A SIMPLE AND RAPID METHOD FOR ISOLATING HIGH QUALITY RNA FROM FLOWER PETALS

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Abstract

A simple RNA isolation method based on hot borate and proteinase K was used to extract high-quality RNA from petals of daffodils (Narcissus pseudonarcissus L. 'Dutch Master'). The method was adapted from the isolation technique described by Wan and Wilkins (1994). A single isopropanol precipitation and ethanol wash replaced all the second day steps of the Wan and Wilkins procedure. The RNA isolated by this procedure was of high enough quality to construct a senescence-associated subtracted cDNA library, and the procedure has proved successful in isolation of RNA from petals of several different flowers.

1. Introduction

Isolation of intact RNA is a basic requirement for many molecular studies, but extracting high-quality RNA can be difficult. This has been attributed to the presence in tissues of secondary metabolites such as polyphenolics, lignins, carbohydrates and etheric oils that bind nucleic acids when the tissues are lysed (Dong and Dunstan 1996). In addition the presence of ribonucleases in plant tissues can result in rapid degradation of RNA. Since plant tissues vary in their content of these interfering secondary metabolites and enzymes, it is often found that isolation procedures that work for one tissue do not work for another. This has resulted in the publication of a range of RNA isolation protocols.

Many of the published RNA isolation protocols are based on highly toxic chaotropic agents such as phenol, phenol/chloroform or guanidine thiocyanate (Dong and Dunstan, 1996; Logemann et al., 1987; Maes et al., 1992) which quickly denature the endogenous ribonucleases in tissues. However, these isolation protocols do not always provide adequate yields or quality of RNA. In addition, there are safety and disposal issues to consider, especially with regard to phenol, which is readily absorbed through the skin and can cause nausea, burns and death (Windholz et al., 1983).

The RNA isolation method of Wan and Wilkins (1994) is a lower-toxicity alternative to other RNA isolation protocols. The procedure uses high pH and sodium dodecyl sulfate (SDS) to inhibit ribonuclease activity, proteinase K to degrade ribonucleases, borate to remove carbohydrates and inhibit polyphenolic production and soluble polyvinylpyrrolidone (PVP) to remove the phenolics released by the lysed tissue. Wan and Wilkins (1994) showed that their procedure (unlike others that used phenol (Verwoerd and Dekker, 1989) or guanidine (Logemann et al., 1987)) recovered intact, high quality RNA from cotton (Gossypium hirsutum L.), a species notorious for high levels of polysaccharides, phenolics, terpenes and other secondary metabolites.

We are interested in studying changes in gene expression during flower senescence and have found that the hot borate isolation procedure of Wan and Wilkins (1994) can be successfully used to isolate RNA from petal tissues. We also found that the method could be considerably simplified without compromising the quality or yield of
the isolated RNA. In this paper we detail the methodology of this shortened procedure for isolation of RNA from petals, which we have found to be simple, fast and reliable.

Abbreviations used: EGTA, [Ethylenebis-(oxyethylenenitrilo)]-tetraacetic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol, PVP-40, polyvinylpyrrolidone (Mr 40 000); IGEPAL, Octylphenylpolyethylene glycol

2. Materials and methods

2.1. Plant material

Petals of one and four-day old flowers of daffodil (*Narcissus pseudonarcissus* L. cv Duth Master) were frozen in liquid nitrogen, powdered, and stored at -80°C until required.

2.2. Borate Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>0.2 M sodium borate decahydrate</td>
<td></td>
<td>Sigma (# S9640)</td>
</tr>
<tr>
<td>30 mM EGTA</td>
<td></td>
<td>Fisher Scientific (# O2783-100)</td>
</tr>
<tr>
<td>1% (w/v) SDS</td>
<td></td>
<td>Fisher Scientific (# BP166-500)</td>
</tr>
<tr>
<td>1% (w/v) sodium deoxycholate</td>
<td></td>
<td>Sigma (# D6750)</td>
</tr>
</tbody>
</table>

Dissolve the Borate Buffer in prewarmed nanopure water. Cool and adjust to pH 9.0 with 5 N NaOH then autoclave to sterilise.

2.3. Chemicals to be added to the Borate Buffer on day of extraction to give final concentrations of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>10 mM DTT</td>
<td></td>
<td>Fisher Scientific (# BP 172-5)</td>
</tr>
<tr>
<td>2% (w/v) PVP-40</td>
<td></td>
<td>Sigma (#PVP-40)</td>
</tr>
<tr>
<td>1% (v/v) IGEPAL CA-630</td>
<td></td>
<td>Sigma (# CA-630)</td>
</tr>
</tbody>
</table>

2.4. Other chemicals required:

- Proteinase K (VWR # EM 24568-2) (20 mg mL⁻¹ in sterile water - store stock at -20°C)
- 4 M LiCl
- 2 M KCl
- 3 M sodium acetate
- Isopropanol
- 70% ethanol

2.5. Extraction Protocol

2.5.1. The extraction solution is prepared by adding DTT, PVP-40 and IGEPAL to the Borate Buffer. For 1 g of petal tissue combine:

- 5 mL XT
- 50 µL of 1 M DTT
- 50 µL of IGEPAL
- 0.1 g of PVP

Transfer the solution to a sterile 14 mL Falcon 2059 tube (Fisher Scientific # 14 959-11b) and heat to 80°C in a fume hood.

2.5.2. Add crushed petal tissue (ground under liquid nitrogen) to hot buffer (facilitated with a precooled plastic funnel)

2.5.3. Vortex 30 s
2.5.4. Add 37.5 ul (0.75 mg per 5 mL) of proteinase K, cap the tube, seal around the cap with parafilm, and incubate the tube horizontally on a shaking air incubator for 1.5 h at 42°C.

2.5.5. Add 0.08 volumes of 2 M KCl (i.e., 400 uL per 5 mL) and incubate horizontally on ice with shaking for 30 min.

2.5.6. Centrifuge tubes at 26 000 x g for 20 min at 4º C and decant supernatant into fresh sterile Falcon 2059 tube.

2.5.7. Add one volume of 4 M LiCl and incubate overnight at 4 ºC or alternatively incubate for 1 h at -20ºC.

2.5.8. Pellet the precipitated RNA by centrifugation at 26 000 x g for 30 min at 4ºC.

2.5.9. Resuspend RNA pellet in 630 uL of water/70 uL of 3 M sodium acetate and precipitate the RNA with 1 volume of isopropanol.

2.5.10. Pellet the precipitated RNA by centrifugation at 16 000 x g for 30 min at 4ºC.

2.5.11. Wash with 70 % ethanol and resuspend in the appropriate volume of sterile RNase-free water.

2.6. Isolation of mRNA and synthesis of cDNA

Polyadenylated (polyA+) mRNAs were isolated from total daffodil petal RNA using the PolyATtract™ mRNA Isolation System IV kit (Promega). First and second strand cDNA sequences were synthesised from 1 µg of polyA+ mRNA as described in the PCR-Select manual (# K1804-1, ClonTech) except that a ribonuclease inhibitor (RNAguard®, Pharmacia Biotech) was added to the first strand reaction mix.

3. Results

3.1. RNA yield

The protocol described here yielded 220 µg RNA /g f. wt. from one day old daffodil petals (Table 1). As anticipated, the RNA content of the petals declined as they started to senesce, and incipiently-senescent 4 day old petals yielded only 129 µg of total RNA.

3.2. RNA quality

The quality of the RNA from both one and four day old petals was similar, with an $A_{260/230}$ absorbance ratio greater than 20 indicating no significant contamination with polysaccharides, buffer salts or other impurities (Dong and Dunstan, 1996; Teare et al., 1997). RNA from both one and four day old petals had an $A_{260/280}$ absorbance ratio of 1.8 and 1.75 respectively, indicating that there was also no significant contamination with proteins (Fox, 1998).

The integrity of the RNA was examined by separation through a 1 % denaturing agarose gel (Fig 1). The 18S and 28S ribosomal ribonucleic acids stained with ethidium bromide as sharp bands and the fluorescence intensity of the 28S ribosomal band was greater than that of the 18S band suggesting little degradation of the total RNA.

The size distribution profile of the cDNA sequences synthesized from mRNA isolated from the total petal RNA of between ca. 1.0 and 12 Kb (visualized by ethidium...
bromide staining) indicated that the extraction procedure isolated predominantly intact mRNA (Fig 2). Synthesis of double stranded cDNA from human skeletal polyA+ mRNA (provided by the PCR-select kit, Clontech) (Lane 2) was used as a control for the cDNA synthesis procedure.

4. Discussion

Ideally, an RNA isolation protocol should be simple, fast, non-toxic and give good yields of high quality RNA. The procedure described in this manuscript appears to satisfy many of these requirements. An important advantage of this method over many that have appeared in the literature is that it is simple and fast. For instance, there are only two tube changes for each sample in the eleven simple steps of the procedure. This reduces the chance for researcher-induced error and allows multiple RNA isolations to be performed with minimal effort. Using this procedure, multiple RNA isolations can easily be completed within 5 h, when the 1 h precipitation at -20ºC with 2 M LiCl is used.

A further important advantage of the method described here is that it isolates high quality RNA. The A$_{260/230}$ and A$_{260/280}$ ratios obtained from RNA isolated from newly opened and incipiently senescent daffodil petals indicate minimal contamination with carbohydrates, proteins, salts and other impurities.

The integrity of the RNA produced by the Hot Borate method was also excellent as judged by both the sharpness of the 18S and 28S ribosomal RNA bands and by finding that the 28S ribosomal RNA transcripts were present in greater abundance than the 18S which suggested that the RNA was not significantly degraded (ClonTech PCR-select user manual; Salzman et al., 1999). Another indicator that the isolated RNA was predominantly intact was the high molecular weight (mostly > 1.6 Kb) double stranded cDNA synthesized from the isolated mRNA (Fig 2).

RNA quality can also be judged by its ability to be used in RT-PCR (Salzman et al., 1999). RNA isolated by this procedure from petals of *N. pseudonarcissus*, *Mirabilis jalapa*, and *Gladiolus purpurea* has been used successfully as a template in RT-PCR to amplify 500 bp cDNA fragments that encode putative expansin proteins (D. Hunter and C. Pham, unpublished data).

Recently, this procedure has been used to isolate high yields of intact RNA (up to 500 ug/g fw.) from the petals of *Alstroemeria samora* (C. Wagstaff, School of Biological Sciences, Cardiff University, Cardiff, pers. comm.). The isolation protocols previously attempted (Tri-Reagent (Sigma); Whitelaw et al., 1999; Ainsworth 1994; Grierson et al., 1985) produced only low yields of poor quality RNA from *Alstroemeria samora* petals, presumably due to the presence of large quantities of carbohydrates and polyphenols.

The majority of the RNA isolation procedures in contemporary are based on the use of toxic chaotropic agents such as phenol and guanidine. Exposure to phenol vapor causes nausea, and the liquid results in painful skin burns. Ingestion of as little as 1 g can kill a human and fatal doses can be absorbed through the skin (Windholz et al., 1983). Guanidine thiocyanate has also been classified as an acute and chronic health hazard (Promega Z512, Material Safety and Data Sheet). The method described in this study does not use these dangerous materials and is therefore more suited to use in the classroom as well as the research laboratory. It does contain a high concentration of sodium borate, and it should be noted that sodium borate is somewhat toxic. Ingestion or absorption of sodium borate may cause nausea, vomiting, diarrhoea and abdominal cramps, and ingestion of as little as 5 to 10 g has caused death in children (http://infoventures.com/e-hlth/pesticide/borax.htm). Therefore, normal precautions should be taken when using the borate buffer to extract the RNA.
References


Tables

1. Yield and absorbance ratios of total RNA isolated from 1 day old and 4 day old Narcissus pseudonarcissus petals

<table>
<thead>
<tr>
<th>Flower Age</th>
<th>A_{230}</th>
<th>A_{260}</th>
<th>A_{280}</th>
<th>A_{260}/A_{230}</th>
<th>A_{260}/A_{280}</th>
<th>Conc. (µg/ ml)</th>
<th>Total RNA (µg/ g. fw.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>0.733</td>
<td>1.653</td>
<td>0.920</td>
<td>2.26</td>
<td>1.8</td>
<td>661.2</td>
<td>220</td>
</tr>
<tr>
<td>4 days</td>
<td>0.455</td>
<td>0.965</td>
<td>0.552</td>
<td>2.12</td>
<td>1.75</td>
<td>386</td>
<td>129</td>
</tr>
</tbody>
</table>

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Figures

1. Visualisation of total RNA isolated from 1 and 4 day old petals separated on a 1 % (w/v) denaturing agarose gel and stained with ethidium bromide.

2. Visualisation of double stranded (ds) cDNA produced from 1 ug of 1 and 4 day old petal polyA⁺ mRNA populations and from control (skeletal muscle) Poly A⁺ mRNA. The cDNA populations were separated on a 1 % (w/v) agarose gel and stained with ethidium bromide. Lane 1. DNA size markers; Lane 2. human skeletal muscle control cDNA; Lane 3. 1 day old tepal ds cDNA; Lane 4. 4 day old tepal ds cDNA.