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Dormant and non-dormant *Castanea sativa* Mill. buds require different polyvinylpyrrolidone concentrations for optimal RNA isolation

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ABSTRACT

Recovering RNA of high quality and sufficient quantity is a prerequisite for ensuring proper representation of expressed genes in a cDNA library. Like Arabidopsis seeds, *Castanea sativa* buds contain polyphenols, polysaccharides and lipids that often interface with RNA, restricting its yield and quality. The hot borate RNA isolation described for Arabidopsis seeds was used to extract RNA from *C. sativa* buds. The optimal concentration of polyvinylpyrrolidone in the extraction buffer differed between dormant (14.3 mg/ml) and non-dormant buds (4.3 mg/ml) for RNA yield. Furthermore, differences were found in RNA integrity and purity between different dormancy stages in buds. In the case of secondary dormant and non-dormant Arabidopsis seeds differences were found attending to the RNA yield but not integrity or purity. The optimal concentration for secondary dormant seeds was 10.0 and 14.3 mg/ml for non-dormant seeds. RNA of *C. sativa* buds proved suitable for RT-PCR and the construction of cDNA libraries. The different optima for dormant and non-dormant tissues likely reflect a different chemical composition. Unexpectedly, the total phenol content in dormant and non-dormant tissues did not explain these different requirements for polyvinylpyrrolidone. The reasons for this are discussed. Optimization of the PVP concentration is recommended as a standard operating procedure for the development of RNA extraction protocols.

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1. Introduction

Obtaining high quality, intact RNA is the first and often the most critical step in performing many fundamental molecular biology experiments, including Northern analysis, nuclease protection assays, RT-PCR, RNA mapping, *in vitro* translation and cDNA library construction. RNA isolation is difficult in the presence of high levels of phenolics, polysaccharides, and endogenous RNases. Phenolics are readily oxidized to form covalently linked quinones and easily bind nucleic acids [1]. In addition to phenolics and other secondary metabolites polysaccharide contamination hinders re-suspension of precipitated RNA, interferes with absorbance based quantification, and may inhibit enzymatic manipulations [2]. Several methods are commonly used for isolation of RNA [3–8]. Each of these methods can be modified to be applicable to a particular plant species [9]. Extracting RNA from plant tissues can be difficult and often requires the modification of existing protocols or the development of new procedures. This is particularly true for woody species such as *Castanea sativa* or seeds because they contain high levels of extractable phenols and polysaccharides. Commercially available kits do not always deliver suitable results.

Polyvinylpyrrolidone (PVP) is an inhibitor of polyphenol oxidase that has been described to prevent browning during RNA isolation and its use in the removal of secondary metabolites from nucleic acids has been widely reported [6,10,11]. PVP presumably prevents the co-precipitation of phenols during RNA extraction, thus preventing their interference with RNA quality [12]. A correlation between phenol content and bud dormancy has been found [13]. Therefore, adaptation and optimization of RNA extraction protocols for buds should be performed with each of the different dormancy stages that form the subject of a study.

In this report we describe a simple extraction protocol that provides high-quality and high-quantity RNA suitable for constructing good-quality cDNA libraries in different dormancy stages from *C. sativa* buds. It was decided to optimize the concentration of PVP in the RNA extraction buffer of dormant and non-dormant tissues separately. This protocol is a modification of the RNA extraction method described by Wan and Wilkins [14] and used successfully for Arabidopsis seeds [15]. The optimal PVP

Abbreviations: dw, dry weight; GAE, gallic acid equivalents; PVP, polyvinylpyrrolidone; RT-PCR, reverse-transcription-polymerase chain reaction.

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concentration in the extraction buffer differed between dormant and non-dormant *C. sativa* buds and Arabidopsis seeds. Total phenol also differed, but did not explain the differences in optimal PVP concentration in the RNA extraction buffer.

2. Materials and methods

2.1. Plant material

Castanea sativa Mill. buds for RNA isolation were sampled in 2008 from twenty 5-year-old trees growing in Villaviciosa in Asturias (51250O, 431280N) and collected in the spring (bud burst; non-dormant, ND) and autumn (bud set; dormant, D). Upon collection the buds were frozen in liquid nitrogen and stored at −80 °C until further use. Per sample 100 mg plant material was used and four replicates per treatment. Water contents were determined for dormant and non-dormant buds and used for correction in the RNA yield.

Arabidopsis plants of accession Landsberg *erecta* were grown in a greenhouse in the summer of 2005. Seeds were harvested, dried at 15% RH and 15 °C and stored under the same conditions. Maximum viability was attained. Seeds were imbibed and incubated for 1 day in the dark at 20 °C and these seeds were non-dormant (ND). Seeds incubated for 10 days in the dark at 20 °C were secondary dormant (D) [15]. Upon incubation seeds were frozen in liquid nitrogen and stored at −80 °C until further use. Per sample 30 mg plant material was used and four replicates per treatment.

2.2. RNA isolation

Solutions were made up with double-distilled, DEPC-treated water. All consumables were prepared RNase free. The RNA isolation was performed according to Wan and Wilkins [14] with modifications as described by Toorop et al. [15]. Briefly, buds and seeds were ground to a fine powder with a mortar and pestle in liquid nitrogen. The homogenate was suspended in 700 μl XT-buffer (0.2 M sodium tetraborate decahydrate, 30 mM EGTA, 1% SDS and 1% sodium deoxycholate, pH 9.0) with 1 mg dithiothreitol and varying amounts of soluble polyvinylpyrrolidone (Fisher Scientific, product code 22754, Mw 58 kDa; 1.4, 4.3, 10.0, 14.3, 20.0, and 30.0 mg PVP per ml buffer), added to 1 mg proteinase K and incubated at 42 °C for 11/2 h. Nucleic acids were precipitated with potassium chloride (final concentration 0.15 M) on ice for 1 h. Samples were centrifuged in a microfuge at 4 °C for 20 min and lithium chloride was added to the supernatant in a final concentration of 2 M. The samples were incubated overnight on ice, specifically to precipitate the RNA. Samples were centrifuged in a microcentrifuge at 4 °C for 20 min to pellet the RNA. The pellet was suspended in 2 M cold lithium chloride and the samples were centrifuged at 4 °C for 20 min. Upon decanting the supernatant the pellet was washed in 2 M cold lithium chloride twice by re-suspending the pellet and centrifuging at 4 °C for 10 min. The pellets were re-suspended in 10 mM Tris, potassium acetate was added to a concentration of 0.2 M and samples were centrifuged at 4 °C for 10 min to precipitate polysaccharides. To the supernatant, cold ethanol was added and the RNA was precipitated for 3 h at −80 °C. The samples were centrifuged at 4 °C for 30 min to pellet the RNA. The pellet was washed with 70% cold ethanol, dried and re-suspended in water. For further purification, sodium acetate, in a final concentration of 0.3 M, and ethanol were added, and RNA was precipitated overnight at −20 °C. The samples were centrifuged at 4 °C for 20 min to pellet the RNA. The pellet was washed with 70% cold ethanol, dried and re-suspended in 50 μl water with RNAsecure (Ambion, Applied Biosystems, Warrington, UK).

2.3. RNA analysis

The quantities of the RNA were assessed with a UV/vis spectrophotometer (WPA Lightwave, Taunton, UK) at 260 and 280 nm and the ratio (A_{260}/A_{280}) was calculated to assess purity. In order to verify RNA integrity 1 μg RNA was subjected to gel electrophoresis in a 1.5% agarose gel, stained with ethidium bromide and visualized using a UV transilluminator and gel doc system (Syngene, Cambridge, UK). The 28S rRNA and 18S rRNA band quantities were determined using Genetools software (Syngene) and the ratio (28S/18S) was used as a parameter for RNA integrity.

2.4. Reverse transcription

Total RNA was purified using the RNeasy Plant Mini Kit (Qiagen, Crawley, UK) and reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Burgess Hill, UK). PCR was performed with Taq polymerase (Roche, Burgess Hill, UK) and PCR primers for the amplification of actin. The sequences were 5′-TCCATCATGAAGTGCGATGT-3′ for the forward and 5′-AACCTCC-GATCCAGACACTG-3′ for the reverse primer, producing a 188 bp amplicon. The PCR conditions were 35 cycles of 30 s at 94 °C, 30 s at 63.3 °C and 40 s at 72 °C in a DNA engine DYAD peltier thermal cycler (MJ Research). Two controls were carried out: water (no template) and 1 μg of the total RNA (no RT) instead of cDNA.

2.5. Hybridization subtraction library

RNA isolated from non-dormant and dormant buds with the optimal PVP concentrations (4.3 mg/ml for non-dormant buds and 14.3 for dormant buds) was used to generate two subtraction libraries, one enriched with dormancy-associated gene transcripts and the other enriched with non-dormancy-associated gene transcripts, using the Clontech PCR-Select cDNA Subtraction Kit. Samples were analysed by gel electrophoresis as described under Section 2.3.

2.6. Phenol quantification

C. sativa buds and Arabidopsis seeds were sampled as above. Upon collecting samples were frozen in liquid nitrogen, and stored at −80 °C until further use. The samples were freeze-dried and finely ground with a pestle and mortar.

Plant extracts were prepared according to Kirca and Arslan [16] with modifications regarding the amounts of plant material. Dried plant material (0.075 g) was weighed in a centrifuge tube and 1.5 ml of methanol was added. The suspension was shaken at 1000 rpm at room temperature on an orbital shaker (thermomixer compact, Eppendorf) for 1 h. The suspension was centrifuged at 5204 × *g* for 10 min, and the supernatant was transferred to an 8 ml volumetric flask. The residue was re-extracted with 1.5 ml methanol using the same procedure. After this procedure was repeated three times all extracts were pooled and the volume adjusted to 7.5 ml with methanol. This crude extract was filtered though a filter paper and kept in amber-coloured glass bottles at −18 °C until analysis.

The total phenolics were determined according to the Folin-Ciocalteu method [17]. Sample extract (50 μl) was mixed with distilled water (450 μl) and 0.2N Folin-Ciocalteu reagent (2.5 ml). Two milliliters of saturated sodium carbonate was added, and the resulting mixture was vortexed for 1 min. After incubation at room temperature for 2 h, absorbance of the solution was measured at 765 nm using a UV/vis spectrophotometer (Beckman coulter DU® 800). The total phenolic content was calculated based on a standard curve of gallic acid, which was linear within a range of 100–2000 mg l^{-1} (R^2 = 0.9954). Results were presented as the mean of

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