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ScienceDirect
South African Journal of Botany 76 (2010) 375–379
SOUTH AFRICAN JOURNAL OF BOTANY
www.elsevier.com/locate/sajb

Short communication

A small-scale RNA isolation protocol useful for high-throughput extractions from recalcitrant plants

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Received 18 September 2009; received in revised form 16 November 2009; accepted 4 January 2010

Abstract

Many plants indigenous to South Africa are rich in secondary and oxidizing compounds such as pigments, complex polysaccharides and polyphenols. This makes isolation of high quality RNA for analysis of gene expression difficult. Here we describe a cost-effective isolation protocol suitable for RNA extraction from recalcitrant plant species. This method uses small amounts of tissue, so is useful when material is limited, and is easy to process large numbers of samples at once. We have used the method successfully with mature leaves of Protea hybrid 'Sylvia', and species *P. repens, Leucospermum* hybrid 'Succession', resurrection plants *Xerophyta humilis* and *Craterostigma pumilum,* and mature needles of Pine (*Pinus radiata*). RNA was analyzed spectrophotometrically and was found to be of high purity with low levels of contaminating compounds. Electrophoretic analyses on denaturing formaldehyde agarose gels and an Agilent 2100 Bioanalyzer confirmed the presence of RNA of high integrity. This is the first description of plant RNA integrity number (RIN) values for these plants using the algorithm designed for analyses of plant RNA containing multiple ribosomal bands. The RNA could successfully be used for reverse transcription and gene amplification.

Keywords: RNA isolation; Gene expression; RIN value; *Proteaceae*; Pine; Resurrection plants

1. Introduction

Understanding the genetics of development and adaptive responses of plants relies on the use of molecular biology. Gene expression analyses by real-time quantitative polymerase chain reaction (qPCR) and semi-quantitative PCR are preceded by the isolation of high quality RNA from large numbers of samples. This can be laborious and can lead to large technical variation between samples if the purity and quality of the RNA extracted is variable. Extraction of high quality RNA, free from contaminating compounds, is further impeded by the presence of secondary products in plants. Proteaceae and other plants indigenous to South Africa have tough leathery leaves and high contents of complex polyphenols, polysaccharides which have hindered reliable nucleic acid extraction and the processing of large numbers of samples simultaneously.

Although the use of RNA isolation kits simplify and accelerate the extraction of RNA from plant tissue these tend to be less cost-effective for large sample numbers and may not be successful for use on all plant species. Extractions that use large buffer volumes (3–15 ml) and tissue weights (1–2 g) result in high RNA yields from recalcitrant plant species (Azevedo et al., 2003; Claros and Canovas, 1998; Hu et al., 2002; Rubio-Pina and Vazquez-Flota, 2008; Wang et al., 2005), but are not amenable to high-throughput studies or where sample material is limited. Previously described protocols that use small volumes, up to 2 ml, and fresh tissue weights, from 50 mg to 200 mg (Onate-Sanchez and Vicente-Carbojosa, 2008; Portillo et al., 2006; Verwoerd et al., 1986) are not suitable for the extraction of RNA from recalcitrant plant tissue. A simple high-throughput RNA extraction protocol for use in a wide range of recalcitrant plants has been the limitation to gene expression studies in many non-model systems.

Here we describe a low cost, high-throughput RNA extraction protocol modified from Azevedo et al. (2003) that can be used to study gene expression in many plant species. By reducing buffer and tissue volumes, extraction could be performed in 1.5 and 2 ml

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doi:10.1016/j.sajb.2010.01.002

Eppendorf tubes, significantly increasing throughput number. The protocol was made more cost-effective by reducing the volumes of buffer and organic solvents, and excluding Proteinase K. Increasing the buffer volume during LiCl precipitation of RNA leads to a reduction in genomic DNA (gDNA) contamination. RNA was successfully extracted from leaves and meristems of Proteaceae species and cultivated hybrids, rich in polysaccharides and phenolic compounds studied for their commercial and ecological significance; two resurrection plants, *Xerophyta humilis* and *Craterostigma pumilum*, routinely used for drought resistance studies and known to contain complex sugars. Pine (*Pinus radiata*) was included as it was used in the original paper (Azevedo et al., 2003) and is known to contain a significant amount of secondary products. RNA quantity was assessed spectrophotometrically and electrophoretically by denaturing formaldehyde gel and a Bioanalyzer (Agilent Technologies, Wilmington, DE). We tested the plant RNA algorithm available on the 2100 expert software (Rev. B.02.07 Si 482 (Beta)) on RNA isolated from mature leaf tissues known to contain multiple ribosomal bands. RNA integrity number (RIN) and equivalents such as RNA Quality Indicator (RQI; Bio-Rad Laboratories, Inc., Hercules, CA) values are becoming accepted as the "standard" for gauging RNA quality and integrity (Imbeaud et al., 2005). RNA extracted using the described protocol yielded RIN values suitable for downstream applications such as reverse transcription, cDNA library construction, qPCR and micro-array analyses.

2. Materials and methods

2.1. Plant tissue

Pine needles from the lower branches of a *P. radiata* tree growing on the slopes of Devil's Peak (latitude 33°91′S; longitude 18°42′E, Cape Town, South Africa) were collected and frozen in liquid nitrogen before extraction. *X. humilis* and *C. pumilum* were collected from Pilanesberg, South Africa (latitude 24°98′S; longitude 28°1′E) re-potted in 30×20 cm trays and grown under glasshouse conditions at the University of Cape Town as described (Dace et al., 1998). *P. radiata*, *X. humilis* and *C. pumilum* were harvested on the day of extraction. *Proteaceae* samples were collected from 'Protea Heights', a farm on the outskirts of Stellenbosch (latitude 33°54′S; longitude 18°40′E), South Africa. 'Sylvia' and 'Succession' are cultivated on the farm and grown in rows 4 m apart with 1 m spacing between plants without fertilization or irrigation. *P. repens* grows unmanaged on the farm. *Proteaceae* leaf and floral meristem tissues were flash frozen in liquid nitrogen immediately after excision from the plant and stored at −80 °C prior to analysis.

2.2. RNase removal treatments

Mortar and pestles were washed with Extran detergent, rinsed well with deionized water and subsequently wiped with chloroform to remove any residual proteins. Gel electrophoresis apparatus were soaked in 0.1 M NaOH and 10 mM EDTA for at least 20 min, rinsed well with diethyl pyrocarbonate (DEPC)-treated water or sprayed with RNase-Free (GeneChoice, Maryland, USA), wiped and then rinsed with DEPC-treated water.

2.3. RNA isolation

Aliquots of 1 ml RNA extraction buffer (100 mM Tris, pH 8.0, 2% (w/v) N-cetyl-N,N,N-trimethylammonium bromide (CTAB), 30 mM ethylenediaminetetraacetic acid (EDTA), 2 M NaCl, 2% (w/v) polyvinylpolypyrrolidone (PVPP), 0.05% (w/v) spermidine and 2%(v/v) β-mercaptoethanol) in 1.5 ml tubes were prewarmed to 42 °C before addition of the plant tissue. Concentrated stocks of the buffer components were prepared in millipore quality (resistance, 10 MΩ-cm) water and autoclaved prior to preparation of the extraction buffer. A 5% (w/v) spermidine stock was prepared in millipore water and sterilised using a 0.22 μm syringe filter. Plant tissue was ground to a fine powder in liquid nitrogen by means of physical maceration in a mortar and pestle. A spatula pre-cooled in liquid nitrogen was used to add the powdered tissue to the extraction buffer. Approximately 3–4 spatula tips equating to ca. 100 mg tissue was used per 1 ml extraction. After addition, tubes were tightly capped and vortexed to suspend the tissue in extraction buffer before returning the tubes to the 42 °C waterbath. Samples were incubated at 42 °C for at least 90 min with intermittent vortexing, before centrifuging for 15 min at top speed (approximately 13 000×*g*) in bench-top microcentrifuge. All centrifugations were performed at 13 000×*g* at 4 °C. The nucleic acid-containing aqueous phase was removed into a fresh 2 ml tube and extracted twice with an equal volume of chloroform:iso-amyl alcohol (24:1) by vortexing well after the addition of solvent and centrifuging for 15 min. The final aqueous phase was removed into a 1.5 ml tube and modified extraction buffer (100 mM Tris, pH 8.0, 2% CTAB, 30 mM EDTA, 2 M NaCl) added to a final volume of 1 ml if the aqueous phase volume was 700 μl or more; or to a final volume of 800 μl if the volume of the aqueous phase was less than 700 μl. After the addition of the buffer, 10 M LiCl was added to a final concentration of 2 M. RNA was allowed to precipitate at −20 °C overnight. Following RNA precipitation, samples were centrifuged for 20 min and the aqueous phase removed without disturbing the RNA pellet. The RNA pellet was washed with 500 μl 70% (v/v) ethanol by vortexing to dislodge the pellet from the tube wall. After centrifugation for 20 min the ethanol was carefully removed by pipetting and the RNA dried in a 37 °C heating block and subsequently dissolved in 20 μl DEPC-treated or sterile millipore water.

2.4. Assessment of RNA quantity and quality

RNA quantity and purity were determined spectrophotometrically using a NanoDrop® (ND1000, Thermo Scientific, Delaware, USA). RNA was visualized on a denaturing formaldehyde agarose gel using a Gel/Chemi Doc with Universal hood II (Bio Rad Laboratories, Inc., Milan, Italy). RNA was also separated electrophoretically on an Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip (Agilent Technologies, CA, USA) using the 2100 Expert Software (Rev. B. 02. 07 Si 482 (Beta)) to calculate plant RIN values.