA amplification profiles obtained with UBC 808, 834 and 845 ISSR primers. Lanes 1, 2 and 3: mango genomic DNA; Lanes 4, 5 and 6: *K. pinnata* genomic DNA; Lanes 7 and 8: 1.2-kb chloroplastic RbcL gene amplified in mango and *K. pinnata*, respectively; Lanes 9 and 10: size variations among introns of mitochondrial *nad*1 gene of mango and *K. pinnata*, respectively; Lane M: 1-kb DNA size ladder.

used for various downstream applications.

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