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ARTICLE

A Rapid and Efficient Method for Purifying High Quality Total RNA from Peaches (*Prunus persica*) for Functional Genomics Analyses

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ABSTRACT

Prunus persica has been proposed as a genomic model for deciduous trees and the *Rosaceae* family. Optimized protocols for RNA isolation are necessary to further advance studies in this model species such that functional genomics analyses may be performed. Here we present an optimized protocol to rapidly and efficiently purify high quality total RNA from peach fruits (*Prunus persica*). Isolating high-quality RNA from fruit tissue is often difficult due to large quantities of polysaccharides and polyphenolic compounds that accumulate in this tissue and co-purify with the RNA. Here we demonstrate that a modified version of the method used to isolate RNA from pine trees and the woody plant *Cinnamomun tenuipilum* is ideal for isolating high quality RNA from the fruits of *Prunus persica*. This RNA may be used for many functional genomic based experiments such as RT-PCR and the construction of large-insert cDNA libraries.

Key terms: cDNA library, fruit, fruit trees, functional genomics, peach, polysaccharides, *Prunus persica*, RNA isolation, *Rosaceae*, RT-PCR.

Abbreviations: DEPC: diethyl pyrocarbonate; RT-PCR: reverse transcription-polymerase chain reaction; EtBr: ethidium bromide; FW: tissue fresh weight.

INTRODUCTION

"The rose is a rose, and was always a rose. But now the theory goes that the apple's a rose, and the pear is, and so's the plum, I suppose.
The dear only knows what will next prove a rose..."

Robert Frost (Poem lyrics of the Rose Family)

Rosaceae (the rose family) includes economically important fruit crops such as apples (genus *Malus*), pears (genus *Pyrus*), raspberries/blackberries (genera *Rubus*), strawberries (genus *Fragaria*), and stone-fruits such as peaches, plums, cherries, almonds, apricots (genus *Prunus*). Among the species within the *Rosaceae* family, it has been proposed that *Prunus persica* (peach) may serve as an ideal genomic model (Christensen, 2003; Georgi et al., 2002; Sosinski et al., 2000). The characteristics of peach that make it a very interesting candidate as a genomic model are that it is a self-pollinating diploid (2n=16), has a small genome size of approximately 300Mb haploid size (approximately twice the size of Arabidopsis), and a relatively short juvenile stage for a perennial tree species (Arumuganathan and Earle, 1991; Baird et al., 1994; Georgi et al., 2002; Sosinski et al., 2000). Additionally, numerous efforts have been made to create tools to assist in developing peach as a genome model, and molecular markers, genetic linkage maps and BAC libraries have been developed (Dirlewanger et al., 1998; Georgi et al., 2002; Sosinski et al., 2000; Wang et al., 2002a, b).

To further develop peach as a genomic model, it is necessary to optimize protocols for isolating total RNA so that cDNA libraries may be constructed to identify EST sequences and to allow gene expression analyses to be performed to begin to analyze putative gene functions. To this end, we have compared the efficiency of three different protocols for RNA isolation. The first two protocols: (1) A phenol:chloroform extraction and LiCl precipitation protocol (Das et al., 1990) and (2) A guanidinium thiocyanate-phenol-chloroform extraction protocol (Chomczynski and Sacchi, 1987), resulted in total RNA that was contaminated with large quantities of polysaccharides and phenolic compounds as determined by spectrophotometer analyses. The third protocol (Peach protocol) is one that we have developed to optimize RNA isolation from peach fruits containing high levels of polysaccharides and poly-phenolic compounds. This protocol is a modification of several different RNA isolation protocols from plants. (Chang et al., 1993; Salzman et al., 1999; Zeng and Yang, 2002). The Peach protocol uses CTAB, high salt concentrations (NaCl), and polyvinylpyrrolidone (PVP40) (PVP is an antioxidant able to remove phenolic compounds). This protocol yields high-quality total RNA from peach/nectarine fruits, suitable for functional genomics analyses such as RT-PCR analysis and cDNA library construction.

MATERIALS AND METHODS

Plant material

Prunus persica (O'Henry variety) fruits at four different post-harvest stages were collected, peeled, sliced in wedges, quickly frozen in liquid nitrogen, and stored at -80°C until further use.

Solutions and reagents

Solutions were treated with DEPC as described by Sambrook et al. (1989) and autoclaved. Tris-HCl (pH 8.0), prepared with DEPC-treated water, was added to the appropriate solutions post-autoclaving.

Extraction buffer: 2% (w/v) CTAB, 2% (w/v) PVP (mol wt 40,000), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.05% spermidine trihydrochloride, 2% ß-mercaptoethanol (added just before use).

SSTE: 1 M NaCl, 0.5% SDS, 10 mM Tris HCL (pH 8.0), 1 mM EDTA (pH 8.0)
Chloroform-isoamyl alcohol (24:1, v/v)
10 M LiCl
Absolute Ethanol
70% Ethanol (in DEPC water)

Peach Protocol for RNA isolation

Extraction # 1

Preheat 10 mL extraction buffer to 65°C in a water bath. Using a mortar and pestle, grind 4 g of fruit mesocarp in the presence of liquid nitrogen. Quickly transfer the frozen powder to the warm extraction buffer. Mix completely by vortexing the tube. Incubate samples at 65°C for 15 min. Be sure to vortex the sample several times during this incubation period to avoid separation of the tissue and extraction buffer. Add an equal volume of chloroform-isoamyl alcohol (24:1) and vortex vigorously. Centrifuge at 12,000 g for 20 min., or longer if the phases are not clearly separated. Transfer the supernatant to a new tube; it will be very viscous at this stage. Re-extract the sample with an equal volume of chloroform-isoamyl alcohol. (24:1). Centrifuge as previously described. Carefully transfer the supernatant to a new tube. To ensure that high quality RNA is obtained, avoid transferring the interphase or chloroform. If problems arise, the samples can be centrifuged again as previously described.

Precipitation #1:

Add 0.25 volumes of 10 M LiCl to the supernatant. Mix well by inverting the tube. Incubate samples overnight at 4°C. Pellet the RNA by centrifuging the sample at 12,000 g for 35 min at 4°C. Carefully discard the supernatant. Resuspend the pellet with 500 µl SSTE. Transfer the resuspended pellet to a microfuge tube

Extraction #2:

Extract the sample with an equal volume of chloroform:isoamyl-alcohol to further reduce contaminants. Vortex, centrifuge at 14,000 g for 10 min at 4°C, and recover the aqueous phase.

Precipitation #2:

Add 2 volumes of 100% ethanol to the sample. Precipitate the RNA at -80°C for 30 min. Centrifuge at 14,000g for 20 min, at 4°C. Discard the supernatant and wash pellet with 1 ml 75% ethanol. Centrifuge at 14,000 g for 10 min at 4°C. Remove supernatant and dry the pellet at room temperature. Dissolve the RNA in DEPC-treated water (pH 5.0). Store at -80°C until future use.

Quantification and Quality Control

Calculate RNA quality and quantity spectrophotometrically by analyzing absorption ratios: A260/240 and A260/280. Absorption ratios A260/240 and A260/280 detect polysaccharide/polyphenolic contaminants and protein contaminates, respectively (Asif, 2000; Logemann et al., 1997; Manning, 1990). Confirm RNA quality by an EtBr stained 1.5% agarose gel containing 3% formaldehyde.

RT-PCR

cDNA synthesis was prepared using Revert Aid ™ H Minus M-MuLV Reverse Transcriptase (MBI Fermenta) according to the manufacturer's specifications. RT-PCR amplifications were performed using actin specific primers designed using the nucleotide sequence of *Prunus persica* actin (Accession #AB046952). The sequence of the actin forward primer is 5´- gATTCTggTgATggTgTgAgTCA - 3´. The actin reverse primer is 5´- gAgAgATggCTggAAgAggACTT -3´. The PCR reaction was carried out for 40 cycles, under the following cycle conditions: denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1.5 min. The final extension time was 10 min at 72°C.

Library Constructions

mRNA isolated from the total RNA was reverse transcribed into cDNA. This cDNA was enriched for large fragments and subsequently cloned directionally into pDNR-1r vector (Clontech) at the XhoI-SmaI sites (Agencourt library construction service). The percentage of recombinants and the clone range of these libraries were analyzed by PCR analysis of 24 randomly selected clones (Agencourt library construction service).

RESULTS AND DISCUSSION

Studying gene expression in fruit trees can be technically complicated due to the large quantity of polysaccharides and polyphenolic compounds that accumulate during ripening and/or in response to environmental stimuli. These polysaccharides and polyphenolic compounds often co-precipitate and contaminate the RNA during the extraction, thereby affecting both the quality and quantity of RNA isolated (Asif et al., 2000; Logemann et al., 1987).

With the aim of developing *Prunus persica* as a genomic model for the family *Rosaceae*, we analyzed three different RNA isolation protocols using peach fruits (tissues that contain very high levels of polysaccharides and polyphenolic compounds). The first protocol uses commercially-available TRIzol (Gibco-according to manufacturer's specifications. This protocol is based upon a guanidinium thiocyanate-phenol-chloroform extraction method originally described by Chomczynski and Sacchi (1987). The second protocol is based upon a phenol:chloroform extraction and LiCl precipitation protocol as describe by Das et al. (1990). These protocols have been used

to isolate RNA from leaf tissue in model plants such as arabidopsis, tobacco and maize (Das et al., 1990; Meisel and Lam, 1996; Silva et al., 1999). However, use of such protocols on peach fruits yielded poor quality RNA with high levels of polysaccharides, polyphenolic compounds, and protein contaminants as determined by A240/260 and A260/280, respectively (Logemann et al., 1987; Manning 1990) (Table I).

Due to the high level of contaminants detected in the RNA isolated using the two previously described protocols, we developed an optimized RNA isolation protocol for peach fruits based on several protocols developed for isolating RNA from tissues containing high levels of polysaccharides and polyphenolic compounds such as grape berries, pine trees, and woody plants *Cinnamomum tenuipilum* (Chang et al., 1993; Salzman et al., 1999; Zeng and Yang 2002). The peach protocol consists of two extractions and two precipitation steps.

The first extraction is similar to the RNA isolation method for *Cinnamomum tenuipilum* described by Zeng and Yang (2002) and is performed in a buffer containing 2% CTAB, 100 mM Tris-HCL (pH8.0), 25 mM EDTA, 2 M NaCl, 0.05% spermidine trihydrochloride and 2% ß-mercaptoethanol. However, the peach extraction buffer contains 2% PVP, which has a molecular weight of 40,000, whereas the method by Zeng and Yang (2002) uses PVP (molecular weight 25,000). Following the first extraction, the RNA was precipitated using LiCl, resuspended and transferred to a microfuge tube so a second extraction and precipitation could be performed efficiently, reducing the volume of reagents and time of extraction and precipitation.

Using the Peach protocol we obtained good RNA yield (24 µg/g FW) with low levels of polysaccharides, polyphenolic compounds and protein contaminants as determined by A240/260 and A260/280, respectively (Logemann et al., 1987; Manning 1990) (Table I). Furthermore, this RNA was not degraded, as was demonstrated by visualizing the ribosomal RNA of the samples on a denaturing 1.5% agarose /1.1% formaldehyde gel (Ausubel et al., 1997; Fig. 1A). As seen in Figure 1A, four different RNA extractions from fruits at different stages post-harvest resulted in 28S ribosomal RNA bands that are approximately twice as intense as the 18S ribosomal RNA band. RT-PCR analysis of actin in these RNA extracts also demonstrated that there are no contaminants that interfere with reverse transcription or PCR reactions (Fig. 1B). The negative control for the RT-PCR reaction (H_20) did not show any amplification (data not shown).

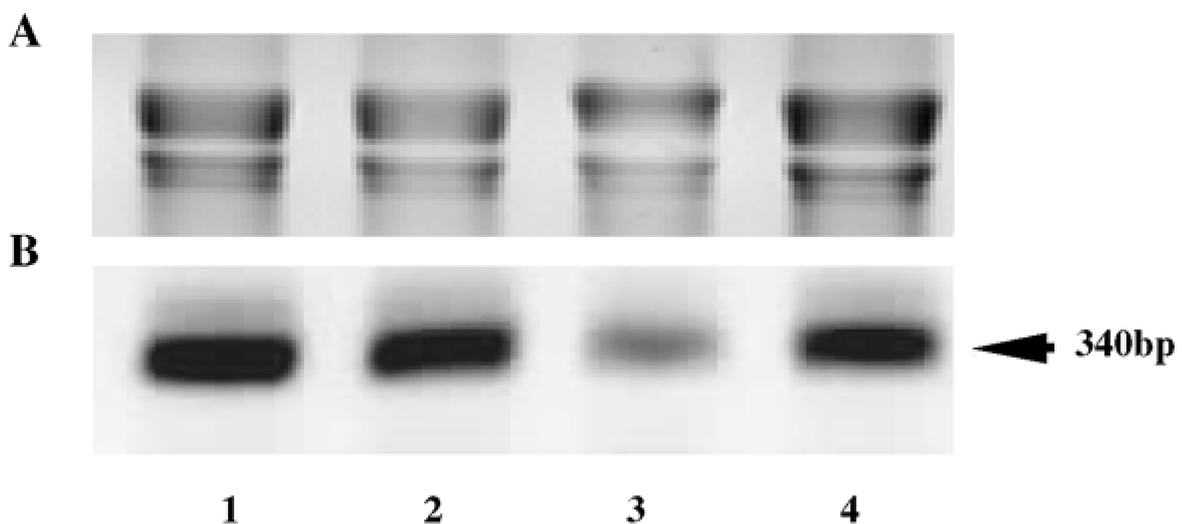

Figure 1. Peach extraction protocol yields high quality RNA as determined by agarose gel and RT-PCR analyses. (A) Total RNA isolated using the peach extraction protocol was separated on an denaturing 1.5% agarose/1.1% formaldehyde gel and staining with ethidium bromide. (B) RT-PCR of actin transcripts from total RNA isolated using the peach extraction protocol. Lanes represent extraction from fruits at four different stages post-harvest.

TABLE I

Spectrophotometer analysis of the purity of RNA isolated from three different extraction protocols

Protocols	**$A_{260/240}$ ***	**$A_{260/280}$ ***	**Yield(µg RNA/g FW)**
TRIzol	0.6	1.4	22.5†
Phenol: Chloroform	1.5	1.6	7†
Peach Protocol	**1.6**	**2.0**	**24**

* Results are expressed as the mean of three extractions
†Yield values are not accurate due to the low A260/A280 ratio

The RNA isolated using the Peach method was also used to create four different cDNA libraries. As summarized in Table II, the RNA isolated using the peach method was successfully used in creating large-insert cDNA libraries with a high number of recombinants.

TABLE II

Quality of cDNA libraries constructed using RNA isolated by the peach extraction protocol

Library	**% Recombination**	**Clone range**
1	92	0.8 2.6 Kb
2	96	0.7 2.2 Kb
3	96	0.7 3.0 Kb
4	100	0.7 2.9 Kb

The analysis of the RNA quality by spectrophotometric analysis, agarose gel analysis of the intensity of the ribosomal RNAs, RT-PCR analysis, and the construction of four large-insert cDNA libraries demonstrate that the Peach RNA isolation protocol presented in this paper can be used to efficiently isolate high quality RNA with low levels of contaminants suitable for functional genomic analyses.

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