ACTA ABP BIOCHIMICA POLONICA

Vol. 59, No 3/2012
391–393
on-line at: www.actabp.pl

Regular paper

An improved method for RNA isolation from plants using commercial extraction kits

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Isolation of RNA from plants rich in secondary metabolites using commercial kits often results in contaminated preparations which are not suitable for downstream applications. Although many specific protocols appropriate for plants with a high content of phenolics, anthocyanins and polysaccharides have been developed, these are often expensive, time consuming and not applicable to different types of tissues. This study presents a simple and efficient modification of RNA extraction from different types of tissues using two commercial reagent kits. By simple improvement, we routinely obtained high-quality RNA of the following plants: the blackcurrant bush, black chokeberry bush, pear tree, apricot tree, apple tree, hardy kiwi, tangerine tree, highbush blueberry and cranberry plant.

Keywords: total RNA, secondary metabolites

Received: 15 March, 2012; revised: 28 May, 2012; accepted: 16 July, 2012; available on-line: 24 July, 2012

INTRODUCTION

The extraction of high-quality RNA from tissues with a high level of secondary metabolites using commercial kits is often difficult. One of the biggest issues during RNA extraction concerns phenolic compounds, which oxidize and form quinones. Aromatic compounds bind RNA, which hinders downstream applications (Loomis, 1974). Although many specific protocols appropriate for plants rich in secondary metabolites have been developed, they are often expensive, time consuming and not applicable to woody tissues (Chomczynski & Sacchi, 1987; Ding *et al*., 2007; Camacho-Villasana *et al*., 2002; Liao *et al*., 2004; Tong *et al*., 2012).

Initially we tried to obtain RNA from highbush blueberry (*Vaccinium corymbosum* L.) tissues using two commercial kits: the Spectrum RNA Kit (Sigma Aldrich, St. Louis, MO, USA) and the RNeasy® (Qiagen Inc., Valencia, CA, USA) procedure as modified by MacKenzie *et al*. (1997) (available on the Qiagen website), but in both cases the quantity or/and quality of RNA obtained was unsuitable for RT-PCR. After the failed RNA isolation with commercial kits, we tried LiCl- (Verwoerd *et al*., 1989) and phenol-based protocols (Ghawana *et al*., 2011). Unfortunately, the RNA pellets were yellowish in both, suggesting contamination with non-nucleic acid compounds. Therefore, we reasoned that a modified homogenization method of samples could improve the quality and yield of commercial RNA extraction kits. In this study, we modified the RNA extraction procedures of two commercial reagents: the Spectrum™ Plant Total RNA Kit and an alternative procedure of RNA isolation from plant tissues rich in secondary metabolites using the RNeasy® Plant Mini Kit developed by MacKenzie *et al*. (1997).

MATERIAL AND METHODS

Plant material. The highbush blueberry was grown under normal environmental conditions on the Blueberry Experimental Farm in Błonie near Prażmów belonging to the Department of Pomology of the Warsaw University of Life Sciences (SGGW). The blackcurrant bush (*Ribes nigrum* L.), black chokeberry bush (*Photinia melanocarpa* Michx.), pear tree (*Pyrus communis* L.), apricot tree (*Prunus armeniaca* L.), apple tree (*Malus* sp. Mill.), hardy kiwi (*Actinidia arguta* Siebold & Zucc.) and cranberry plant (*Vaccinium macrocarpon* L.) were collected from an experimental field located at the university campus. The tangerine tree (*Citrus reticulata* Blanco) was acquired at a local flower shop. Mature leaves, fruits and barks were collected and stored at –80°C until further use.

RNA extraction. All the glassware, mortars and pestles were treated with RNaseZAP™ (Sigma Aldrich) and autoclaved. Plasticware (tips and tubes) were certified as RNase- and DNase-free. To limit the exposure to noxious components of extraction buffers (e.g., β-mercaptoethanol (B-ME), sodium lauroyl sarcosinate, guanidine thiocyanate) the homogenization was conducted in a fume hood.

Modification of the Spectrum™ Plant Total RNA Kit protocol. Samples (up to 50 mg) were ground under liquid nitrogen to fine powder using a mortar and a pestle. Subsequently, 500 µl of Lysis Solution was added, and all was ground to obtain a homogenous mixture. Trituration was carried on until the sample was completely thawed. Then, 300 µl of Lysis Solution were added and mixed in by grinding. The mixture (~800 µl) was transferred to a 2-ml collection tube and incubated without shaking at room temperature for 10 minutes. The following steps of RNA extraction were conducted using the Spectrum™ Plant Total RNA Kit protocol A.

Modification of the alternative procedure of the RNeasy®Plant Mini Kit protocol (MacKenzie *et al*., 1997). Samples (up to 50 mg) were ground under liquid nitrogen to fine powder using a mortar and pestle. Then, 500 µl of extraction buffer (4 M guanidine thiocyanate, 0.2 M sodium acetate, pH 5.0, 25 mM EDTA, 2.5% (w/v), polyvinylpyrrolidone (PVP) and 1% (v/v) B-ME) was added, and the grinding was continued to obtain a

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Abbreviations: B-ME, β-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; PVP, polyvinylpyrrolidone; PVPP, polyvinylpolypyrrolidone; RT-PCR, reverse transcription-polymerase chain reaction

Table 1. Yield and purity of RNA isolated from blueberry plant parts using different methods

Extraction method	Tissue	$A_{260/280}$	Yield (µg/50 mg FW)
Present study (modification of Spectrum™ Plant Total RNA Kit protocol)	Bark	2.09±0.02	21.61±0.34
	Mature leaf	2.09±0.02	20.62±0.15
	Flower	2.06±0.03	69.74±2.19
Spectrum™ Plant Total RNA Kit basic protocol	Bark	2.16±0.06	0.27±0.04
	Mature leaf	2.16±0.02	1.13±0.07
	Flower	2.14±0.04	1.75±0.05
RNeasy® alternative procedure (MacKenzie *et al.*, 1997)	Bark	2.16±0.08	2.76±0.05
	Mature leaf	1.59±0.02	0.47±0.02
	Flower	2.08±0.05	0.45±0.03

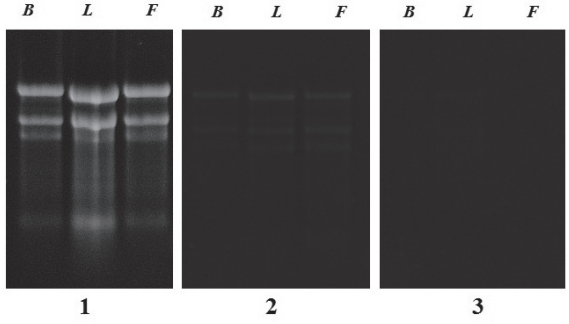

Figure 1. Denaturing gel electrophoresis of RNA extracted from highbush blueberry using different protocols.
(1) present study – modification of Spectrum™ Plant Total RNA Kit protocol; **(2)** original Spectrum™ Plant Total RNA Kit protocol; **(3)** RNeasy® procedure modified by MacKenzie *et al.* (1997). RNA was purified from: bark (B), leaf (L) and flower (F).

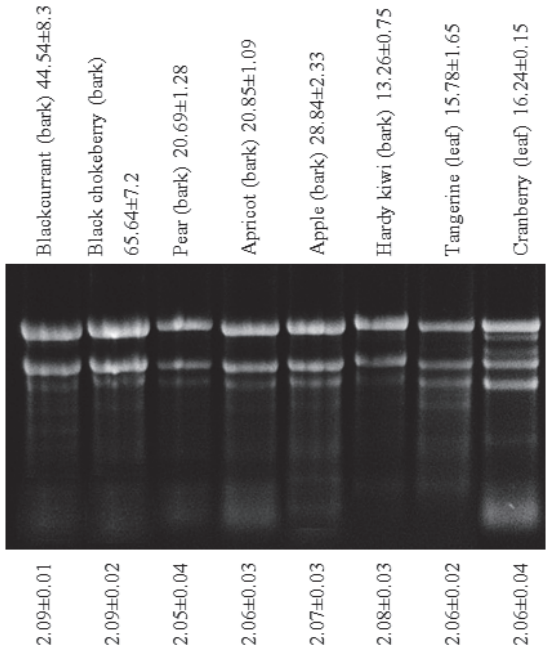

Figure 2. Denaturing gel electrophoresis of RNA isolated from different plants using modification of the alternative procedure of the RNeasy®Plant Mini Kit protocol (MacKenzie *et al.*, 1997)).
The A260/280 ratios, plant and tissue types along with RNA yield (µg/ 50 mg) are given below and above each panel, respectively.

homogenous mixture and carried on until the sample was completely thawed. Next, 300 µl of extraction buffer and 60 µl of 20% sodium lauroyl sarcosinate were added and mixed in by further grinding. The mixture (~860 µl) was transferred to a 2-ml collection tube and incubated with vigorous shaking at room temperature for 10 minutes. The following steps of RNA isolation were performed in accordance with the instructions of the MacKenzie's alternative procedure (MacKenzie *et al.*, 1997).

RNA quantity, quality and RT-PCR. RNA was analyzed primarily by running 1 µl of each sample on 1.2% denaturing-formaldehyde agarose gel stained with ethidium bromide. The concentration and purity of the extracted RNA were analyzed by means of a Tecan Infinite® 200 PRO NanoQuant spectrophotometer (Tecan Group Ltd., Switzerland).

Total RNA from blueberry leaves, flowers and bark were used for one-step RT-PCR. Amplification was performed using the Titan One Tube RT-PCR System (Roche, Germany) and the primers specific to the ribulose 1,5-bisphosphate carboxylase chloroplast (*Rbc1*) gene (Sanchez-Navarro *et al.*, 2005).

RESULTS AND DISCUSSION

Most of the published RNA isolation protocols employing denaturing organic solvents (phenol or chloroform), denaturing agents (guanidine thiocyanate), reducing agents (B-ME) and polymers that bind contaminants (PVP, polyvinylpolypyrrolidone (PVPP)) can successfully be used to obtain RNA from plant material with a high level of secondary metabolites. Unfortunately, LiCl- and phenol-based protocols as well as kits with reagents dedicated to plants rich in secondary metabolites did not result in obtaining high-quality RNA from blueberry tissues. Therefore, we presumed that during the first step of RNA isolation tissues with a high content of secondary metabolites could have had insufficient contact with extraction buffers.

Homogenization with extraction buffer improved the quality and quantity of RNA when using both the Spectrum™ Plant Total RNA Kit and the alternative procedure of the RNeasy®Plant RNA Mini Kit developed by MacKenzie *et al.* (1997). The quantity and purity of blueberry RNA preparations were found to be superior when using this simple modification as compared to the original kit protocols (Table 1). RNA concentration was at least ten times improved. Electrophoresis on agarose formaldehyde gel resolved 26S and 18S rRNA bands

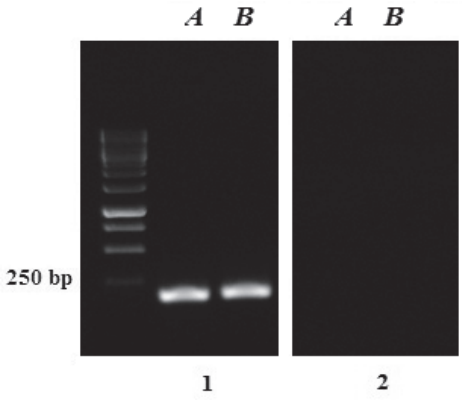

Figure 3. Agarose gel analysis of RT-PCR assays with the *Rbc1*-specific primers on total RNA extracts from highbush blueberry bark tissue.
(1A) RNA obtained with a modified Spectrum™ Plant Total RNA Kit protocol; **(1B)** RNA obtained with a modified alternative procedure of the RNeasy®Plant Mini Kit protocol (MacKenzie *et al.,* 1997); **(2A)** RNA obtained with the Spectrum™ Plant Total RNA Kit protocol; **(2B)** RNA obtained with an alternative procedure of the RNeasy®Plant Mini Kit protocol (MacKenzie *et al.,* 1997).

with no DNA contamination in all samples (Fig. 1 and Fig. 2). Additionally, RNA preparations from nine different woody plants did not reveal the high level of RNA degradation that may occur during isolation. The A260/280 ratios of RNA extracts indicated there was no protein, phenol or other contaminants that absorb strongly at or near 280 nm. RNA extracts obtained with our modification were suitable for RT-PCR. Amplified fragments of the blueberry *Rbc1* gene (186 bp) were visible on agarose gel, whereas the quality and quantity of RNA extracts obtained in accordance with the manufacturer's instructions were not applicable to amplification (Fig. 3). The results of this study indicate that, apart from the choice of chemical reagents, the method of tissue homogenization is a critical step during RNA extraction from "difficult" sources.

Acknowledgements

This work was supported by grant N N310 036038 from the Ministry of Science and Higher Education.

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