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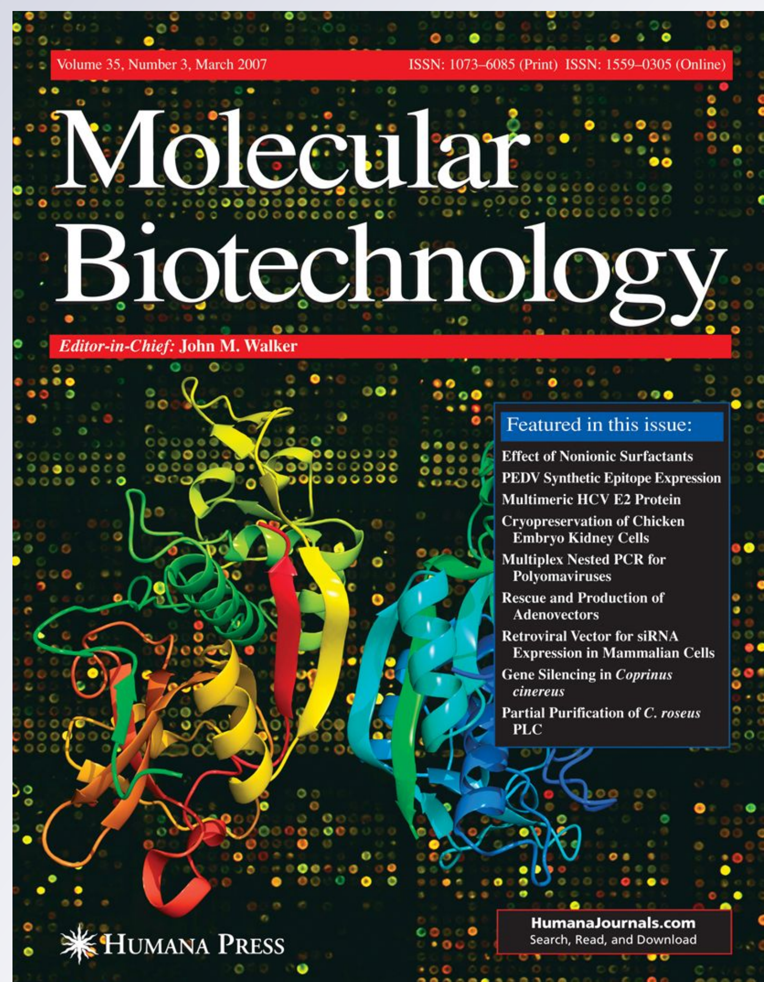

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Rapid and Efficient Isolation of High Quality Nucleic Acids from Plant Tissues Rich in Polyphenols and Polysaccharides

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Abstract Isolation of high quality nucleic acids from plant tissues rich in polysaccharides and polyphenols is often difficult. The presence of these substances can affect the quality and/or quantity of the nucleic acids isolated. Here, we describe a rapid and efficient nucleic acids extraction protocol that in contrast to other methods tested, effectively purify high quality nucleic acids from plant tissues rich in polysaccharides and polyphenolic compounds such as different grape tissues and fruit tissue of fruit trees. The nucleic acids isolated with this protocol were successfully used for many functional genomic based experiments including polymerase chain reaction, reverse transcription polymerase chain reaction (RT-PCR), cloning, and semiquantitative RT-PCR.

Keywords Fruit trees · Nucleic acids extraction · Polyphenols · Polysaccharides · RT-PCR

Abbreviations

Chl:Iaa	Chloroform-isoamylalcohol
CTAB	Cetyltrimethylammonium bromide
DEPC	Diethyl pyrocarbonate
EDTA	Ethylenediaminetetra acetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
FW	Fresh weight
PVPP	Polyvinylpolypyrrolidone
PVP	Polyvinylpyrrolidone
NaOAc	Sodium acetate
RT-PCR	Reverse transcription polymerase chain reaction

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Introduction

Isolation of high quality nucleic acids from plant tissues rich in polysaccharides and polyphenols is often a difficult task. The presence of these substances can affect the quality and/or quantity of the nucleic acids isolated [1]. Polyphenolic compounds (particularly tannins) are readily oxidized to form covalently linked quinones, and can irreversibly bind proteins and nucleic acids to form high molecular weight complexes [2, 3], whereas polysaccharides tend to co-precipitate with nucleic acids in low ionic strength buffers [4, 5]. In addition, these contaminating substances severely interfere with RNA-dependent reverse transcriptase, DNA polymerase, DNA restriction endonuclease activities, and absorbance-based quantification [3, 6]. In contrast to other tissues, nucleic acids extraction from fruit can be further complicated by high levels of DNase and RNase activity and substances that co-precipitate or form a covalent complex with nucleic acids [3].

So far many procedures have been published for the extraction of nucleic acids from plant tissues that vast majority are not completely satisfying because they are time-consuming [1, 6], have been optimized for a specific species [7–10], are technically complex [3, 5], and require ultracentrifugation steps [11, 12] or the use of phenol and polyvinylpolypyrrolidone (PVPP) [12, 13]. Here, we present an optimized method for nucleic acids (DNA and RNA) extraction based on the cetyltrimethylammonium

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bromide (CTAB)-polyvinylpyrrolidone (PVP) that in contrast to the other methods, allows efficient isolation of high quality nucleic acids from different grape tissues and fruit tissue of fruit trees. In this method, phenolic compounds are bound to soluble PVP, then eliminated by isopropanol precipitation of the nucleic acids. The solubility of polysaccharides is also increased by high NaCl molarity of the extraction buffer and are subsequently removed by LiCl precipitation. The extraction method described here is rapid, simple and efficient that does not require ultracentrifugation and phenol. Besides, this method is widely applicable to extract nucleic acids from different plant tissues.

Materials and Methods

Plant Materials

Berries, leaves, petioles, clusters, stems, roots, and seeds from grape (*Vitis vinifera* L. cv. Askari) were collected from plants grown in the grape collection of the Grape Research Station, Takistan-Qazvin, Iran. Berries at three ripening stages such as pre-veraison, veraison, and post-veraison, and leaves at three stages of development including young, mid, and old were harvested. Fruit tissues from apple (*Malus domestica*), banana (*Musa acuminata*), pear (*Pyrus communis*), cherry (*Prunus avium*), peach (*Prunus persica*), and apricot (*Prunus armeniaca*) trees were also collected from native plants in Qazvin. All samples were immediately frozen in liquid nitrogen upon collection and then stored at –80°C. Grape seeds were removed from the berries at veraison stage by gently breaking open the berries under liquid nitrogen, then pericarp and seed portions were stored separately until use.

Solutions and Reagents

1. Extraction buffer: 300 mM Tris–HCl pH 8.0, 25 mM ethylenediaminetetra acetic acid (EDTA) pH 8.0, 2 M NaCl, 2% (w/v) soluble PVP (Sigma, MW 40000), and 2% (w/v) CTAB (Sigma).
2. 2% (v/v) β-mercaptoethanol.
3. Chloroform-isoamylalcohol (Chl:Iaa) (24:1, v/v).
4. 3 M sodium acetate (NaOAc) pH 5.2.
5. Isopropanol.
6. 8 M LiCl.
7. 70% (v/v) ethanol (EtOH).
8. Diethyl pyrocarbonate (DEPC) treated and autoclaved distilled water contained 20 µg/ml DNase I enzyme.
9. TE buffer or dionozed water contained 20 µg/ml DNase-free RNase A enzyme.

Nucleic Acids Extraction Protocol

1. For fruit or other watery tissues[1], use a ratio of 0.1 g tissue/1 ml extraction buffer[2]. For dry tissues[3] (e.g. leaf, petiole, cluster, stem, root, and seed) use a maximum of 0.05 g tissue/1 ml buffer[4] .
2. Prepare extraction buffer and prewarm it to 65°C for at least 20–30 min. Before use, add 2% (v/v) β-mercaptoethanol and keep the buffer warm while grinding tissue.
3. Grind samples in liquid nitrogen using a prechilled mortar and pestle[5]. Add the still-frozen ground tissue into a 2-ml Eppendorf tube containing 1 ml of the extraction buffer. Shake and vortex gently.
4. Incubate tubes in a 65°C water bath for 20–30 min. Shake and vortex gently every 5 min.
5. Cool the tubes to room temperature and add an equal volume of Chl:Iaa (24:1, v/v). Shake and vortex gently until the two phases form an emulsion, then centrifuge at 14,000 rpm for 5 min.
6. Transfer the aqueous phase into a new tube and re-extract with an equal volume of Chl:Iaa (24:1, v/v) and centrifuge as above.
7. Collect the aqueous phase and add 0.1 volume of 3 M NaOAc pH 5.2 and an equal volume of isopropanol, mix by inversion and then store at –80°C for 30 min.
8. Collect nucleic acids (DNA and RNA) pellet by centrifugation at 14,000 rpm for 30 min. To extract genomic DNA, wash the pellet with 70% EtOH, air-dry[6], dissolve in an appropriate volume of TE buffer or deionized water[7], and store at –20°C. To extract total RNA, dissolve the pellet in 200 µl DEPC-treated water and follow the 9th and 10th steps.

[1] Pestles, mortars and all glassware used in the isolation of total RNA from plant material are baked for 2 h at 180°C. Also, 2 ml Eppendorf tubes and tips are immersed overnight in 0.1% DEPC treated water and then autoclaved for 30 min.

[2] For isolation of total RNA, EDTA, NaCl, NaOAc, and LiCl are treated with 0.1% DEPC solution and stored overnight, then are autoclaved for 30 min. Tris–HCl and 70% EtOH are prepared with 0.1% DEPC-treated, autoclaved distilled water.

[3] Isolation of total RNA should carry out at 4°C, whereas genomic DNA extraction is performed at room temperature.

[4] The extraction procedure can be scaled up or down depending on the amount of initial plant tissue available and the quantity of nucleic acid required.

[5] In the isolation of total RNA, tissues should grind to a fine powder, but for genomic DNA extraction, it is not necessary that tissues grind completely.

[6] At this point, the pellet is white and clear. Do not let the pellet dry completely. Immediately resuspend the pellet in an adequate volume of TE buffer or DEPC-treated water.

[7] If the pellet is unclear, centrifuge at 14000 rpm for 1 min to remove any trace of plant material in nucleic acid solution.

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9. To selectively precipitate total RNA, add 0.3 volume of 8 M LiCl and store the tubes overnight at 4°C.
10. Collect the RNA pellet by centrifugation at 14,000 rpm for 30 min at 4°C[8], then wash the pellet with ice cold 70% EtOH, air-dry for 10 min (see foot note 6), and dissolve in an appropriate volume of DEPC-treated water (see foot note 7). Store RNA at –80°C.

Quantification and Quality Control

The extracted nucleic acids were quantified with a spectrophotometer set (Labomed-U.K) at wavelengths of 230, 260, and 280 nm (A_{260}/A_{230} and A_{260}/A_{280} ratios). The integrity of total RNA and DNA were verified by running samples on a 1.5% non-denaturing agarose gel containing ethidium bromide (EtBr) (5 μg/ml) and a 0.8% agarose gel after staining with EtBr (1 μg/ml), respectively.

cDNA Synthesis and RT-PCR

To test the quality of the RNA obtained, 5 μg of total RNA treated with DNase I (Fermentas) were used as a template using Oligo $(dT)_{18}$ primer (1 μg/μl, Qiagen) for 5 min at 70°C. Then, reaction mixture was incubated with RevertAid^{TM} M-MuLV Reverse Transcriptase (200 u/μl, Fermentas) for 60 min at 42°C. The reaction was inactivated by heating the mixture at 70°C for 10 min. RT-PCR amplification was performed to demonstrate that the RNA could also be used for other analyses. The oligonucleotide primers were designed on the basis of the conserved sequence for a grape thioredoxin *h* gene, called Vv*CxxS*2 (NCBI GenBank accession number HM370528), including an addition of three nucleotides and of a *Bam*H I restriction site at the 5′-ends (Table 1). Following initial denaturation at 94°C for 3 min, the RT-PCR reaction was carried out in a thermal cycler programmed (Techene, U.K) for 35 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min. Reaction products and DNA size markers (100 bp DNA ladder, Sibenzyme) were resolved on the 1.2% TBE-agarose gels and visualizes under UV light following EtBr staining.

Restriction Analysis of DNA and PCR Amplifications

Five micrograms of each genomic DNA sample measured by spectrophotometer were incubated at 37°C for 1 h with 20 U of *Eco*R I (Fermentas) in a total volume of 100 μl and were analyzed on the 0.8% agarose gels using 20 μl aliquots of the reaction mixture. By using the genomic DNA isolated from different grape tissues, PCR amplifications were performed in a total 25 μl consisting 1× PCR buffer, 0.2 mM dNTP, 10 pM of each primer (Table 1), 50 ng template DNA from different tissues, and 0.25 U *Pfu* DNA polymerase (Fermentas) using the following profile: a 3 min denaturation at 94°C and 35 cycles of 30 s denaturation at 94°C, 1 min annealing at 58°C, and a 2 min extension at 72°C, followed by a final extension at 72°C for 5 min. The PCR products were resolved by electrophoresis in 1.2% agarose gels, stained with EtBr, and observed under UV light.

Cloning of DNA Fragments

The amplified products were separated on 0.8% agarose gels, and then excised from the gels and purified using GF-1 PCR Clean Up Kit (Vivantis). The purified fragments were digested with *Bam*H I enzyme (Fermentas) and then were cloned into a pUC19 plasmid vector (Fermentas) to generate the pVTRXh-3 plasmid. The ligation samples were directly used to transform the competent *Escherichia coli* strain DH5α as described in Sambrook and Russell [14]. After screening, the recombinant plasmids were isolated and run on 1.2% agarose gel.

Gene Expression Analysis

The expression of the Vv*CxxS*2 gene was analyzed in different grape tissues at different developmental stages by semi-quantitative RT-PCR. The PCR reaction was performed using 1/20 of the reverse transcription reaction in a final volume of 20 μl containing 10 pmol of specific primers for coding region of Vv*CxxS*2 gene (Table 1). As a control, the primers specific to *Arabidopsis* actin gene (At*Act*2; NCBI GenBank accession number AF485783) were used (Table 1). PCR was carried out under the following conditions: 3 min at 94°C followed by 30 cycles of 30 s at 94°C, 1 min at 58°C, and 30 s at 72°C, with a final extension for 5 min at 72°C. A negative control lacking template was included for each set of RT-PCR reactions. Reactions were performed in triplicate. Five microliters of amplification products were separated by 1.2% agarose gel electrophoresis, stained with EtBr, and observed under UV light.

Results and Discussions

Quantity and Quality of Isolated Nucleic Acids

The nucleic acids extraction from fruit trees can be technically complicated due to the large quantity of

[8] At this point, it is important to remove all supernatant to prevent carryover of contaminating substances.

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Table 1 Nucleotide sequence of oligonucleotide primers used for PCR and RT-PCR amplifications

Primer Name	Sequence (5'-3')	Melting Temperature (°C)	Size of Amplicon (bp)
Primers used for cloning			
VTrx3F	tacggatccATGGAAAATCAGGAGCCG	78.7	866[a]–381[b]
VTrx3R	atcggatccCTAGGCTACATACACGCGAAA	78.2	
Primers used for sq.RT-PCR			
Sq–VTrx3F	ATGGAAAATCAGGAGCCG	62.6	381
Sq–VTrx3R	CTAGGCTACATACACGCGAAA	61.7	
AtAct2F	GTTAGCAACTGGGATGATATGG	66.8	530
AtAct2R	AGCACCAATCGTGATGACTTG	69.6	

The oligonucleotide primers used for cloning include an addition of three nucleotides (small letters) and of a *Bam*H I restriction site (underlined) at the 5′-ends

[a] The length of amplified DNA segment

[b] The length of amplified ORF

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 nucleic acids during the extraction, thereby affecting both the quality and quantity of isolated nucleic acids [1, 10]. Development of a single robust nucleic acids isolation protocol for plant tissues rich in polysaccharides and polyphenolic compounds might be useful for molecular biology and functional genomic experiments. The protocol reported here is based on the methods of CTAB [12, 15] with modifications, and resulted in the rapid and efficient isolation of high quality nucleic acids from plant tissues rich in polysaccharides and polyphenols. Also, this method is highly useful for different grape tissues at different developmental stages.

In our proposed protocol, the following modifications were considered: elimination—the needs for lyophilization, applying PVP into the extraction buffer instead of PVPP, simplification—the extraction step by using only Chl:Iaa (24:1, v/v), eliminating phenol, concentration—the nucleic acids in solution by an isopropanol precipitation (step prior

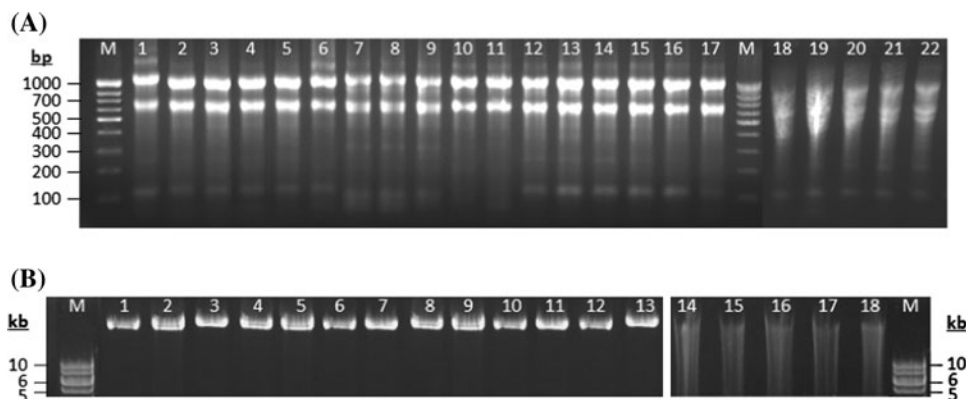

Fig. 1 Electrophoretic analysis of nucleic acids isolated from different plant tissues. **a** Visualization of total RNA isolated from different plant tissues by the new protocol (*lanes 1–17*) and other methods tested by using grape berry tissue (*lanes 18–22*). The RNA samples were separated on 1.5% non-denaturing agarose gel containing EtBr and photographed under UV light. *M* 100 bp DNA ladder; grape: *lanes 1–3* berry at three developmental stages including pre-veraison, veraison, and post-veraison, respectively, *lanes 4–6* leaf at three developmental stages such as young, mid, and old, respectively, *lane 7* petiole, *lane 8* cluster, *lane 9* stem, *lane 10* root, *lane 11* seed, *lane 12* apple, *lane 13* banana, *lane 14* pear, *lane 15* cherry, *lane 16* peach, *lane 17* apricot, *lane 18* Ainsworth et al. [16] method, *lane 19* Fillion et al. [17] method, *lane 20* Asif et al. [1] method, *lane 21* Iandolino et al. [3] method, *lane 22* Reid et al. [12] method. **b** Visualization of genomic DNA isolated from different plant tissues by the new protocol (*lanes 1–13*) and other methods tested by using grape leaf tissue (*lanes 14–18*). The DNA samples were separated on 0.8% agarose gel, stained with EtBr, and observed under UV light. *M* 1 kb DNA ladder; grape: *lane 1* berry, *lane 2* leaf, *lane 3* petiole, *lane 4* cluster, *lane 5* stem, *lane 6* root, *lane 7* seed, *lane 8* apple, *lane 9* banana, *lane 10* pear, *lane 11* cherry, *lane 12* peach, *lane 13* apricot, *lane 14* Cheng et al. [7] method, *lane 15* Xu et al. [20] method, *lane 16* Hanania et al. [8] method, *lane 17* Hameed et al. [21] method, *lane 18* Kang and Yang [22] method

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to selectively precipitating the RNA with LiCl), and eliminating the need for ultracentrifugation. PVPP has moderate effect, and is bulky, and often requiring multiple extraction steps accompanying with low product of nucleic acids. However, these problems can be avoided by using soluble PVP (MW 40000), because of a crucial and more immediate contact with the polyphenolic compounds upon homogenization and incompatible with phenol extraction, consequently has more effects on rapid binding to nucleic acids [1, 2].

The nucleic acids isolation procedures can be judged by the quantity, quality, and the integrity of the nucleic acids obtained. This method was compared with five different RNA isolation protocols using mature grape berries (tissues that contain very high levels of polysaccharides and polyphenolic compounds) [1, 3, 12, 16, 17]. The RNA samples prepared by this method demonstrated the intact sharp 28S and 18S ribosomal RNA (rRNA) bands and the lack of RNA degradation on agarose gels, indicating high quality of obtained total RNA (Fig. 1a, lanes 1–17). Whereas, other procedures were exhibited a failure production of a high quality RNA due to the serious degradation of the RNA as indicated by the degradation of 28S and 18S rRNA as well as a smear of smaller sized RNAs (Fig. 1a, lanes 18–22). As well, RNA quality was measured by means of spectrophotometric ratios that relate differences in absorption spectra maxima of pure nucleic acids (A_{max} = 260 nm), proteins (A_{max} = 280 nm) and polysaccharides (A_{max} = 230 nm). In contrast to other methods [1, 3, 12, 16, 17], the A_{260}/A_{230} ratio for all RNA samples prepared by our protocol was higher than 1.82. This indicated that the RNA samples were of high purity and without polyphenol and polysaccharide contamination. Also, the A_{260}/A_{280} ratios ranged from 1.80 to 2.15, indicating a low protein contamination (Table 2). On the other hand, the average A_{260}/A_{230} of grape berry RNA prepared by other protocols ranged from 1.24 to 1.77, which indicated that, the samples contained polyphenol and polysaccharide contamination. Furthermore, protein contamination in these samples was noted as the average ratio at A_{260}/A_{280} of grape berry RNA prepared by other protocols ranged from

Table 2 Yield and purity of total RNA prepared by the new protocol and other methods evaluated by UV light absorption spectra and ratios of A_{260}/A_{230} and A_{260}/A_{280}

Method	Plant	Tissue	Developmental stage	Absorbancy ratio[a]		RNA yield[a, b] (μg/g FW[c])
				A_{260}/A_{230}	A_{260}/A_{280}	
The new protocol	Grape	Berry	Per-veraison	2.06 ± 0.05	2.01 ± 0.04	98.84 ± 5.23
			Veraison	2.08 ± 0.04	2.08 ± 0.01	81.60 ± 5.46
			Post-veraison	2.02 ± 0.02	1.86 ± 0.04	28.43 ± 3.31
		Leaf	Young	2.10 ± 0.05	2.02 ± 0.02	658.80 ± 6.78
			Mid	1.98 ± 0.02	2.03 ± 0.01	505.12 ± 5.64
			Old	1.99 ± 0.01	1.98 ± 0.04	509.43 ± 3.29
		Petiole	Young	2.11 ± 0.03	2.02 ± 0.02	115.28 ± 4.59
		Cluster	Pre-veraison	2.09 ± 0.02	2.00 ± 0.04	184.96 ± 4.90
		Stem	Green	2.12 ± 0.03	1.90 ± 0.02	134.40 ± 2.08
		Root	Young	2.01 ± 0.06	1.81 ± 0.01	153.80 ± 3.15
		Seed	Veraison	1.86 ± 0.04	2.10 ± 0.05	94.85 ± 4.67
	Apple	Fruit	Mature	1.98 ± 0.03	1.91 ± 0.01	22.66 ± 2.43
	Banana	Fruit	Mature	1.88 ± 0.02	1.83 ± 0.03	23.76 ± 3.12
	Pear	Fruit	Mature	1.95 ± 0.06	1.91 ± 0.02	27.04 ± 2.06
	Cherry	Fruit	Mature	1.92 ± 0.03	2.02 ± 0.02	21.75 ± 2.08
	Peach	Fruit	Mature	1.96 ± 0.02	1.81 ± 0.01	27.50 ± 1.89
	Apricot	Fruit	Mature	1.97 ± 0.04	2.00 ± 0.04	32.25 ± 2.71
Ainsworth [16]	Grape	Berry	Post-veraison	1.35 ± 0.11	1.38 ± 0.09	10.47 ± 2.56
Fillion et al. [17]	Grape	Berry	Post-veraison	1.59 ± 0.12	1.59 ± 0.10	11.75 ± 1.70
Asif et al. [1]	Grape	Berry	Post-veraison	1.58 ± 0.15	1.49 ± 0.11	13.33 ± 1.21
Iandolino et al. [3]	Grape	Berry	Post-veraison	1.69 ± 0.08	1.65 ± 0.10	15.67 ± 2.34
Reid et al. [12]	Grape	Berry	Post-veraison	1.68 ± 0.06	1.62 ± 0.05	14.78 ± 4.75

[a] Results are the mean of three samples (standard deviation)

[b] RNA yields using other methods were significantly different from yields with the new protocol at the 95% confidence level

[c] *FW* fresh weight

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1.29 to 1.75, less than the accepted A_{260}/A_{280} value of 1.80. These results indicated that RNA samples prepared by other protocols were low in purity (Table 2).

The average RNA yields ranged from 19.67 to 665.58 µg per gram fresh weight (g FW) tissue. Grape leaves yielded the highest amount of total RNA (499.48–665.58 µg/g FW), and the lowest amount from post-veraison berries and mature fruits (19.67–34.96 µg/g FW) (Table 2). Nevertheless, the average RNA yields from other protocols were far less and ranged from 7.91 to 19.53 µg/g FW (Table 2). The differences observed in the average RNA yields of different tissues were likely related to the developmental, structural, and metabolic properties distinct tissues [6, 12]. The amount of RNA extracted by the present method from grape leaves is higher than the amounts obtained in grapes (167.00 and 600.00 µg/g FW) [3, 12] and also other plant species such as pear (96.00 µg/g FW) [18], kiwifruit (527.00 µg/g FW) [19], and apple (540.00 µg/g FW) [6]. For post-veraison berries and mature fruits, values are comparable to fruits of other important crop species, e.g., apples (20.00 and 18.90 µg/g FW) [9, 19] and peach (24.00 µg/g FW) [10].

The nucleic acids extraction protocol was also compared with five different genomic DNA isolation methods using young grape leaves [7, 8, 20–22]. In contrast to other methods tested, the genomic DNA samples isolated by this protocol were analyzed on agarose gels and showed the clear, sharp, and high molecular weight DNA bands without degradation, indicating a high quality of prepared genomic DNA samples (Fig. 1b, lanes 1–13). The DNA samples prepared by this protocol were of high purity with low polysaccharide and protein contamination, which was indicated by the A_{260}/A_{230} and A_{260}/A_{280} ratios [14], which ranged from 1.85 to 2.13 and 1.80 to 2.08, respectively (Table 3). However, these obtained amounts are comparable to the A_{260}/A_{230} and A_{260}/A_{280} ratios of grape leaf DNA prepared by other methods (Table 3). The average genomic DNA yields ranged from 14.55 to 676.84 µg/g FW. The highest and lowest amount of genomic DNA is related to grape leaves (664.16–676.84 µg/g FW) and mature fruits (14.55–29.26 µg/g FW), respectively (Table 3). However, the average genomic DNA yields from other methods ranged from 350.22 to 448.68 µg/g FW for young grape leaf (Table 3). The amount of

Table 3 Yield and purity of genomic DNA prepared by the new protocol and other methods evaluated by UV light absorption spectra and ratios of A_{260}/A_{230} and A_{260}/A_{280}

Mehtod	Plant	Tissue	Developmental stage	Absorbancy ratio[a]		DNA yield[a, b] (µg/g FW[c])
				A_{260}/A_{230}	A_{260}/A_{280}	
The new protocol	Grape	Berry	Post-veraison	1.98 ± 0.02	1.91 ± 0.01	26.02 ± 3.24
		Leaf	Young	1.93 ± 0.03	1.98 ± 0.05	670.50 ± 6.34
		Petiole	Young	2.10 ± 0.01	1.90 ± 0.03	130.68 ± 5.89
		Cluster	Pre-veraison	1.98 ± 0.01	1.84 ± 0.02	161.44 ± 4.63
		Stem	Green	1.95 ± 0.04	1.85 ± 0.03	144.84 ± 4.25
		Root	Young	1.90 ± 0.02	1.82 ± 0.01	86.24 ± 3.06
		Seed	Veraison	2.11 ± 0.02	2.06 ± 0.02	60.64 ± 2.81
	Apple	Fruit	Mature	2.03 ± 0.04	1.90 ± 0.03	23.12 ± 1.78
	Banana	Fruit	Mature	2.10 ± 0.02	1.91 ± 0.06	25.55 ± 2.59
	Pear	Fruit	Mature	1.99 ± 0.01	1.95 ± 0.04	24.90 ± 3.08
	Cherry	Fruit	Mature	2.11 ± 0.02	1.85 ± 0.05	19.21 ± 1.26
	Peach	Fruit	Mature	2.10 ± 0.01	1.91 ± 0.04	21.95 ± 2.39
	Apricot	Fruit	Mature	1.89 ± 0.04	1.83 ± 0.01	19.72 ± 5.17
Cheng et al. [7]	Grape	Leaf	Young	1.64 ± 0.05	1.66 ± 0.07	356.13 ± 5.91
Xu et al. [20]	Grape	Leaf	Young	1.66 ± 0.11	1.70 ± 0.03	383.22 ± 4.32
Hanania et al. [8]	Grape	Leaf	Young	1.65 ± 0.05	1.64 ± 0.07	432.54 ± 8.12
Hameed et al. [21]	Grape	Leaf	Young	1.75 ± 0.04	1.59 ± 0.03	441.90 ± 6.78
Kang and Yang [22]	Grape	Leaf	Young	1.69 ± 0.04	1.72 ± 0.04	371.43 ± 10.48

[a] Results are the mean of three samples (standard deviation)

[b] DNA yields using other methods were significantly different from yields with the new protocol at the 95% confidence level

[c] *FW* fresh weight

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genomic DNA isolated by this protocol from grape leaves is comparable to the amounts obtained in soybean leaves (45.00 μg/g FW), sorghum leaves (43.00 μg/g FW) [23], and Brassica leaves (376.75 μg/g FW) [24].

Restriction Analysis of Genomic DNA

Genomic DNA from seven fruit trees was restricted by *Eco*R I (4 U of enzyme/μg of DNA, kept 1 h at 37°C). The restricted DNA was produced a good pattern on 0.8% agarose gel, indicating suitable digestion of DNA samples extracted from this method (Fig. 2). Therefore, we concluded that the purity and quality of the genomic DNA was sufficient for enzyme digestion and could be used for Molecular markers and Southern blot analysis.

Cloning of Isolated DNA Fragments

To confirm the quality of total RNA and genomic DNA extracted by this method, the full-length open reading frame (ORF) and genomic DNA sequences of the grape thioredoxin *h* gene (Vv*CxxS*2) were amplified by PCR using oligonucleotides primers VTrx3F and VTrx3R (Table 1). The PCR products were separated on the 1.2% agarose gels, obtained the fragments with the expected size. The ORF and genomic DNA sequences of Vv*CxxS*2 gene are 381 bp and 866 bp, respectively, that were amplified from different grape tissues (Figs. 3a, b). The purified DNA fragments were cloned into a pUC19 cloning vector and it has been demonstrated all colonies contained plasmids larger than pUC19 after screening and subculture, confirming the presence of an insert (Fig. 3c). Thus, total RNA and genomic DNA isolated by this procedure is recomended for cloning.

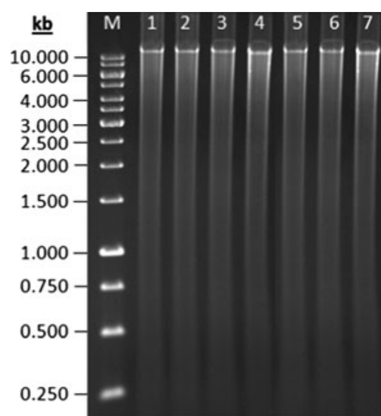

Fig. 2 Restriction analysis of genomic DNA samples isolated from different fruit tissues. The DNA samples were restricted by *Eco*R I enzyme and separated on 0.8% agarose gel, stained with EtBr, and observed under UV light. *M* 1 kb DNA ladder, *lane 1* grape, *lane 2* apple, *lane 3* banana, *lane 4* pear, *lane 5* cherry, *lane 6* peach, *lane 7* apricot

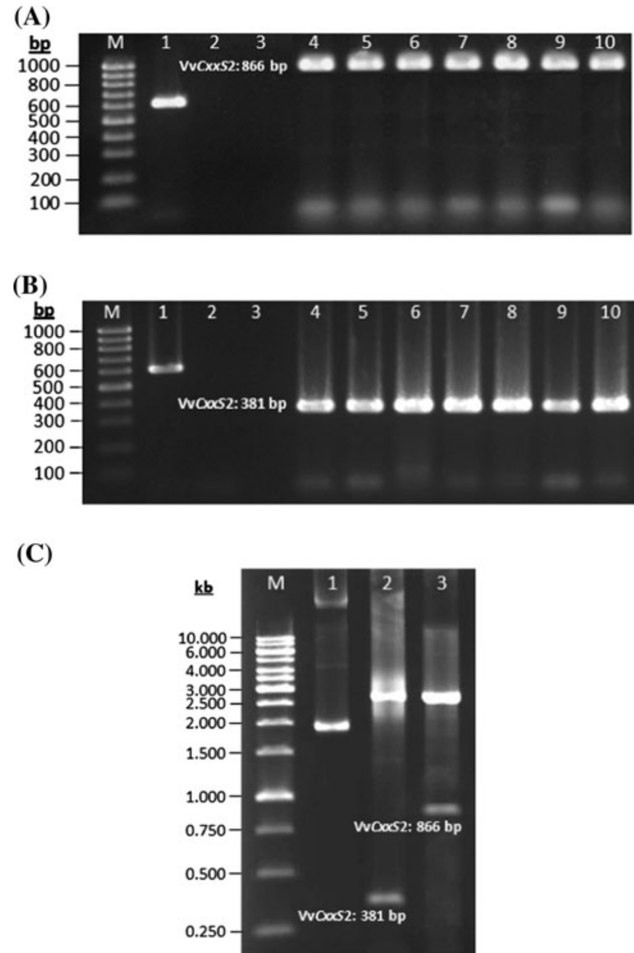

Fig. 3 PCR and RT-PCR amplification products from different grape tissues and cloning of DNA fragments. **a** Agarose gel electrophoretic analysis of PCR amplified DNA fragment of the Vv*CxxS*2 gene in different grape tissues. **b** RT-PCR analysis of different grape tissues with Vv*CxxS*2 gene. *M* 100 bp DNA ladder, *lane 1* positive control, *lane 2* negative control, lake of primers, *lane 3* negative control, lake of template, *lane 4* berry, *lane 5* leaf, *lane 6* petiole, *lane 7* cluster, *lane 8* stem, *lane 9* root, *lane 10* seed. **c** Recombinant clones carrying ORF and genomic DNA fragments of the Vv*CxxS*2 gene. *M* 1 kb DNA ladder, *lane 1* original plasmid pUC19, *lane 2* recombinant plasmid carrying ORF fragment of the Vv*CxxS*2 gene, *lane 3* recombinant plasmid carrying genomic DNA fragment of the Vv*CxxS*2 gene

Semiquantitative RT-PCR

Apart from the integrity of the ribosomal bands, the intactness of the RNA can also be monitored by RT-PCR. The expression of the grape thioredoxin *h* gene (Vv*CxxS*2) was analyzed by semiquantitative RT-PCR in various tissues at different developmental stages (Fig. 4). Transcripts of Vv*CxxS*2 were present in all tissues at different developmental stages, although it appears to express in berry, with slight modifications, more than other tissues. The expression of this gene was represented as a single band

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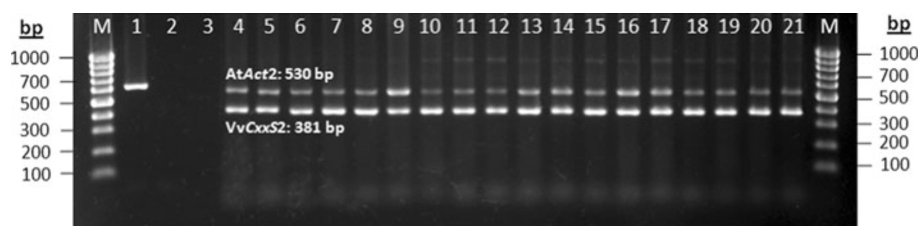

Fig. 4 Expression analysis of Vv*CxxS2* gene in different grape tissues at different developmental stages by semiquantitative RT-PCR. *M* 1 kb DNA ladder, *lane 1* positive control, *lane 2* negative control, lake of primers, *lane 3* negative control, lake of template, *lanes 4–9* berries at six developmental stages, *lanes 10–12* leaves at three developmental stages including young, mid, and old, respectively, *lanes 13–15* petioles at three developmental stages such as young, mid, and old, respectively, *lanes 16–18* clusters at three developmental stages including pre-veraison, veraison, and post-veraison, respectively, *lane 19* stem, *lane 20* root, *lane 21* seed

without any smearing, indicating the integrity of the isolated RNA that even a low expression of the gene can be monitored, and also that this procedure is not affected by different tissues and developmental stages.

Conclusion

We report a rapid, efficient, and reliable nucleic acids extraction method for plant tissues rich in polysaccharides and polyphenolic compounds. In contrast to other methods tested, the nucleic acids prepared by this protocol were of high quality and quantity, and were successfully used for reverse transcription reaction, PCR, RT-PCR, cloning, and gene expression analysis. We believe that nucleic acids isolated by this procedure could be applied for other molecular biology techniques and functional genomic experiments including Northern blot, Southern blot, Molecular markers, and Microarray analysis.

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