

A Modified Protocol for RNA Isolation from High Polysaccharide Containing *Cupressus arizonica* Pollen. Applications for RT-PCR and Phage Display Library Construction

Yago Pico de Coaña · Nuria Parody ·
Enrique Fernández-Caldas · Carlos Alonso

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Abstract RNA isolation is the first step in the study of gene expression and recombinant protein production. However, the isolation of high quantity and high-quality RNA from tissues containing large amounts of polysaccharides has proven to be a difficult process. *Cupressus arizonica* pollen, in addition to containing high polysaccharide levels, is a challenging starting material for RNA isolation due to the roughness of the pollen grain's walls. Here, we describe an improved technique for RNA isolation from *C. arizonica* pollen grains. The protocol includes a special disruption and homogenization process as well as a two-step modified RNA isolation technique which consists of an acid phenol extraction followed by a final cleanup using a commercial kit. Resulting RNA proved to be free of contaminants as determined by UV spectrophotometry. The quality of the RNA was analyzed on a bioanalyzer and showed visible 25S and 18S bands. This RNA was successfully used in downstream applications such as RT-PCR and phage display library construction.

Keywords RNA isolation · Recombinant allergen · Pollen · Phage display · Polysaccharide rich tissue · Arizona cypress (*Cupressus arizonica*)

Introduction

Species of the *Cupressaceae* family are an important cause of allergic pollinosis in Mediterranean countries, Japan and South Western United States [1]. Diagnostic and therapeutic strategies against these allergies have traditionally relied on the use of non-standardized pollen extracts. The use of these extracts has not produced satisfactory therapeutic results, mainly due to high variability in extract composition [2]. New approaches for the development of diagnostic and therapeutic tools include production of known major allergens as recombinant proteins [3–5] and the use of cDNA libraries to identify new allergenic candidates. For library construction, in particular, high-quality total RNA and high quantities of mRNA are required. Although protocols for total RNA isolation from *Cupressus arizonica* pollen have already been described [3], they are not practical for large-scale mRNA purification, especially due to low resulting yields. *C. arizonica* pollen has an extremely high polysaccharide content (around 85% w/w) [6] which, in addition to the toughness of the pollen grain, prevents the use of traditional tissue disruption and RNA purification techniques. Presence of polysaccharides in the tissue sample leads to contamination in the final isolation product; therefore, removal of these contaminants is essential for precise quantification and for the use of isolated RNA in downstream applications. In this article, we report a method for the isolation of large quantities of high-quality total RNA and mRNA from *C. arizonica* pollen. We also describe the construction of a T7 Phage display

Y. Pico de Coaña (✉) · N. Parody · C. Alonso
Centro de Biología Molecular Severo Ochoa, Consejo Superior
de Investigaciones Científicas, Universidad Autónoma de
Madrid, Nicolás Cabrera 1, 28049 Madrid, Spain
e-mail: ypico@cbm.uam.es

N. Parody
e-mail: nparody@cbm.uam.es

C. Alonso
e-mail: calonso@cbm.uam.es

N. Parody · E. Fernández-Caldas
Research & Development Department, Laboratorios
LETI S.L, Madrid, Spain
e-mail: efernandez@leti.com

library of *C. arizonica* pollen. The data presented indicate that the isolated total and mRNA obtained can be used for RT-PCR and that the library may be useful for the identification and characterization of new allergens from *C. arizonica* pollen.

Materials and Methods

Cupressus arizonica Pollen

Pollen was generously donated by Dr. Paolo Raddi from the Institute for the Pathology of Forest Plants (Florence, Italy). Pollen was either frozen at -70°C upon recollection or stored in a glass container prior to use.

Solutions and Equipment

All RNA isolation protocols were carried out in RNase-free conditions: solutions were treated with diethyl pyrocarbonate (DEPC). Glassware was thoroughly rinsed in DEPC-treated water and baked for 6 h at 180°C . Chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Total RNA Isolation

Frozen pollen (2 g) was ground with 2 g of quartz sand in liquid nitrogen for 5–7 min in a large mortar. After disruption, 15 ml of lysis solution (8 M Guanidine hydrochloride, 20 mM MES pH 7, 20 mM EDTA, β -mercaptoethanol added prior to use at a final concentration of 200 mM) was added to the mortar and frozen again using liquid nitrogen. The resulting mixture was ground again until a fine powder was obtained. Frozen powder was transferred to a 50-ml falcon tube and incubated for 10 min at 70°C . After incubation at 70°C , the volume was adjusted to 25 ml by the addition of lysis solution. Then, 1 vol of chloroform was added and shaken vigorously for 15 s, and it was left on ice for 5 min and centrifuged for 10 min at $3000\times g$ in a SS34 rotor.

After centrifugation, the resulting thick semisolid interphase and the aqueous phase were collected, mixed, and homogenized by passing them five times through a 20-G needle. One volume of water saturated phenol:chloroform:isoamyl alcohol 25:24:1 was added to the mixture and shaken vigorously for 15 s. The mixture was left on ice for 10 min and vigorously shaken every 2 min. The sample was divided into two 30 ml Corex[®] tubes and centrifuged for 15 min at $12000\times g$ at 4°C in a pre-chilled SS34 rotor. The phenol:chloroform:isoamyl alcohol extraction was repeated two more times. The resulting aqueous phase was precipitated at -70°C for 2–3 h after addition of 0.1 vol of 3 M sodium acetate pH 5.2 and 1 vol of isopropanol. The gel-like

precipitate was washed twice with ice-cold 70% ethanol and resuspended in 9 ml of the lysis solution from the commercial Talent Total Quick RNA kit (Talent S.R.L., Italy). After incubation for 10 min at 65°C , the solution was processed according to the manufacturer's instructions.

RNA was analyzed for purity and integrity using a Nanodrop ND-1000 spectrophotometer and an Agilent 2100 Bioanalyzer, respectively.

Poly(A)⁺ mRNA Purification

The protocol used for Poly(A)⁺ mRNA selection has been previously described [7]. In brief, Poly(A)⁺ mRNA is bound to oligo dT fixed in a cellulose matrix, eluted after salt removal, precipitated in ethanol, and resuspended in DEPC-treated water. Poly(A)⁺ mRNA was also analyzed for purity and integrity using a Nanodrop ND-1000 spectrophotometer and an Agilent 2100 Bioanalyzer, respectively.

Reverse Transcription and cDNA Synthesis

Total RNA (5 μg) was reverse transcribed in 30 μl reactions primed with Oligo dT¹⁶ using SuperScript[™] II RNase H[−] Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions.

T7 Phage Display Library Construction

Poly(A)⁺ mRNA (4 μg) primed with random hexamers was used for cDNA synthesis with a Novagen OrientExpress[™] cDNA Synthesis and Cloning System. The resulting cDNA was used to construct a phage display library of *C. arizonica* using a Novagen T7 Select 1-1b system as per the manufacturer's instructions.

PCR Analysis

Forward and reverse primers were designed based on known *C. arizonica* sequences of major allergen Cup a 1 (GenBank accession numbers AJ243570 and AJ278498) [3, 5]. These primers either spanned the whole 1044 bp sequence [forward primer 5'-GGGGATCCTCTGATAATCCCATAGACAG-3'; reverse primer 5'-GGCTGCAGTTATGCTACA-ACTCCAGCATTT-3' (Bam HI and PstI restriction sites are underlined)] or divided the reported Cup a 1 sequence in two fragments: Cup a 1 50a (forward primer 5'-GGGGATCCTCTGATAATCCCATAGACAG-3'; reverse primer 5'-GGCTGCAGCCATCAGAACAAATCGGAGAGA-3') and Cup a 1 50b (forward primer 5'-GGGGATCCTCATAATTCTCTCCGATTGTTCT-3'; reverse primer 5'-GGCTGCAGTTATGCTACA-ACTCCAGCATTT-3'), spanning 526 and 550 bp of the Cup a 1 sequence, respectively.

Primers were used in PCR reactions using cDNA obtained from total RNA reverse transcription or a sample from the *C. arizonica* T7 phage display library in order to test RNA quality in downstream applications.

For analysis of the T7 phage display library, ten randomly selected individual lysis plaques were scraped after library titration, eluted in deionized water and used as template for PCR reactions using T7 specific primers (Novagen, Nottingham, UK) that surround the vector's multiple cloning site.

Cloning and Sequencing of Cup a 1 cDNA

PCR fragments were purified from a 1% agarose gel using the WizardTM PCR Preps DNA Purification System (Promega, Madison, WI, USA), digested with BamHI and EcoRI, inserted into pBluescriptII KS[−] vector (Stratagene, La Jolla, CA, USA) and transformed into *Eschericia coli* XL-1 Blue competent cells. Positive clones were selected by PCR and sequenced in an ABI Prism 3700 automatic sequencer (Applied Biosystems, San Jose, CA, USA). The obtained sequences were aligned using the ClustalW algorithm [8].

Results and Discussion

Fully ruptured homogenates of pollen grains proved to be more difficult to obtain than expected. Disruption of pollen grains in the presence of liquid nitrogen in a mortar was not efficient and addition of quartz sand was necessary (Fig. 1). If RNA purification is to be performed at larger scales (i.e., more than 5 g of starting material), disruption and homogenization using a French press has proven to be the best choice. Previously reported plant RNA protocols [9] or commercial kits and reagents (Trizol, Qiagen RNeasy, Talent Total Quick RNA) were unable to produce total RNA of acceptable purity. Figure 2 shows UV absorbance spectra of polysaccharide contamination with characteristic peaks at 230 and 270 nm. The A260/280 ratio in these samples was never higher than 1.55 as opposed to the accepted values of highly pure RNA ranging from 1.8 to 2.0 [10].

Polysaccharide contamination was eliminated by using the protocol proposed by Logemann et al. [9] with the following modifications: (1) Incubation of the lysate at 70°C followed by a chloroform extraction reduced lysate viscosity, (2) Solubilization of the gel-like pellet that resulted after acid phenol extraction and precipitation was achieved by resuspending it in the Talent Total Quick RNA kit lysis solution followed by incubation at 65°C. After processing the sample with the Talent Total Quick RNA

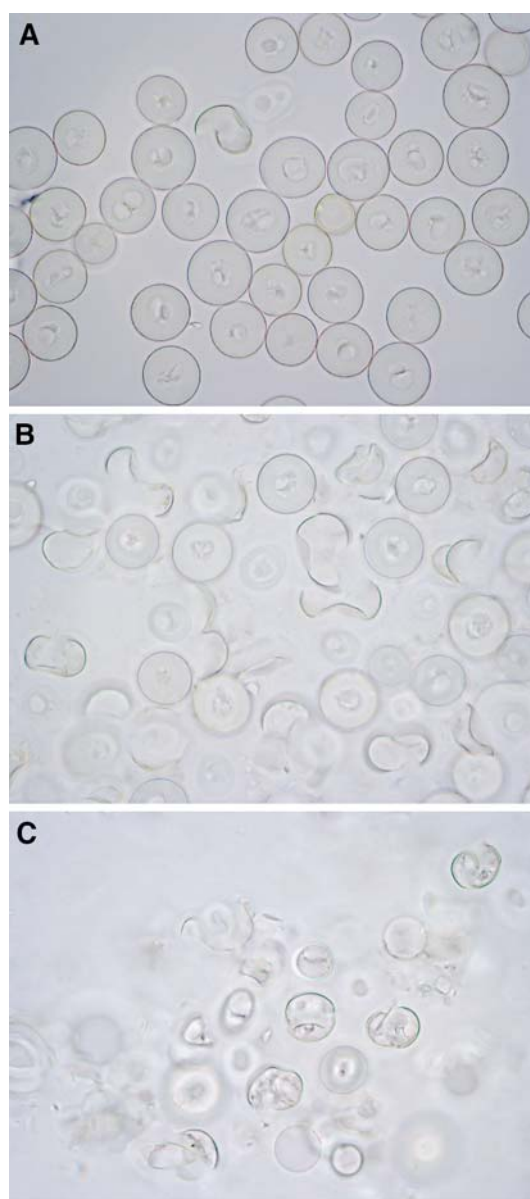


Fig. 1 *C. arizonica* pollen. **a** Intact pollen resuspended in lysis solution. **b** Partially disrupted pollen, some grains still intact. **c** Pollen disrupted with mortar and pestle using quartz sand. Practically all grains are broken

kit, pure RNA was obtained (Fig. 2), having A260/280 and A260/230 ratios of 1.93 and 2.17, respectively. These ratios indicate absence of protein and polysaccharide contamination [10, 11].

RNA integrity was analyzed with the Agilent 2100 Bioanalyzer (Fig. 3) and subsequently with the RNA integrity number (RIN) algorithm [12]. Traditional protocols and commercial kits yielded degraded RNA with no visible ribosomal bands (Fig. 3a, lanes 1–4). RIN in these samples ranged between 1 and 2.2. When the modified protocol was applied to unfrozen pollen, only the 18s band

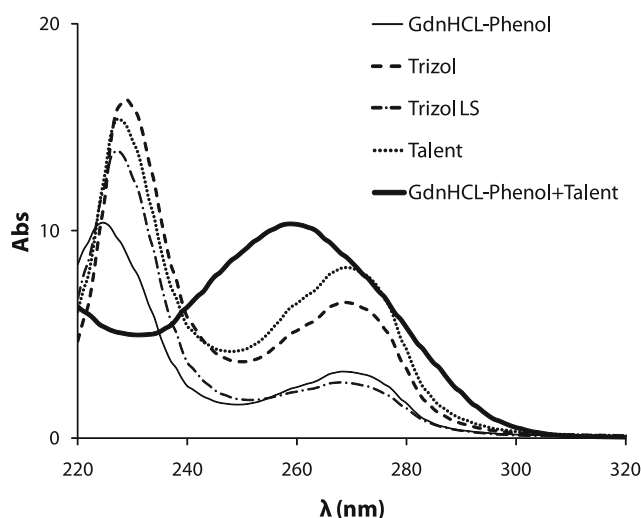


Fig. 2 UV absorption spectra from different total RNA samples. Contaminated samples show absorption peaks around 220–230 nm (polysaccharides) and at 268 nm. The pure sample obtained by our modified protocol (GdnHCL-Phenol + Talent) displays the characteristic absorption spectrum of nucleic acids with a single peak at 258 nm and A260/280 A260/230 ratios (1.93 and 2.17) that show absence of phenolic, polysaccharide, or protein contamination

was visible, but the RNA proved to be of slightly higher quality (RIN = 3.5–4) (Fig. 3a, lane 5). If commercial protocols such as Trizol were applied to *C. arizonica* leaves, both rRNA bands were noticeable. However, RNA in this sample (Fig. 3a, lane 6) was partially degraded as revealed by its RIN (5.6). Only when the modified protocol was applied to pollen that had been frozen at -70°C upon recollection, high-quality RNA was obtained (RIN = 7.8–8.2) (Fig. 3a, lanes 7–8). These data suggest that even if *C. arizonica* pollen retains its allergenic activity after long-term storage at room temperature [13], the RNA may have been degraded and biological viability may be compromised.

In RT-PCR reactions using primers to amplify *C. arizonica* major allergen Cup a 1, products of the expected size (1044 bp) could be obtained using RNA samples from frozen and unfrozen pollens even though the RNA from the unfrozen pollen was of low quality (Fig. 4). Non-reverse-transcribed RNA showed no amplification, excluding the possibility of genomic DNA contamination (Fig. 4). Sequenced clones (GenBank accession number EF079863) confirmed the identity of the amplification product. When aligned with previously reported nucleotide sequences [3, 5] (accession numbers AJ243570 and AJ278498), 26 substitutions were observed at the nucleotide level (data not shown). The deduced amino acid sequence alignment contained 11 substitutions (Fig. 5).

In order to construct the phage display library, Poly(A)⁺ mRNA was purified from high-quality total RNA samples by affinity chromatography with an oligo dT-cellulose

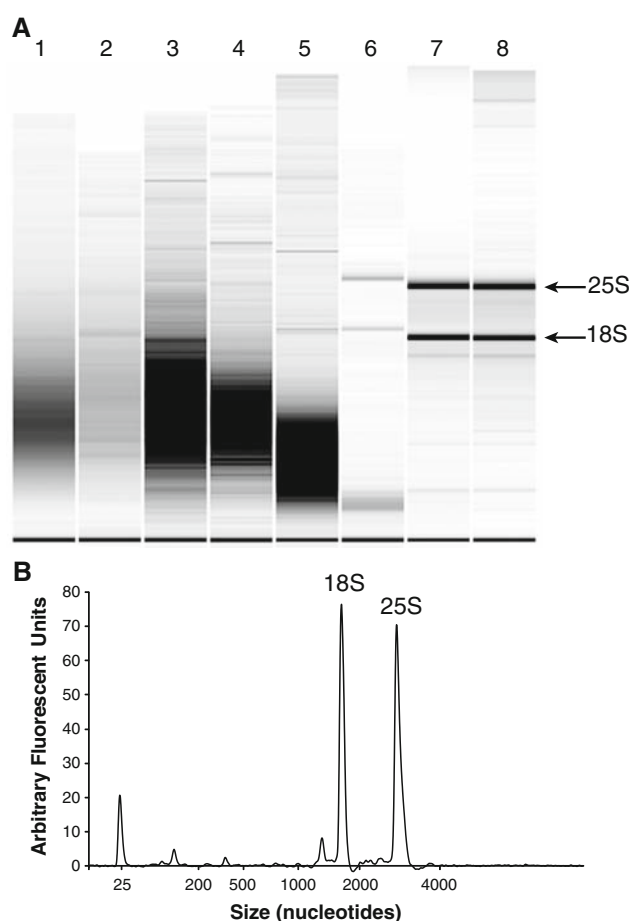


Fig. 3 a Gel view of analyzed RNAs. Lanes 1–5 degraded RNAs obtained from unfrozen pollen using several techniques [1 Trizol, 2 Trizol LS, 3 Talent Quick RNA, 4 GdnHCL-phenol, 5 GdnHCL-phenol + Talent (modified protocol)]. Lane 6 Total RNA obtained from *C. arizonica* leaves by Trizol; 18S and 25S ribosomal bands are clearly visible. Lanes 7, 8 Total RNA purified by the modified protocol from frozen pollen. b Electropherogram view of lane 6 total RNA purified by the modified protocol with a RIN of 8.2

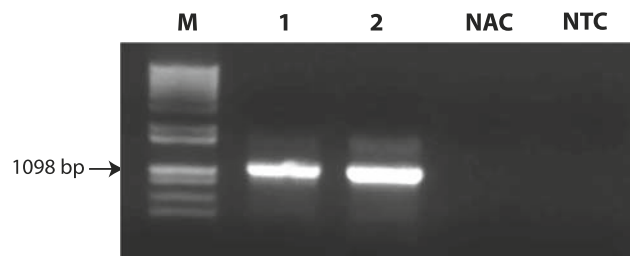


Fig. 4 PCR amplification of *C. arizonica* cDNA. M marker, 1 cDNA obtained from unfrozen pollen RNA, 2 cDNA obtained from frozen pollen RNA, NAC no amplification control: non-reverse-transcribed total RNA was used as a template, NTC no template control

column. Poly(A)⁺ mRNA purity was confirmed by UV spectrophotometric analysis showing A260/280 and 260/230 ratios within expected ranges 1.8–2.0 and 2.0–2.3, respectively (data not shown). Poly(A)⁺ mRNA integrity

Fig. 5 ClustalW multiple sequence alignment of previously reported sequences Cup a 1(3) and Cup a 1.02(5) with Cup a 1.03, the sequenced clone described in this article. * = Identical residues; - = different residues; · = semiconserved substitutions; : = conserved substitutions

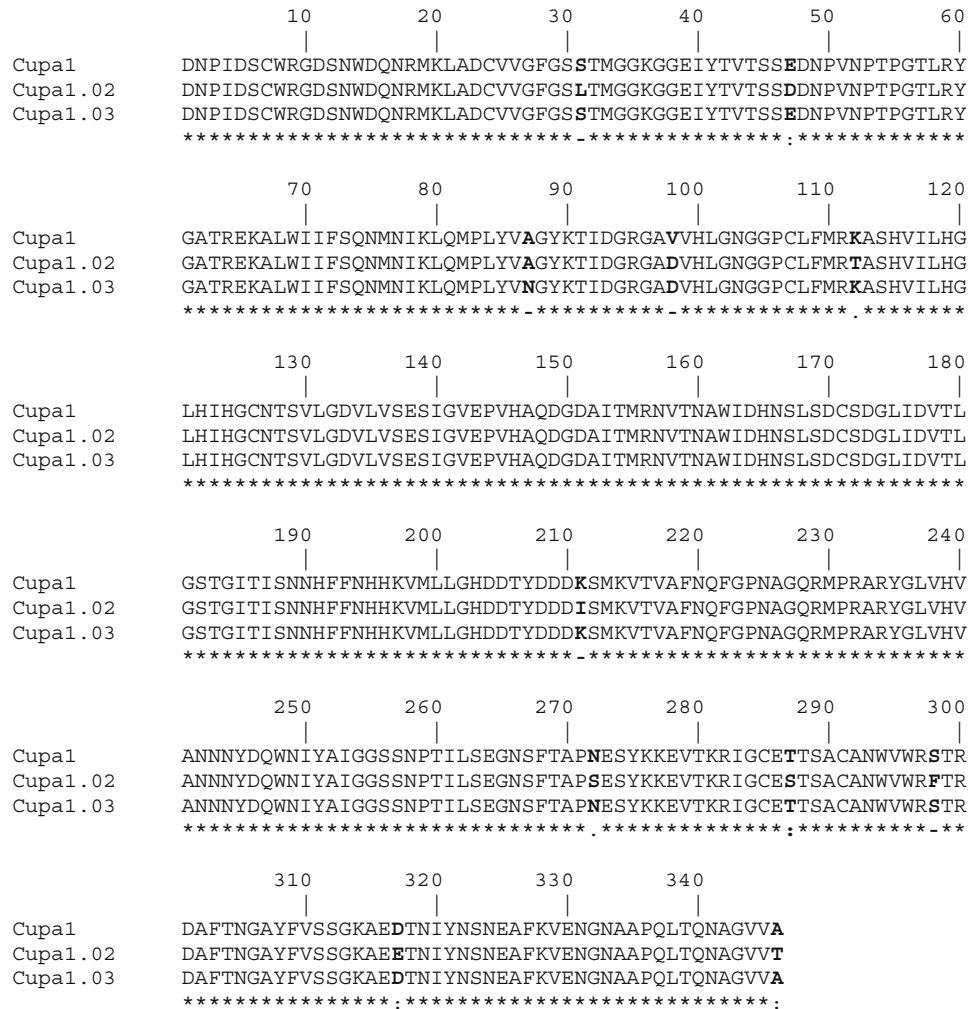
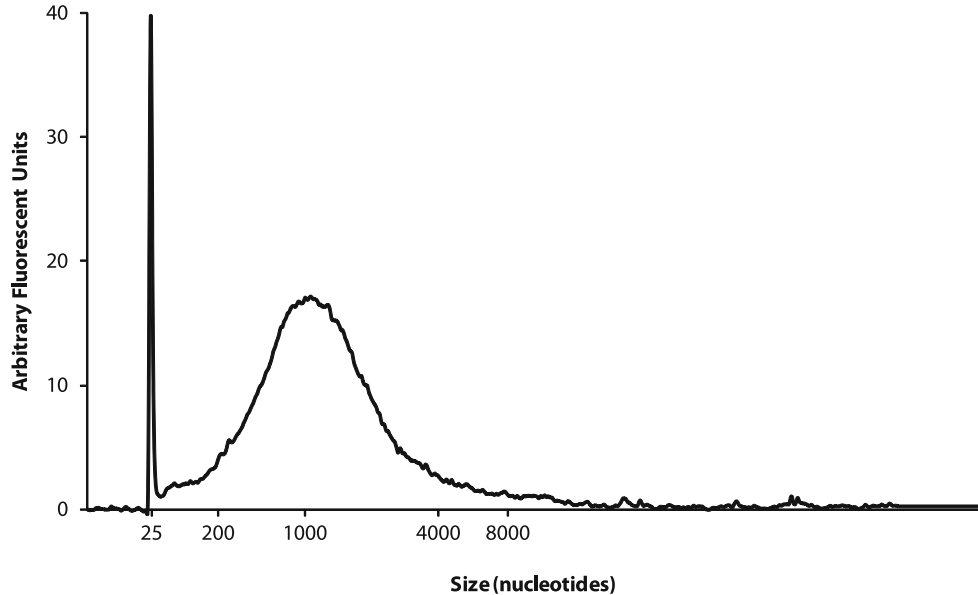


Fig. 6 Chromatogram view of Poly(A)⁺ mRNA analyzed with the Agilent 2100 Bioanalyzer. Poly(A)⁺ mRNA size ranges from ~100 to 4000 nucleotides. Absence of rRNA is denoted by the smooth curve and lack of rRNA peaks. The first peak corresponds to the Agilent RNA 6000 Nano Marker



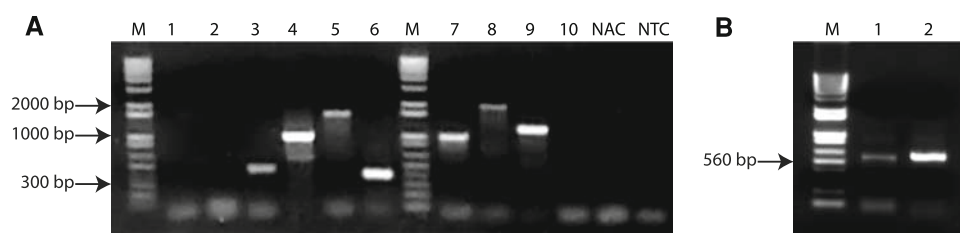


Fig. 7 **a** Individual plaque PCR of *C. arizonica* T7 phage display library. *M* molecular weight markers, 1–10 random clones, *NAC* no amplification control, *NTC* no template control. **b** Cup a 1 fragment

amplification using amplified library as template. *M* molecular weight markers. 1 Cup a 1 50a, 2 Cup a 1 50b

and absence of rRNA contaminants were analyzed with the Agilent 2100 Bioanalyzer (Fig. 6). The quality of the Poly(A)⁺ mRNA was also confirmed when it was used for phage display library construction. Initial library titer was 2.5×10^6 pfu/ml and contained ~70% recombinant clones ranging from ~300 to ~2000 bp (Fig. 7a). This library was amplified to a final titer of 2.6×10^9 pfu/ml. Successful PCR amplification of fragments Cup a 1 50a and 50b using amplified library samples as template further confirmed the library's quality (Fig. 7b).

Our modified protocol yielded 50–70 µg of high-quality total RNA per gram of a polysaccharide-rich tissue such as *C. arizonica* pollen which was successfully used in RT–PCR and the generation of a phage display library.

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