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Isolation of High-Quality RNA from Apple (*Malus domestica*) Fruit

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It is difficult to isolate sufficient quantities of high-quality RNA from apple fruit. An abundance of polyphenolic compounds and polysaccharides and a relatively low concentration of RNA in the fruit tissue create conditions that hamper RNA isolation when standard techniques are used. We have developed two RNA isolation methods that include an initial homogenization and extraction with acetone or ethanol. These in turn remove the interfering compounds and precipitate the protein and nucleic acids for subsequent RNA extraction. The quality of RNA was satisfactory with both acetone and ethanol preparations; however, the acetone powder produced consistently higher quantities of RNA.

KEYWORDS: RNA isolation; apple fruit; RT-RCR

INTRODUCTION

The isolation of high-quality RNA is a prerequisite for the study of gene expression during fruit development and ripening. Unfortunately, however, the isolation of RNA from certain fruits can be difficult because they contain high quantities of metabolites, e.g., phenolics and polysaccharides. Pectins can be especially problematic in some fruits. Several publications describe extraction techniques for specific plant tissues that pose problems for the isolation of large quantities of high-quality RNA (*1*−*8*).

Apples contain large amounts of both phenolics (*9*, *10*) and pectins (*11*). Ripe apple fruit have a particularly high concentration of soluble pectins that can form gels and are commonly used to thicken fruit preserves (*11*). The pectin gel can encapsulate large molecules such as nucleic acids and proteins, which can greatly reduce the yield and quality of protein and nucleic acid extracts. Furthermore, apple fruit are often stored for several months at low temperature and under reduced oxygen and/or high CO_2. All of these treatments reduce the metabolic rate of the fruit, which in turn is expected to decrease the RNA content (*12*−*14*). The very low concentration of RNA in stored apple fruit and the high content of phenolics and soluble pectins in ripening fruit make RNA isolation a difficult task. At present, there are no commercial kits available that efficiently extract RNA from apples (*2*). Moreover, our early attempts to isolate RNA with standard procedures, i.e., guanidinium thiocyanate−phenol−chlorophorm (*15*) or modified hot borate methods (*16*), failed to provide a sufficient quantity of high-quality RNA. Therefore, we developed and tested two procedures for isolating apple fruit RNA that consistently produced greater quantities of high-quality RNA than did standard published procedures.

MATERIALS AND METHODS

Acetone Extraction. (1) Apples were sliced into liquid N_2 and therein ground to a fine powder. The powder was suspended in 90% acetone, which had been precooled with the addition of dried ice pieces (30 mL of acetone per gram of tissue) and homogenized briefly with a Polytron. Homogenization with organic solvents should be done in the hood, and precautions should be taken to avoid combustion. (2) The mixture was poured into a sintered glass funnel whose base was covered with a number 1 Watman filter paper and inserted into a conical vacuum flask connected to a water vacuum pump. (3) To this, 90% acetone was added continuously until the tissue looked completely white. The cleared dehydrated tissue can be stored for long periods at −70° C. (4) To 1 g of cleared, powdered tissue, 10 mL of warm extraction buffer (65° C), consisting of 100 mM Tris-HCl (pH 8), 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA) (pH 8), 2% cetyl-methylammonium bromide (CTAB), and 2% β-mercaptoethanol, was added. The contents were then mixed by vortexing the tube and incubated at 65° C for 1 h, with gentle vortexing at 15 min intervals. (5) The tube was cooled to room temperature, and an equal volume of chloroform/isoamyl alcohol (24:1) was added and vortexed gently until the two phases formed an emulsion. The emulsion was centrifuged at 14500*g* for 15 min at room temperature. (6) The upper aqueous phase was collected, and an equal volume of chloroform/isoamyl alcohol (24:1) was added again, mixed, and centrifuged as above. (7) The aqueous phase was collected, and 10 M LiCl was added to a final concentration of 3 M LiCl. The sample was stored at 4° C overnight to allow for RNA precipitation. (8) The RNA was recovered by centrifugation at 14500*g* at 4° C for 20 min. The pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water, and the aqueous phase was sequentially extracted with equal volumes of phenol, phenol-chloroform/

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isoamyl alcohol (24:1), and finally chloroform/isoamyl alcohol (24:1). At each step, the tubes were centrifuged for 10 min at room temperature in an Eppendorf centrifuge and the upper aqueous phase was collected. (9) RNA in the aqueous phase was precipitated by the addition of $^1/_{10}$ volume of 3 M Na acetate (pH 5.2) and 3 volumes of 100% ethanol. The sample was then stored at −20° C overnight to allow the RNA to precipitate. (10) The RNA was recovered by centrifugation in a microfuge at 4 °C for 20 min. The pellet was washed with 70% ethanol, centrifuged as above, and vacuum-dried and dissolved in 50 μL of sterilized DEPC-treated water. (11) The purity of the RNA was determined by calculating the ratio of the absorbance at 260 and 280 nm. The quantity of RNA was calculated using an extinction coefficient of 40 μg/mL for absorbance of 1 optical density (OD) at 260 nm. The spectrophotometer was blanked with DEPC-treated water in a quartz cuvette, and 1 μL of the RNA solution was pipetted into a matching quartz cuvette containing 1 mL of DEPC-treated water.

Ethanol Extraction. (1) Apples were sliced into liquid N_2 and therein ground to a fine powder. The powder was added to a solution consisting of 85% ethanol, 0.1% sodium dodecyl sulfate (SDS), and 0.1% sodium thiosulfate and homogenized with a Polytron for 30−40 s. The suspension was centrifuged at 10086*g* at 4 °C for 15 min. (2) The supernatant was discarded, and the pellet was washed by suspending it in a solution of 75% ethanol, 0.1% SDS, and 0.1% sodium thiosulfate. It was then centrifuged as above. The residue was kept at −70 °C. (3) Warm extraction buffer (65 °C) was added as described in step 4 of the instructions for acetone extraction. The rest of the steps were as described in the same instructions.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Northern Blots. As pointed out above, the quality of RNA is reflected in the ability to clone and study the expression of apple fruit genes. In this regard, we have isolated a number of apple genes using these RNA samples (see GeneBank accessions for *Malus domestica* submitted by T. Solomos et al.). We have also studied the expression of a number of genes using RT-PCR and Northern blot analysis. In this paper, we present some sample results for the expression of transcripts for C_2H_4 biosynthesis enzymes, 1-aminocyclopropene-1-carboxylic acid synthase (ACS 1) and 1-aminocyclopropene-1-carboxylic acid oxidase (ACO1) (*17*), in RNA extracted from "Granny Smith" apples stored at low temperatures, reduced oxygen atmospheres, and/or 1-methylcyclopropene (1-MCP).

Two primers were prepared that synthesize a 1400 kb ACS1 fragment (GeneBank AF312737): Plus 1, ACGGCCAAGACTCCTCCTACTTCTTAGG, and Minus 1, AGACCAGGCTACCTTTCATCTACC. The RNA was extracted using the acetone procedure and then treated with DNAase prior to RT-PCR using the Invitrogen (Carlsbad, CA) Superscript Reverse Transcriptase RT-PCR two-step kit. The labeling of the probes was carried out using the Invitrogen RadPrime cDNA labeling kit and following the instructions.

RESULTS AND DISCUSION

Quantity and Quality of the RNA. The samples that were used for determining the quantity of RNA were prepared by using two fruits that had been stored at 1 °C for 45 days and were close to the C_2H_4 climacteric peak. The liquid N_2 powder from each individual fruit was prepared as described earlier. To extract RNA, 10 g of the powder was used for each procedure. The quantity of RNA was better with the acetone extraction than with the ethanol extraction procedure (**Table 1**). It should be noted that the fruit used for this comparison were stored at 1 °C for 45 days, and it was expected that the RNA concentration per gram of tissue would be relatively low compared to other plant tissues, e.g., leaves. In high-quality RNA, the value of the 260/280 OD ratio varies between 1.8 and 2.1. It may be seen from **Table 1** that although the yield was higher with the acetone method the quality was satisfactory with both extraction procedures. The quality of the RNA was further assessed by gel electrophoresis. The samples were electrophoresed on agarose gels following previously published methods (*18*). The ethidium bromide fluorescence of the two ribosomal RNAs (28S and 18S) indicated that the RNA was not degraded and was of good quality using either protocol (**Figure 1**).

Table 1. Comparison of the Spectral Quality and Quantity of RNA Extracted Using the Two Methods Described above[a]

sample	A_{260}	A_{280}	ratio of A_{260}/A_{280}	yield (μg/g FW)
acetone method	0.3108	0.1585	1.96	41
alcohol method	0.1115	0.0606	1.84	22

[a] Apple fruit were stored at 1 °C for 45 days, peeled, sliced into liquid N_2, and pulverized. A total of 10 g of the pulverized, frozen tissue was used for each extraction procedure.

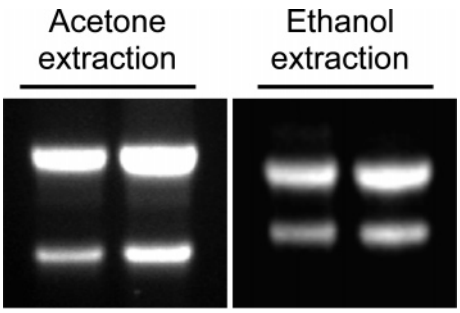

Figure 1. Gel electrophoresis of apple fruit RNA prepared from either acetone- or ethanol-extracted tissue as described earlier in the text. Two fruits were used for each treatment. Fruits were stored for 45 days at 1 °C. A total of 20 μg of total RNA from each sample was run on the gel. The two primary ethidium-bromide-stained bands in each lane are the 28S and 18S ribosomal RNAs.

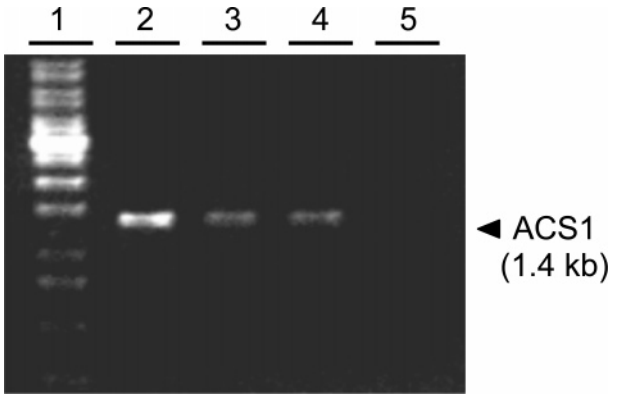

Figure 2. RT-PCR for ACS1 using acetone-extracted fruit tissue. "Granny Smith" fruit were stored for 61 days postharvest at 6.5 °C. Lane 1, *Eco*RI-digested λDNA; lane 2, air control; lane 3, 2 μL/L 1-MCP; lane 4, 1.013 kPa O_2; lane 5, 1.013 kPa O_2 + 2 μL/L 1-MCP.

RT-PCR and Northern Blots. The RNA used for the RT-PCR was prepared from "Granny Smith" fruit that were stored at 6.5 °C for 61 days postharvest in air, 2 μL/L 1-MCP, 1.013 kPa O_2, or 2 μL/L 1-MCP + 1.013 kPa O_2. **Figure 2** shows a clean PCR amplification where the size of the amplified fragments met expectations. Moreover, the fluorescence intensity of the PCR products correlated with the rate of C_2H_4 evolution for the treatments (data not shown).

In addition to RT-PCR, the quality of the RNA was further demonstrated by Northern blot analysis using RNA from both extraction procedures. The RNA used for the Northern blots of ACS1 were prepared from fruits that were stored at 6.5 °C, in air (controls), 2μL/L 1-MCP, 1.013 kPa O_2, and 1.013 kPa O_2

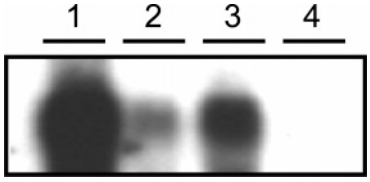

Figure 3. Northern blot for ACS1 using acetone-extracted fruit tissue. RNA was from fruits that were stored for 61 days at 6.5 °C. Lane 1, air control; lane 2, 2 μL/L 1-MCP; lane 3, 1.013 kPa O_2; lane 4, 1.013 kPa O_2 + 2 μL/L 1-MCP. Each lane was loaded with 20 μg of total RNA.

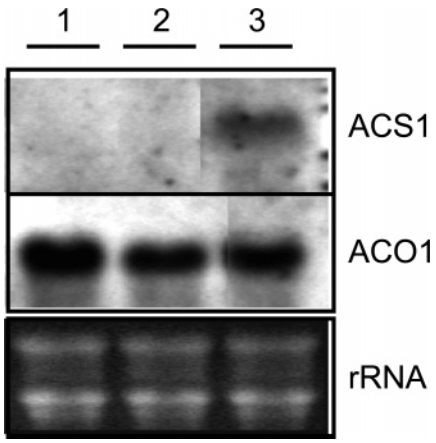

Figure 4. Northern blot for ACS1 and ACO1 using ethanol-extracted fruit tissue. "Granny Smith" fruit were stored for 22 days at 1 °C. Lane 1, air control; lane 2, 2 μL/L 1-MCP; lane 3, 1.52 kPa O_2. Each lane was loaded with 20 μg of total RNA.

+ 2μL/L 1-MCP. The fruits used for the ethanol extraction results displayed in **Figure 4** were stored at 1 °C for 22 days in air, 2 μL/L 1-MCP, and 1.52 kPa O_2. At the sampling time, only the apples stored in air expressed detectable amounts of ACS1 transcripts (**Figure 4**). This was reflected in a measurable amount of C_2H_4 being synthesized in these fruit (data not shown). In apple fruits, the expression of ACS1 is closely linked to ripening and the amount of ethylene being synthesized (**Figures 3** and **4**). ACO1, on the other hand, is constitutively expressed in mature apple fruit (**Figure 4**).

In summary, the results demonstrate that the quality of RNA was similar with both procedures but the yield of RNA was greater using the acetone extraction protocol. Furthermore, although we typically store the dehydrated acetone powder at −70 °C, it is expected that it could be safely stored at higher temperatures, e.g., −20 or possibly 4 °C.

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