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Genetic Resources

High-Quality RNA, cDNA, and Derived EST Libraries From Grapevine (*Vitis vinifera* L.)

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Abstract. The implementation and generation of unbiased results from RNA-based techniques in functional genomic studies requires the isolation of high-quality RNA. In grapevines, the yield and quality of RNA can be significantly reduced by contaminants such as polyphenols, polysaccharides, and proteins, which are abundant during different stages of berry and leaf development. We describe an optimized cetyltrimethylammonium bromide (CTAB)–based RNA extraction protocol that allows effective extraction of high-quality total RNA from a wide range of grapevine tissues, including difficult organs such as symptomatic, pathogen-infected leaves and mature berries. Total RNA extracted with this protocol was successfully used for cDNA library construction as well as reverse transcription–polymerase chain reaction (RT-PCR) amplification. This protocol can also be easily scaled to accommodate different amounts of tissue.

Key words: CTAB, grapevine, polyphenols, polysaccharides, RNA extraction, *Vitis vinifera*

Abbreviations: CTAB, cetyltrimethylammonium bromide; DEPC, diethyl pyrocarbonate; EtBr, ethidium bromide; PVP, polyvinylpyrrolidone; RT-PCR, reverse transcription–polymerase chain reaction.

Introduction

A critical step in the successful implementation of RNA-based techniques for molecular biology and functional genomic experiments (e.g., reverse transcription–polymerase chain reaction [RT-PCR], generation of expressed sequence tags, microarray analysis) is the routine isolation of high-quality RNA. Throughout development, grapevine (*Vitis vinifera* L.) organs undergo significant changes in the quantity of a wide range of structural and soluble metabolites, including polyphenols and polysaccharides, as well as changes in the concentration of specific proteins (Boss and Davies, 2001). Polyphenolic compounds (particularly tannins) can irreversibly bind proteins and nucleic acids to form high-molecular-weight complexes (Newbury and Possingham, 1977), whereas polysaccharides tend to co-precipitate with RNA (Lodhi et al., 1994; Richards et al., 1994), severely

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interfering with RNA-dependent reverse transcriptase, DNA polymerase, and DNA restriction endonuclease activities. In addition, abiotic stresses such as water and nutritional deficiencies or pathogen infection can significantly enhance the biosynthesis and accumulation of secondary metabolites, particularly flavonoids (Winkel-Shirley, 2002). RNA extraction from fruit can be further complicated by high levels of RNAse activity and substances that can co-precipitate or form a covalent complex with nucleic acids (Rodriguez-Pousada et al., 1990).

It is preferable to use a single RNA extraction protocol to reduce the technical bias in downstream RNA-based methodologies. Although several protocols have been developed for the extraction of total RNA from grapes (Newbury and Possingham, 1977; Tesniere and Vayda, 1991; Wang and Vodkin, 1994; Boss et al., 1996; Loulakis et al., 1996; Geuna et al., 1998; Thomas and Schiefelbein, 2002), the vast majority result in low RNA yield, are time-consuming, have been optimized for a specific tissue/organ, and, in some cases, are technically complex. Here we present an optimized RNA extraction protocol that allows efficient isolation of high-quality total RNA from a wide range of grapevine tissues and organs, spanning different developmental stages as well as exposure to biotic stress. The protocol can be easily scaled to accommodate different quantities of plant material and RNA yield requirements.

Materials and Methods

Plant material

Petioles, stems, flowers, and berries from grapevine (*Vitis vinifera* L. cv. Cabernet Sauvignon clone 8) were collected at different developmental stages from field-grown vines at the University of California Experimental Vineyard, Davis, California. Leaves were collected from vineyards in the Napa Valley of California. Leaves of *Vitis* species (a *V. arizonica* × *V. rupestris* hybrid) artificially inoculated with the Pierce disease pathogen, *Xylella fastidiosa*, were collected from potted greenhouse vines. All samples were frozen in dry ice at the time of collection and then stored at –80°C until total RNA was isolated.

Material, solutions, and reagents

Pestles, mortars and all glassware used in the isolation of total RNA from plant material were baked for 2 h at 180°C or alternatively treated with Rnase Away (Molecular Bioprod) and then triple rinsed with 0.1% DEPC-treated water (Sambrook et al., 1989). Both 50- and 30-mL centrifuge tubes (chloroform resistant) (Oak Ridge, Nalgene, Rochester, NY) were immersed overnight in 0.1% DEPC-treated water and then autoclaved.

- Lysis and extraction buffer: 100 mM Tris-HCl[1] (pH 8.0) from a 1 M Tris-HCl[1] stock solution, 25 mM EDTA (pH 8.0) from a 0.2 M EDTA stock solution, 2% CTAB[2] (w/v) (Sigma), 2.5 M NaCl, 2% soluble PVP (w/v) (Sigma, MW 40,000), and 0.5 $g \cdot L^{-1}$ spermidine (Sigma)
- β-mercaptoethanol
- Chloroform-isoamylalcohol (24:1)
- 10 M LiCl (DEPC-treated and autoclaved)

- Ice-cold 70% ethanol prepared with 0.1% DEPC-treated, double-distilled water
- Room temperature 95% ethanol
- 0.1% DEPC-treated, double-distilled autoclaved water
- Qiagen RNAeasy mini or midikit (Qiagen, Valencia, CA) or equivalent silica-gel column-based clean-up kit
- Miracloth (Calbiochem, La Jolla, Calif) or QIAShredder column (Qiagen, Valencia, CA) for small-scale extraction
- 50-mL Nalgene Oak Ridge polypropylene tubes (Nalge Nunc International, Rochester, NY)
- 30-mL Nalgene Oak Ridge Teflon FEP tubes (chloroform resistant) (Nalge Nunc International, Rochester, NY)
- 50-mL Falcon tubes (Fisherbrand)

RNA isolation protocol

1. Use 6.5-8.5 mL of extraction buffer per gram of fresh weight tissue.[3] The ratio of buffer volume to fresh weight depends on the actual water content of extracted tissues. Use a larger volume of extraction buffer for tissues with reduced water content (e.g., stems, flowers, leaves) and select smaller volumes for succulent fruit tissues. For example, for berries at stage II of berry development, weigh approximately 3.0-3.2 g of frozen fruit and extract in 24 mL of extraction buffer.
2. Prewarm extraction buffer to 65°C for at least 20-30 min. Before use, add 2% β-mercaptoethanol and keep the buffer warm while grinding tissue.
3. Grind samples to a fine powder in liquid nitrogen with a pestle and mortar. Add the still-frozen powdered tissue to a 50-mL Falcon tube containing 24 mL of the extraction buffer. Shake and vortex vigorously.
4. Incubate sample for 30 min at 65°C. Shake and vortex vigorously every 5 min.
5. Centrifuge samples at 5,000*g* for 10 min at 4°C with a swinging-bucket centrifuge.
6. Pour the supernatant through a Miracloth filter directly into a 50-mL Oak Ridge tube.[4] Keep tube on ice.
7. Centrifuge at 13,000*g* for 10 min at 4°C to remove any trace of plant material in suspension.
8. Combine equal volumes (~12 mL) of the supernatant and chloroform-isoamylalcohol (24:1) solution in a 30-mL Teflon FEP Oak Ridge tube. Mix gently for 1 min by inverting the tube.
9. Centrifuge at 13,000*g* at 4°C for 5 min.
10. Pipette supernatant into a new pair of 30-mL Teflon FEP Oak Ridge tubes.
11. Add an equal volume of chloroform-isoamylalcohol (24:1), mix gently, and centrifuge at 13,000*g* for 5 min at 4°C.
12. Combine the supernatants into one 50-mL Oak Ridge tube and add 1/4 vol of 10 M LiCl to supernatant, mix gently, and precipitate overnight at 4°C.
13. The next day, centrifuge at 13,000*g* for 30 min at 4°C.
14. Decant the supernatant by pipetting without disturbing the pellet.[5]

15. Wash pellets once with 1 mL of 70% ethanol and centrifuge again at 13,000*g* at 4°C for 5 min. Repeat this step if the pellet does not look white or clear. Pipette to remove residual ethanol.
16. Do not let the pellet dry. Immediately resuspend the pellet in an adequate volume of double-distilled DEPC-treated water.[6]
17. Once the pellet is resuspended, keep sample on ice and proceed immediately with clean-up protocol as indicated by the manufacturer (e.g., QIAGEN RNAeasy MiniKit or MidiKit – RNA clean-up protocol or equivalent silica-gel-based membrane clean-up kit).[7]
18. After final clean up, the RNA is ready for use or can be stored at –80°C for up to 1 mo.[8]

RNA analysis and RT-PCR

Concentration, yield, and quality control indices based on absorbance at 230, 260, and 280 nm ($A_{260/230}$ and $A_{260/280}$ ratios) were performed with 10 µL of resuspended total RNA. Five microliters of the total RNA solution was loaded on a 0.9% agarose gel, electrophoresed to separate RNA, stained with ethidium bromide (EtBr), and visualized under UV light to check size distribution of the total RNA and the integrity of ribosomal bands. Total RNA was reverse transcribed with Omniscript reverse transcriptase according to manufacturer's instruction (Qiagen, Valencia, Calif) using specific primers of interest. Reaction products and DNA size markers (1 kbp DNA ladder, Gibco BRL) were resolved on 2% agarose TAE gels and visualized under UV light following EtBr staining.

Notes

1. Tris-HCl and EDTA are made from a stock solution, autoclaved, and then combined.
2. CTAB, NaCl, PVP, and spermidine are directly dissolved in Tris-EDTA (pH 8).
3. The extraction procedure can be scaled up or down depending on the amount of initial plant tissue available and the quantity of RNA required. For example, RNA can be extracted from 100 mg of leaf blade tissue by using 1.5-mL Eppendorf tubes instead of the Oak Ridge tubes. The amount of extraction buffer should be scaled down proportional to the fresh weight of tissue used.
4. When working with reduced quantities of extraction buffer/tissue, supernatant can be pipetted into 2-mL round-bottom centrifuge tubes after passage through a QIAShredder column in place of Miracloth.
5. At this point, it is important to remove all supernatant to prevent carryover of phenolics and other contaminant pigments, as well as some proteins. Work quickly during this step to prevent drying of the pellet.
6. The water volume used after LiCl precipitation and clean up with 70% methanol will depend on expected RNA yield and the requirements of the final clean-up protocol.
7. If RNA is to be used for RT-PCR, it should be treated with DNAse to remove possible DNA contaminants.
8. Longer-term storage of RNA can be achieved by addition of 2.5 vol of absolute ethanol and 0.1 vol of DEPC-treated 3 M NaOAc (pH 5.2).

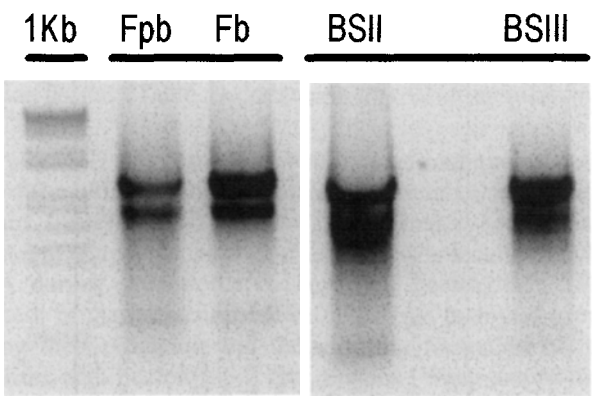

Figure 1. Total RNA extracted from different grapevine tissues. Fpb, flower prebloom; Fb, flower at bloom; BSII, berries at stage II; BSIII, berries at stage III, 23° Brix. The intense ribosomal bands as well as the smear from 0.5 kb up to 4-5 kb are indications of intact RNA.

Results and Discussion

In our laboratory, several standard protocols and commercially available kits for RNA extraction based on the single-step RNA extraction protocol of Chomczynski and Sacchi (1987) gave unsatisfactory results for various grapevine tissues and particularly for berries after the onset of ripening and for symptomatic leaves infected with the bacterial pathogen *X. fastidiosa*. Similar results have been previously reported for grapes (Loulakakis et al., 1996) and other plant species (e.g., pine) that contain high levels of contaminant metabolites (Chang et al., 1993). Here we describe an optimized CTAB-based protocol based on the original methods reported by Chang et al. (1993) and Jakoola et al. (1998). This protocol allows the efficient extraction of high-quality total RNA from several grapevine tissues at various developmental stages as well as from leaves infected with *X. fastidiosa*.

The presence of the intense, well-resolved ribosomal bands and the smear of RNA ranging from 0.5-4 kb found in this study are indicative of high-quality total RNA obtained from different grapevine tissues (Figure 1). RNA yields ranged from 8-352 μg per gram fresh weight tissue (Table 1), with yields dependent on organ, developmental stage, and stress. RNA content in preveraison berries was several-fold higher than that reported by Franke at al. (1995). For postveraison berries, values are comparable to those obtained for fruits of other important crop species, e.g., blackcurrant (15-20 μg/g fresh weight; Woodhead et al., 1997), apple (13 μg/g fresh weight; Lay-Yee et al., 1990), and peach (12-15 μg/g fresh weight; Calaham et al., 1989). The amount of RNA extracted from leaves was similar to that obtained in grapes (200-300 μg/g fresh weight; Loulakakis et al., 1996) and other plant species such as strawberry (324-935 μg/g fresh weight; Mazarra and James, 2000) and aspen (400-600 μg/g fresh weight; Bhalerao et al., 2003). Stem RNA content was also within the previously reported range for grapevines (80-120 μg/g fresh weight; Thomas and Schiefelbein, 2000). RNA quality was measured by means of spectrophotometric ratios that relate differences in absorption spectra maxima of pure RNA (A_{max} = 260 nm), proteins

Table 1. Yield and quality of total RNA and cDNA libraries from different grapevine tissues.

Total RNA Quality Parameters					cDNA Library	
Grapevine Tissue	$A_{260/280}$	$A_{260/230}$	Yield (mg/g Fresh Weight)	Average Insert Size (kb)[1]	Library Name/Unigene Number[2]	No. ESTs[3]
Vitis vinifera[4]						
Flower pre-anthesis	2.32	2.44	195.7	1.3	Cabernet Sauvignon flower prebloom - CAB1/13014	3748
Flower at anthesis	2.42	2.33	125.4	1.4	Cabernet Sauvignon flower bloom - CAB2/14167	4428
Berries at stage I	2.28	2.23	89.1	0.94	Cabernet Sauvignon berry stage I - CAB3/13015	3414
Berries stage II - green hard	2.27	2.2	40.2	1.1	Cabernet Sauvignon berry - CAB4/13016	3836
Berries stage II - green soft	2.31	2.32	33.2	1.19	Cabernet Sauvignon berry - CAB2SG/12754	4429
Berries stage III - 19° Brix	2.24	1.83	15.8	1.3	Cabernet Sauvignon berry postveraison - CAB7/13017	3558
Green stems at anthesis	2.12	2.16	85	1.4	*Vitis vinifera* cv. Cabernet Sauvignon stem - CAST/14168	4700
Petioles	2.03	2.09	44.4	1.23	*Vitis vinifera* cv. Cabernet Sauvignon (clone 8) petiole - CAP/14375	4491
Vitis vinifera leaves, nonsymptomatic	2.19	2.67	167	1.1	Cabernet Sauvignon leaf-CA48EN /12753	2051
V. arizonica × *V. rupestris*						
Leaves, Pierce disease infected	2.15	2.73	352	1.1	*Vitis* sp. (*Vitis arizonica* × *Vitis rupestris*) RR890915I/NA[5]	3656
Leaves, nonsymptomatic	2.18	3.63	180	0.95	*Vitis* sp. (*Vitis arizonica* × *Vitis rupestris*) RR890915N/NA	2877

[1]Average estimated from EtBr gel–based screening.

[2]Available at http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=29760.

[3]Total number of ESTs deposited at Genbank database (http://www.ncbi.nlm.nih.gov/dbEST/index.html).

[4]See *Mullins et al. (1998) for detailed description of flower and berry developmental stages.*

[5]NA, not available.

(A_{max} = 280 nm), and polysaccharides (A_{max} = 230 nm). Normally, a 1:2:1 ratio in the $A_{230:260:280}$ is expected in high-purity RNA isolates. This ratio was observed for all grapevine tissues (Table 1), indicating that RNA extracts were of high purity.

In this protocol, the extraction buffer, precipitation procedure, and final clean-up step differentially remove contaminant metabolites present in grapevine tissues. Tannins (procyanidins) are one of the major contaminants present in excess in different grapevine tissues. These high-molecular-weight polyphenols complex RNA during extraction (Newbury and Possingham, 1977). The PVP present in excess in the extraction buffer binds free hydroxyl groups in tannins, thus preventing their oxidation and the resulting formation of high-molecular-weight complexes with nucleic acids and proteins (Wang and Vodkin, 1994). The high NaCl molarity of the extraction buffer increases the solubility of polysaccharides, reducing their co-precipitation with RNA in later steps of the protocol (Fang et al., 1992; Lodhi et al., 1994). Finally, the constant presence of the strong reductant β-mercaptoethanol and the use of the chaotropic agent NaCl denatures ribonucleases and other contaminating proteins that are released during tissue disruption and homogenization.

The detergent CTAB preserves the integrity of nuclear and organelle membranes yielding total RNA with lower concentrations of unspliced heteronuclear transcripts (hnmRNA) as well as an increased RNA-to-DNA ratio (Mejjad et al., 1994; Dellaporta et al., 1984). One disadvantage of using this detergent is that it may artificially increase absorbance at 260 nm if still present in the final RNA solution (Doyle and Doyle, 1990). The final clean-up step in our protocol takes care of this problem based on selective binding of RNA to various commercially available columns. RNA extracts are further depleted of coextracted DNA during the LiCl precipitation step. The high-molarity LiCl solution not only differentially precipitates RNA from DNA but also favors precipitation of larger transcripts over smaller ones. The quality of purified RNA was further demonstrated by constructing several cDNA libraries with an average insert size of 1.1 kb (Table 1). Although this protocol is expected to yield total RNA samples essentially free of DNA contamination, an additional DNA removal step can be added if the sample is to be used for methods such as RT-PCR. We used this optional step in RT-PCR experiments to characterize the response of grapevine leaves to infection by the bacterial pathogen *X. fastidiosa* (Choi et al., unpublished results) (Figure 2).

Franke and Adams (1995) reported a decrease in total RNA content over the course of berry development, although they were unable to extract RNA from berries after the onset of ripening (i.e., veraison). Our results also revealed a continuous decline in RNA tissue concentration throughout berry development but extended the analysis of Franke and Adams (1995) with the isolation of high-quality RNA up to the point of commercially ripe fruit (23% soluble solids concentration in juice, referred to as ° Brix) (Figure 3). A reduction in total RNA concentration expressed on a fresh-weight basis has also been reported in senescing leaves (Bhalerao et al., 2003; Lers et al., 1998). However, when expressed on a per-berry basis, RNA content remained approximately constant, except for mature berries (Figure 3). The expansion of berries during postveraison is driven primarily by the accumulation of solutes (mostly hexoses) and water in the

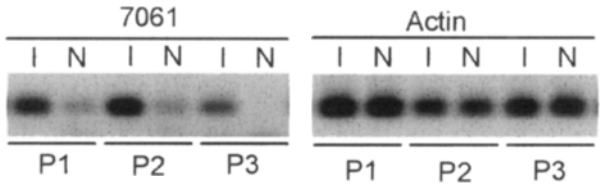

Figure 2. Reverse transcription–polymerase chain reaction (RT-PCR) from RNA extracted from grapevine leaves with the cetyltrimethylammonium bromide (CTAB)–based protocol. 7061, gene differentially expressed in Pierce disease–infected plants (Choi et al., unpublished results); I, Pierce disease–infected plant; N, noninfected plants; P1-P3, primer sets 1-3.

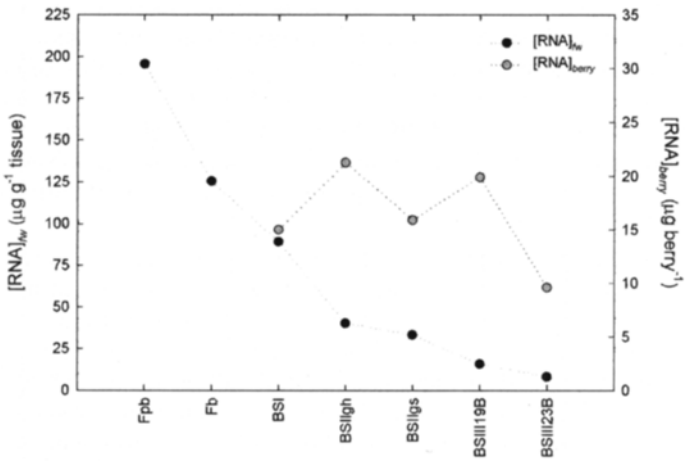

Figure 3. Developmental changes in total RNA concentration (RNA) in grapevine flowers and berries expressed on a fresh-weight and per-berry basis. Fpb, flower prebloom; Fb, flower at bloom; BSI, berries at stage I; BSIIgh, berries at stage II, green hard; BSIIgs, berries at stage II, green soft; BSIII19B, berries at stage III, 19° Brix; BSIII23B, berries at stage III, 23° Brix. Note: The transition from green hard to soft berries represents the first observable event of the onset of ripening in grapevine berries.

vacuoles, while most cell division occurs during early stages of fruit development after berry set (Mullins et al., 1998; Ojeda et al., 1999). We speculate that as berries progress into more advanced stages of ripening, there is a sustained decrease in transcriptional activity. It is also possible that the observed decline in RNA content during later stages of berry development is a reflection of enhanced RNA catabolism, coinciding with increased RNAses activity, similar to that reported for senescing leaves (Lers et al., 1998) and in other fruits (Luo and Liu, 1994). Further experimentation may reveal the metabolic consequences of this observed ripening-linked decline in berry total RNA content.

Conclusion

We have developed an RNA extraction procedure that yields consistently large amounts of high-quality total RNA from a wide range of grapevine tissues, including organs such as symptomatic Pierce disease–infected leaves and mature

berries containing high concentrations of polyphenols, polysaccharides, and contaminating proteins. The protocol takes 4 h distributed over 2 d. Total RNA extracted from these tissues has been successfully used for cDNA library construction and RT-PCR experiments. The protocol can also be easily scaled to accommodate differing amounts of tissue and RNA yield requirements.

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