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Short communication

Establishment of a rapid, inexpensive protocol for extraction of high quality RNA from small amounts of strawberry plant tissues and other recalcitrant fruit crops

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ABSTRACT

Strawberry plant tissues and particularly fruit material are rich in polysaccharides and polyphenolic compounds, thus rendering the isolation of nucleic acids a difficult task. This work describes the successful modification of a total RNA extraction protocol, which enables the isolation of high quantity and quality of total RNA from small amounts of strawberry leaf, root and fruit tissues. Reverse-transcription polymerase chain reaction (RT-PCR) amplification of *GAPDH* housekeeping gene from isolated RNA further supports the proposed protocol efficiency and its use for downstream molecular applications. This novel procedure was also successfully followed using other fruit tissues, such as olive and kiwifruit. In addition, optional treatment with RNase A following initial nucleic acid extraction can provide sufficient quality and quality of genomic DNA for subsequent PCR analyses, as evidenced from PCR amplification of housekeeping genes using extracted genomic DNA as template. Overall, this optimized protocol allows easy, rapid and economic isolation of high quality RNA from small amounts of an important fruit crop, such as strawberry, with extended applicability to other recalcitrant fruit crops.

1. Introduction

The isolation of sufficient quantity and high quality nucleic acids is a prerequisite for conducting analytical studies on genetics, molecular biology and other related physiological investigations in plants (Hu et al., 2002). Northern blot hybridization, RT-PCR and microarray tests, employed for gene expression and transcriptomic analysis of plants under a variety of conditions, require RNA of high quality (Berendzen et al., 2005). However, the isolation of functional RNA from certain plant tissues rich in polysaccharides, polyphenolic compounds and proteins is often a time-consuming and tedious task. Polyphenols are known to get readily oxidized to form quinones which in turn can irreversibly interact with proteins and nucleic acids to form high molecular weight complexes that hinder isolation of good quality RNA (Japelaghi et al., 2011). In turn, polysaccharides tend to co-precipitate with nucleic acids in low ionic strength buffers (Wang and Stegemann, 2010). In addition, these co-precipitated compounds severely restrict RNA reverse transcriptase and DNA polymerase functionality, as well as DNA restriction endonuclease activities, and absorbance-based quantification assays (Moser et al., 2004). Furthermore, stressful conditions for plant growth may further encumber the isolation of high quality RNA, since polyphenols and polysaccharides are bioaccumulated in plant tissues under adverse environmental stimuli (Chang et al., 1993). Therefore, many protocols, along with their modifications, have been published over the years for nucleic acid isolation from recalcitrant plants (Berendzen et al., 2005; Henderson and Hammond, 2013; Hu et al., 2002; Samanta et al., 2011; Sharma et al., 2003; Smart and Roden, 2010; Wang and Stegemann, 2010). However, the majority of them pose certain limitations to researchers as they are time consuming (Moser et al., 2004), tissue-specific (Asif et al., 2000) or technically complex (Wang and Stegemann, 2010).

In recent years, strawberry has received particular attention from the research community due to the dietary value of its fruits and the economic importance of its cultivation (Tulipani et al., 2008). Strawberry tissues are rich in polyphenolic compounds and polysaccharides (Asami et al., 2003); thus, the isolation of good quantity and quality nucleic acids from its tissues is often challenging. Several protocols have been used for RNA isolation from strawberry plant tissues, using phenol (Mazzara and James, 2000), hot borate (Martínez and Civello, 2008), or cetyltrimethylammonium bromide (CTAB) (Palomer et al., 2006) in the isolation procedure. The current study presents an

Abbreviations: EB, extraction buffer; PCI, phenol: chloroform: isoamyl alcohol; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; NaOAC, sodium acetate.

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optimized protocol for the isolation of high quality total RNA from small amounts of strawberry leaf, root and fruit tissue, suitable for downstream molecular applications. This procedure represents a significant improvement in terms of time and amount of tissue needed in comparison with established extraction protocols, requiring ca. 3 h (including nuclease treatment) and 100 mg of tissue for successful completion.

The protocol presented herein is based on a modified version of the SDS/PCI method of Mortaji et al. (2008) for RNA extraction from wheat seeds. For comparative purposes, an established protocol for RNA extraction from strawberry leaves and petioles using LiCl and sodium dodecyl sulfate (SDS) in the extraction buffer (Mazzara and James, 2000), as well as a commercially available, ready-to-use reagent (TRIzol® Reagent, Invitrogen) commonly used for nucleic acid extraction have also been tested. The proposed protocol was subsequently employed for successful RNA extraction from other fruits such as olive and kiwifruit.

2. Materials and methods

2.1. Plant material

Fully expanded leaves, roots and fruit were sampled from six-month-old strawberry (*Fragaria* × *ananassa*) plants grown hydroponically in a constantly aerated half-strength Hoagland nutrient solution in 15 l pots. Conditions in the growth room were 16 h photoperiod (250 μmol m^{-2} s^{-1}), 23 °C/20 °C day/night temperature and 65% relative humidity. Olive fruit (*Olea europaea*) and kiwifruit (*Actinidia deliciosa*) were harvested from commercial orchards at maturity stage. All samples were flash-frozen in liquid nitrogen and stored at −80 °C until needed.

2.2. Solutions and reagents

The RNA extraction buffer (EB) was similar to that proposed by Mortaji et al. (2008), with the exception of the use of 0.5 M instead of 1 M Tris–HCl (Merck) pH 9, along with 1% (w/v) sodium dodecyl sulfate (SDS). Other solutions used were saturated phenol: chloroform: isoamyl alcohol (PCI) 25:24:1 (v/v), 3 M sodium acetate (NaOAC) (Merck) pH 5.6, as well as 100 and 70% (v/v) ethanol. All experiments were carried out in triplicate using independent pools of tissue samples.

2.3. Nucleic acid isolation procedure

Nucleic acid isolation was carried out based on a protocol proposed by Mortaji et al. (2008) for RNA extraction from wheat seeds, following several major modifications:

1. Initially, plant tissue samples were ground into fine powder in a pre-chilled pestle and mortar under liquid nitrogen. Subsequently, 100 mg of homogenate sample (instead of 300 mg) was transferred to 2 ml tubes containing 1 ml ice-cold EB (0.5 M Tris–HCl pH 9, 1% (w/v) SDS), instead of 1.5 ml pre-warmed EB. Sample was agitated immediately and 1 ml of PCI was added to the tube. The sample was gently agitated for an additional 2 min to form an emulsion and then centrifuged at 14,000 ×*g* for 5 min at 4 °C for phase separation.
2. The upper aqueous phase (~800 μl) was collected into a fresh chilled 2 ml tube and equal volume of PCI was added and gently agitated. Sample was centrifuged at 14,000 ×*g* for 7 min at 4 °C.
3. The aqueous phase (~600 μl) was collected into fresh chilled tube and the sample was re-extracted with an equal volume of PCI, agitated and centrifuged as previously described.
4. The upper aqueous phase (~400 μl) was carefully transferred to a fresh chilled tube and centrifuged at 16,000 ×*g* for 7 min at 4 °C to remove traces of phenol.
5. The supernatant was collected into a fresh chilled tube, where 0.1 volume 3 M NaOAC (pH 5.6) and 1 volume (instead of 3) 100% (v/v) ethanol were added, mixed by inversion and subsequently incubated at −80 °C for 20 min for nucleic acid precipitation.
6. Sample was then centrifuged at 16,000 ×*g* for 8 min at 4 °C, supernatant was discarded and 1 ml 70% (v/v) ethanol was added to the tube, centrifuged at 16,000 ×*g* for 3 min at 4 °C and supernatant discarded again.
7. Nucleic acid pellet was air-dried at room temperature (or heated at 50 °C for 2–3 min), and finally dissolved in 20–30 μl ddH_2O, depending on the pellet size.

Alternative isolation protocols included the protocol described by Mazzara and James (2000) and the ready-to-use TRIzol® Reagent (Invitrogen, USA) following the manufacturer's instructions.

2.4. Nucleic acid quantification and quality control

The nucleic acid quantity and purity were determined spectrophotometrically by measuring absorbance ratios A_{260}/A_{230} and A_{260}/A_{280}, indicative of contamination by polyphenols/carbohydrates and proteins, respectively, using the NanoDrop (ND-1000, Thermo Scientific, Delaware, USA). The integrity of extracted nucleic acids was verified by running 1 μg sample in a 1.5% (w/v) agarose gel, stained with GelRed™ (Biotium, Inc., USA).

2.5. DNase and RNase treatment

The DNase I Set (Macherey-Nagel GmbH & Co., Germany) was used to purify RNA from DNA-contaminated samples according to manufacturer's protocol. In turn, in order to eliminate RNA from extracted samples for genomic DNA isolation, nucleic acid samples were incubated with RNase A (Macherey-Nagel GmbH & Co., Germany) according to the manufacturer's instructions.

2.6. Reverse transcription (RT) and polymerase chain reaction (PCR)

For first-strand cDNA synthesis, 1 μg total RNA was reversed transcribed using the Primescript 1st Strand Synthesis kit, as per manufacturer's instructions (Takara Bio Inc., Japan). The cDNA synthesized was then diluted 1:10 (v/v) with DEPC-treated water and stored at −20 °C for PCR amplification. The strawberry *glyceraldehyde 3-phosphate dehydrogenase (FaGAPDH)* gene (Miyawaki et al., 2012) was amplified using both cDNA and genomic DNA (after RNase treatment) templates (1 μg) in a 10 μl reaction mix containing Taq DNA polymerase (Invitrogen Inc.). The initial denaturation step (95 °C for 5 min) in PCR reaction was followed by 40 cycles of 95 °C for 30 s, 56 °C for 45 s and 72 °C for 45 s, and a final extension period at 72 °C for 10 min. The amplified product was visualized by gel electrophoresis in a 1.5% (w/v) agarose gel. Similarly, primers for kiwifruit and olive fruit were used in identical cycles for the amplification of *actin* (*AdACT*; Yin et al., 2010) and *ubiquitin* (*OeUBQ2*; Hernández et al., 2009) housekeeping genes, respectively. Oligonucleotide sequences that were used in this study are listed in Table 1.

Table 1
Oligonucleotides primers used for PCR and RT-PCR.

Gene	Primer	Primer sequence	Reference
FaGAPDH	For	5′-TCCATCACTGCCACCCAGAAGACTG-3′	Miyawaki et al. (2012)
	Rev	5′-AGCAGGCAGAACCTTTCCGACAG-3′	
AdACT	For	5′-TGCATGAGCGATCAAGTTTCAAG-3′	Yin et al. (2010)
	Rev	5′-TGTCCCATGTCTGGTTGATGACT-3′	
OeUBQ2	For	5′-AATGAAGTCTGTCTCTCCTTTGG-3′	Hernández et al. (2009)
	Rev	5′-AAGGGAAATCCCATCAACG-3′	