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Isolation of poly $(A)^+$ mRNA for downstream reactions from some medicinal and aromatic plants

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In the present protocol for extraction of RNA, hexadecyltrimethylammoniumbromide (CTAB) and insoluble polyvinylpyrrolidone were used followed by LiCl precipitation, CsCl ultracentrifugation and finally poly $(A)^+$ mRNA was isolated with the help of oligo(dT)-cellulose columns. The isolated poly $(A)^+$ mRNA was found to be suitable for cDNA-AFLP and suppression subtractive hybridization applications. It is a modified and consolidated protocol based on previously described methods for isolated steps and works better for medicinal and aromatic plants. High yield of poly $(A)^+$ mRNA coupled with its amenability for downstream reactions like RT-PCR, northern blotting and cDNA synthesis for library construction is a key feature of the present protocol.

Keywords: Aromatic, *Catharanthus roseus*, Medicinal, Poly $(A)^+$ mRNA, RNA isolation.

Molecular studies of many interesting phenomena in plant systems has been hampered due to difficulties encountered in isolation of high quality poly $(A)^+$ mRNA. For isolation of poly $(A)^+$ mRNA from animal tissues, the single step acid-guanidinium thiocyanate-phenol-chloroform method[1] is generally used. However, due to the presence of high level of ribonucleases and cell wall components, isolation of pure mRNA from plant tissue is complicated[2]. This is further compounded in recalcitrant systems of medicinal and aromatic plants such as *Catharanthus roseus, Artemisia annua, Capsicum anuum, Papaver somniferum, Mentha arvensis*, *Pelargonium graveolens, etc.* due to presence of secondary metabolites like alkaloids, flavonoids, polyphenols, gummy polysaccharides, terpenes, quinones and other unidentified compounds that interfere in RNA isolation. Phenolic compounds are readily oxidized to form covalently linked quinones and avidly bind nucleic acids. This renders RNA unusable for downstream procedures like reverse transcription and cDNA library construction. Evaluation of many protocols[1–3] for isolation of poly $(A)^+$ mRNA from medicinal and aromatic plants by us has shown their unsuitability as in most

cases the RNA pellet obtained is of dark colour, large and water insoluble. Such RNA pellets give a low yield and degraded poly $(A)^+$ mRNA. An earlier protocol[4] described for RNA isolation from carnivorous plants works better with medicinal and aromatic plants, but the total RNA obtained is accompanied by a large amount of genomic DNA. Such RNA could not be used directly for poly $(A)^+$ mRNA selection using oligo(dT)-cellulose as there might be tracts of poly A in the contaminating DNA also which would cause erroneous results in expression studies. Thus, it is necessary to develop alternative methods for RNA isolation while working with medicinal and aromatic plant tissues. So a protocol was devised using hexadecyltrimethylammoniumbromide (CTAB), insoluble polyvinylpyrrolidone (which binds phenolic compounds effectively) and reducing agent (β-mercaptoethanol) in RNA extraction buffer to overcome these problems. The homogenization step is followed by a LiCl precipitation, CsCl-ultracentrifugation, phenol extraction and selection of poly $(A)^+$ mRNA on oligo(dT)-cellulose columns. This procedure initially standardized for isolation of poly $(A)^+$ mRNA from root and leaf tissues of *Catharanthus roseus* plant routinely works well with the medicinal and aromatic plants tested so far in our laboratory inspite of chemotypic heterogeneity among the plant systems under study.

Materials and Methods

Solutions and reagents used - The RNA extraction buffer comprised of CTAB (2% w/v; Sigma); polyvinylpyrrolidone (2% w/v; mol.wt. 360,000; Sigma), Tris-Cl (100m*M*; *p*H 8.0; Sigma); sodium-EDTA (25m*M*; *p*H 8.0; Sigma); and NaCl (2*M*; Sigma). All the components were mixed except Tris-Cl in less than the total stipulated volume of diethylpyrocarbonate (DEPC) treated un-autoclaved water and autoclaved next day. Required volume of *Tris-Cl (1*M*; *p*H 8.0; Sigma) that was prepared separately in DEPC treated RNase free water was added. Before use, β-mercaptoethanol to a final concentration of 2% (v/v) was also added. Chloroform: isoamyl alcohol (24:1) was freshly prepared in a dark colour bottle. CsCl cushion solution (50 ml) was prepared by adding CsCl (48 g; Sigma); 1.0 ml of EDTA (0.5*M*; *p*H 7.1; Sigma) and deionized water. LiCl (10*M*; Sigma), NaCl (5*M*; Sigma) and the CsCl cushion solutions were treated overnight with DEPC (0.1%) and autoclaved next day. RNase free water was prepared by treating deionized water overnight with DEPC (0.1%) and autoclaving thrice to remove traces of remaining DEPC, which affects the purine bases of mRNA. Phenol: chloroform (1:1; v/v) in which phenol was equilibrated to *p*H 8.0 with Tris-Cl (0.1*M*; *p*H 8.0) was freshly prepared before use in a dark colour bottle. *n*-Butanol (Sigma) was saturated with DEPC treated RNase

free water in a dark colour bottle. Ethidium bromide (10 mg/ml; Sigma) solution was prepared in RNase free water. *Tris-Cl (1*M*; *p*H 7.5; Sigma) stock solution was prepared in DEPC treated RNase free water. Ethanol (100 and 75%) was used from bottles exclusively used for RNA work. Loading buffer (20 ml) was prepared by adding 0.4 ml of Tris-Cl (1*M*; *p*H 7.5); 4.0 ml of NaCl (5*M*) and RNase free water. High salt buffer (20 ml) was prepared by adding 0.2 ml of Tris-Cl (1*M*; *p*H 7.5); 2.0 ml of NaCl (5*M*) and RNase free water. Medium salt buffer (10 ml) was prepared by adding 0.1 ml of Tris-Cl (1*M*; *p*H 7.5); 0.2 ml of NaCl (5*M*) and RNase free water. Low salt buffer (10 ml) was prepared by adding 0.1 ml of Tris-Cl (1*M*; *p*H 7.5) and RNase free water.

*Tris cannot be treated directly with DEPC as it leads to decomposition of DEPC. It has to be prepared in DEPC treated RNase free water.

Materials used – Other materials used for poly $(A)^+$ mRNA isolation were oligo(dT)-cellulose (Stratagene); plasticware/glassware treated overnight with DEPC (0.1%) and autoclaved; RNase free blade; DEPC treated spin columns (Axygen) and tabletop minicentrifuge/DEPC treated 1 ml syringe and glass wool; Optima™ TLX Ultracentrifuge (Beckman); and DEPC treated Quick-Seal™ ultracentrifuge tubes (5.1 ml).

Plant samples for poly(A)$^+$ mRNA isolation - Fresh samples (3 g per preparation) of root and leaf tissues of six months old field grown *Catharanthus roseus* plants were collected on crushed ice and immediately ground in liquid nitrogen. After standardization of the protocol (Fig. 1) on *Catharanthus roseus* root and leaf tissues (Fig. 2a), it was applied on a variety of tissues from other medicinal and aromatic plants like *Artemisia annua* (leaves), *Capsicum anuum* (placenta and fruit wall), *Papaver somniferum* (capsule), *Mentha arvensis* (trichomes), *Pelargonium graveolens* (leaves), *Rosa damascena* (petals), *Ocimum basilicum* (leaves) *etc*.

Protocol options – The RNA yield improved in overnight precipitation instead of 2 hr incubation at -20ºC. Spin columns were washed using a minicentrifuge at 10,000 rpm for 15 sec at room temperature during poly (A)$^+$ mRNA isolation. There were three options for poly (A)$^+$ mRNA capturing step using oligo(dT)-cellulose – (i) Poly (A)$^+$ mRNA selected in an oligo(dT)-cellulose column packed in a spin column and separated using a minicentrifuge as described in the protocol (Fig. 1); (ii) Poly (A)$^+$ mRNA selected in an oligo(dT)-cellulose column packed in a 1 ml syringe and separated by gravity flow; and (iii) Poly (A)$^+$ mRNA selected by binding to oligo(dT)-cellulose and pelleted in a microcentrifuge tube using a

minicentrifuge. It was pelleted in each step and the supernatant was aspirated out. The poly $(A)^+$ mRNA was obtained in the supernatant upon washing with low salt buffer.

Results and Discussion

The guanidine thiocyanate (GTC) based method[1], although highly successful in the case of animal tissues, does not work well in the case of medicinal and aromatic plants. Besides, many a times the accompanying GTC causes a problem with the downstream enzymatic reactions involving the isolated RNA. Even a commercially available kit like PolyATtract® System 1000 (Promega Corporation, USA) which utilizes a magnetic particle separation[5] of poly $(A)^+$ mRNA and yields high amount of poly $(A)^+$ mRNA from animal cells in a short time, does not work well with medicinal and aromatic plants. The yield of poly $(A)^+$ mRNA isolated from medicinal and aromatic plants like *Catharanthus roseus* using the Promega kit was much lower than that obtained using the protocol described in the present study (Fig. 2b and 2c). The current protocol is a modification of the methods described earlier[3,4,6]. GTC has been replaced by CTAB and LiCl precipitation in this protocol. The combination of insoluble polyvinylpyrrolidone and a high ionic strength as a result of the presence of

NaCl (2*M*) was instrumental in separating the contaminating compounds. The ultracentrifugation step completely removed the contaminating DNA, proteins and polysaccharides. Although, some plant RNA isolation protocols prefer to use soluble polyvinylpyrrolidone[7], our experience with medicinal and aromatic plants has shown that insoluble polyvinylpyrrolidone is more effective in countering the problem arising due to presence of phenolic compounds. Although, the present protocol described the use of spin columns for packing the oligo(dT)-cellulose columns for the poly $(A)^+$ mRNA capturing step using a minicentrifuge, there was also a choice of packing the oligo(dT)-cellulose column in a 1 ml syringe (end plugged with DEPC treated glasswool) and separating the components by gravity flow. A different approach was also tried to capture the poly $(A)^+$ mRNA in a microcentrifuge tube containing oligo(dT)-cellulose, whereby using a minicentrifuge the oligo(dT)-cellulose-poly $(A)^+$ mRNA complex was pelleted which finally upon washing with low salt buffer released the poly $(A)^+$ mRNA in the supernatant. However, the yield of poly $(A)^+$ mRNA obtained in this case was lower than the spin column or syringe methods. The syringe method yielded a good quantity of poly $(A)^+$ mRNA but was comparatively slower than the spin column method. The spin column

procedure was the method of choice due to its rapidity and the high yield of poly $(A)^+$ mRNA obtained.

Characterization of poly $(A)^+$ mRNA quality - The poly $(A)^+$ mRNA quality was checked by UV spectrophotometry, ethidium bromide staining and cDNA synthesis. (i) UV Spectrum - The yield of poly $(A)^+$ mRNA (A_{260nm}) was determined to be upto 10-20 µg/g fresh weight plant tissue. This was only 1% of the total RNA isolated. The ratio of A_{260nm}/A_{280nm} was 1.85 ± 0.05 indicating low content of protein impurities. (ii) Ethidium bromide staining - The intactness of the poly $(A)^+$ mRNA and its contamination with DNA were checked through agarose (0.8%) gel electrophoresis on both native (Fig. 2b) as well as denaturing (Fig. 2c) gels. No DNA contamination was present and the poly $(A)^+$ mRNA quality was further found to be good by successful northern blotting experiments. (iii) cDNA synthesis - The isolated poly $(A)^+$ mRNA was successfully used for cDNA synthesis which was further used for library construction, RT-PCR, cDNA-AFLP and suppression subtractive hybridization studies.

Although, the present protocol took a slightly longer time than the commercially available kits, the yield and quality of poly $(A)^+$ mRNA from plant source were better. This protocol could prove beneficial for performing genome-scale analyses of medicinal and aromatic plants by DNA

microarrays if it could be optimized for high throughput performance in future like the RNA isolation methods available for animal systems[8].

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Fig. 1 – Detailed protocol steps

Tissue disruption and lysis

Pre-warm 20 ml of RNA extraction buffer in each of four 40 ml polypropylene tubes to 65ºC. Grind 3 g of plant tissue in liquid nitrogen using a pestle and mortar for each of the 4 preps (i.e. total 12 g plant material required), transfer the powder (3 g) into 20 ml of pre-warmed extraction buffer and mix vigorously by inversion.

⬇

Extraction

Extract twice with an equal volume of chloroform: isoamyl alcohol (24:1), separating the phases each time by centrifuging at 10,000 rpm for 10 min at 4ºC.

⬇

LiCl precipitation

Add 0.25 volume of LiCl (10*M*) to the aqueous phase, mix well and incubate for overnight precipitation at 4ºC. Pellet the RNA by centrifugation at 10,000 rpm for 20 min at 4ºC.

⬇

Ultracentrifugation

Dissolve the RNA pellet in each of the four tubes in 1.6 ml RNase free water. Pool 2 preps at one place and add 1.13 ml of CsCl cushion solution to each of the pooled (3.2 ml) preps. Layer the 4.33 ml CsCl-RNA solution onto 0.765 ml of CsCl cushion solution contained in a 5.1 ml RNase free Quick-Seal™ ultracentrifuge tube. Add 2 µl of ethidium bromide solution (10 mg/ml) to each tube, balance them, seal their tops and centrifuge for 4 hr at 60,000 rpm at 22ºC in a TLA 100.4 rotor. Cut the neck of the tubes with a RNase free blade, discard the supernatant, wash the pink (due to intercalating ethidium bromide) RNA pellet deposited at the bottom of the tubes with ethanol (75%), vacuum dry for 5 min, re-suspend in 500 µl RNase free water and transfer to a microcentrifuge tube.

⬇

Extraction

Extract the ethidium bromide (3 to 4 times) with an equal volume of water saturated *n*-butanol and discard the upper butanol layer carrying the ethidium bromide. Extract the clear RNA solution with an equal volume of phenol: chloroform (1:1; v/v). Re-extract the organic phase with 100 µl RNase free water to increase the recovery.

⬇

Precipitation of total RNA

Precipitate the total RNA in four aliquots (300 µl each) by addition of 1/25volume of NaCl (5*M*) and 2.5 volumes of ethanol (100%) followed by overnight/2 hr incubation at –20ºC. Pellet the total RNA by centrifuging at 12,000 rpm for 20 min at 4ºC. Wash the pellet with ethanol (75%) by spinning at 12,000 rpm for 5 min at 4ºC, vacuum dry for 5 min and dissolve in loading buffer (300 µl per aliquot).

Oligo(dT)-cellulose column packing

Equilibrate oligo(dT)-cellulose which has been kept overnight for swelling [30 mg oligo(dT)-cellulose should be kept for swelling overnight in 300 µl loading buffer] with loading buffer by aspirating out the overlaying loading buffer and adding new loading buffer (300 µl). Repeat this equilibration process three more times. Pack four spin columns to 0.1 ml bed volume by dividing the 30 mg oligo(dT)-cellulose in the four columns [7.5 mg oligo(dT)-cellulose per column]. Provide a spin pulse in the minicentrifuge to wash out the loading buffer. Wash the column with 2 ml high salt buffer.

Poly $(A)^+$ mRNA selection and elution

Heat the total RNA aliquots (300 µl each) at 65ºC for 15 min to relax the secondary structures, cool immediately on ice for 5 min, add equal volume (300 µl) of loading buffer to each and load onto an oligo(dT)-cellulose column. Collect the flow through and load it on the column once again. Repeat this process once more for a total of three loading steps. Wash the loaded column first with 2 ml high salt buffer and then with 2 ml medium salt buffer. Elute the poly $(A)^+$ mRNA bound to the oligo(dT)-cellulose column using 2 ml low salt buffer.

Poly $(A)^+$ mRNA precipitation

Divide the eluted poly $(A)^+$ mRNA sample from each of the four columns into four parts (sixteen 500 µl aliquots). Precipitate the poly $(A)^+$ mRNA by addition of 1/25 volume NaCl (5*M*) and 2 volumes ethanol (100%) followed by overnight/2 hr incubation at –20ºC. Pellet the poly $(A)^+$ mRNA by centrifuging at 20,000 rpm for 1 hr at 2ºC, vacuum dry the pellet for 10 min and dissolve in RNase free water (15 µl per aliquot) for downstream applications. If required, store the dry poly $(A)^+$ mRNA pellet at this stage in ethanol (100%) at –80ºC instead of dissolving in water.

Fig. 2a

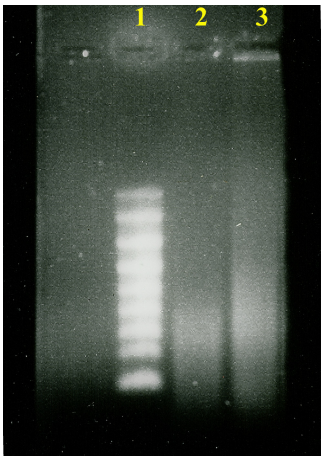

Fig. 2b

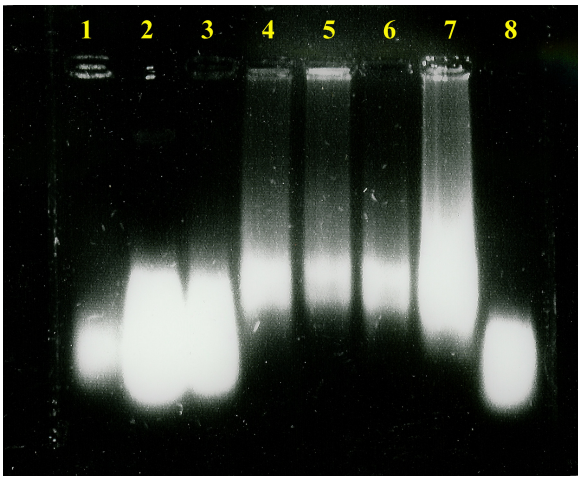

Fig. 2c

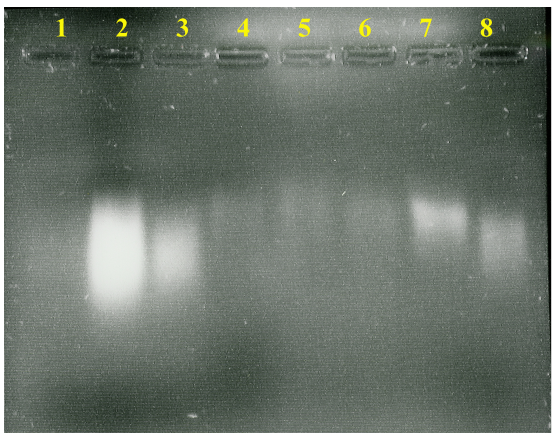

Fig. 2 - Isolation of poly $(A)^+$ mRNA from *Catharanthus roseus*.

Fig. 2a – Characterization of poly $(A)^+$ mRNA from different tissues.

(a) Lane 1 - RNA Marker (0.281kb-6.583kb; Promega); **Lane 2 -** Root tissue poly $(A)^+$ mRNA selected in an oligo(dT)-cellulose column packed in a spin column and separated using a minicentrifuge; and **Lane 3 -** Leaf tissue poly $(A)^+$ mRNA selected in an oligo(dT)-cellulose column packed in a spin column and separated using a minicentrifuge.

Fig. 2b, c – Characterization of poly $(A)^+$ mRNA from the leaf tissue using the different options for oligo (dT)-cellulose column packing.

(b) The samples electrophoresed in a native agarose gel; and **(c)** The same samples electrophoresed in a denaturing formaldehyde gel. [**Lane 1 -** Poly $(A)^+$ mRNA isolated by pelleting oligo(dT)-cellulose-poly $(A)^+$ mRNA complex in a microcentrifuge tube using a minicentrifuge; **Lane 2 -** Poly $(A)^+$ mRNA isolated in an oligo(dT)-cellulose column packed in a spin column and separated using a minicentrifuge; **Lane 3 -** Poly $(A)^+$ mRNA isolated in an oligo(dT)-cellulose column packed in a 1ml syringe and separated by gravity flow; **Lane 4 -** Flow through after poly $(A)^+$ mRNA selection in microcentrifuge tube method; **Lane 5 -** Flow through after poly $(A)^+$ mRNA selection in spin column method; **Lane 6 -** Flow through after poly $(A)^+$ mRNA selection in syringe method; **Lane 7 -** Total cellular RNA; and **Lane 8 -** Poly $(A)^+$ mRNA isolated using the commercially available kit PolyATtract® System 1000 (Promega Corporation, USA)]