tionally accelerate plot drawing and calculations. In the course of cDNA library construction, we measured cDNA concentrations during LD-PCR, after SfiI digestion, and before ligation to vector. It allowed us to generate high-quality cDNA libraries, more than 2 × 10^6 independent clones, 90%–99% of recombinant clones, with less effort.

To confirm the accuracy of the measurements in our assay, we compared data obtained by digital image analysis of gels and by spectrophotometer measurements. Using LD-PCR, we amplified the pool of DNA from constructed placenta cDNA library after 7, 10, 13, 16, or 19 cycles, purified each on a GFX column (Amersham Pharmacia Biotech, Baie d’Urfe, QC, Canada), and eluted products with 50 µL water. The quantities of the resultant PCR products were estimated after electrophoresis (Figure 1B). Using the series of 1-kb ladder dilutions, we created a calibration curve for this gel and calculated the concentrations of PCR samples. In parallel, we quantified the concentrations of PCR products by spectrophotometry on a Hewlett Packard 8451A spectrophotometer. The comparison of spectrophotometer measurements and digital image analysis showed a very good concordance (Table 1).

Remarkably, we could use only 5 µL from 50 µL purified PCR product for digital analysis, but it was necessary to use at least 20 µL of the same product for spectrophotometric measurement. The use of new intercalating dyes such as GelStar® (BioWhittaker, Walkersville, MD, USA) or SYBR® Green (Sigma, St. Louis, MO, USA) for staining of nucleic acids in gels can additionally increase the sensitivity of the entire assay. Digital image analysis not only requires less material than a spectrophotometer but also can be faster, especially when multiple samples are analyzed. This procedure takes only 20 min, including the 10-min gel run.

In addition, the substitution of the DNA ladder for dilutions of total RNA with known concentrations allowed us to use the proposed digital image analysis technique for estimation of the quantity and quality of total RNA samples (Figure 2). Note that multiple experimental samples can be analyzed simultaneously in contrast to spectrophotometric measurements. Data generated by digital imaging for total RNA demonstrated good correlation with spectrophotometer measurements (data not shown).

Digital image analysis is becoming a more powerful instrument for life research. We demonstrate that this technique can be successfully implemented for the measurement of ds-cDNA and total RNA concentrations. In comparison with traditional spectrophotometric measurements, digital image analysis has higher sensitivity, demands less material, and is a timesaving tool.

REFERENCES


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Protocol for Small-Scale RNA Isolation and Transcriptional Profiling of Developing Arabidopsis Seeds

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Seeds provide much of the economic value in agricultural products, and numerous research efforts are aimed toward understanding the regulation of the storage compound accumulation in crop seeds. With its fully sequenced genome, Arabidopsis thaliana (thale cress) has now become the dominant model system in dicot plant science, including seed biology. Unfortunately, Arabidopsis seeds are very small and not always amendable to study. For example, the application of microarray technology to large-scale gene expression profiling in Arabidopsis seeds can be limited because of the relatively large amount of RNA needed to prepare probes. Another potentially restrictive factor is that seeds are difficult material for RNA isolation because of high amounts of secondary metabolites and polysaccharides. In our experience, commercial kits and widespread plant RNA extraction methods are unsatisfactory in preparing microarray probes using Arabidopsis or other seeds. Here, we describe a rigorous procedure that enabled us to isolate high-quality RNA and perform successful microarray experiments using small amounts of developing Arabidopsis seeds.

A. thaliana (ecotype Col-2) seeds were dissected from siliques between 5 and 13 days after flowering, immediately frozen with dry ice, and stored at -80°C. The fresh weight of developing Arabidopsis seeds is in the range of 10–30 µg each, so 3000–10 000 seeds (80–250 siliques) were harvested for each extraction. The extraction buffer presented here is a combination of buffers described in Schultz et al. (10) and Chung et al. (4). Thiourea was used as a chaotropic reagent because RNA extractions from plant material using guanidine buffer often fail (2). The addition of three different detergents (7) also improved both yield and quality of

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Protocol 1. RNA Isolation and Purification (A)

All centrifugation steps are done using a microcentrifuge at maximum speed (13 000 rpm, about 10 000 × g).
1. Grind the tissue (50–100 mg seeds) to fine powder in liquid nitrogen using a mortar and pestle.
2. Add 1.5–2 mL extraction buffer on top of the powder, let freeze, and continue homogenization until the mixture has thawed.
3. Transfer the homogenate to RNase-free microcentrifuge tubes, (a few drops of chloroform can be added to the homogenate to reduce foaming and facilitate the transfer). Incubate at 65°C for 3–5 min, and quickly cool on ice.
4. To the crude extract, add an equal volume of chloroform:isoamyl alcohol (CI, 24:1), and vortex mix for 2 min.
   The sample can be left on ice at this stage, if one is processing several samples at the same time. Centrifuge at room temperature for 10 min, and transfer the supernatant to a new tube.
5. Re-extract the lower organic phase with 200 μL extraction buffer, mix, and centrifuge as before.
6. Combine the aqueous phases, and extract with an equal volume of CI.
   Optional: to increase recovery, you can back-extract the PCI-organic phase with 200 μL extraction buffer or RNase-free water.
8. Extract supernatant with CI.
9. Precipitate RNA by adding 0.1 vol. 3 M neutral sodium acetate (pH 6.2) and 1 vol isopropanol. Incubate at -20°C to -80°C for at least 1 h, and centrifuge at 4°C for 30 min.
10. Rinse the pellet with 300 μL ice-cold 70% ethanol, and air-dry briefly.
11. Dissolve the pellet in 0.5–1 mL 65°C RNase-free water, and add 1 M acid sodium-acetate (pH 4.5) a final concentration of 80 mM and 0.4 vol 2-butoxyethanol.
12. Incubate the samples at 65°C for 7–10 min, and then incubate them on ice for at least 10 min.
13. Pellet the polysaccharides by centrifugation at 4°C for 30 min.
14. Adjust the sodium-acetate concentration of the supernatant to 0.2 M using 3 M neutral sodium acetate (pH 6.2) and add 1 vol isopropanol. Precipitate RNA at -20°C to -80°C for at least 30 min and collect by centrifugation for 30 min at 4°C. Rinse the pellet with 70% ethanol and air-dry.
15. Repeat the 2-butoxyethanol/isopropanol precipitations.
16. Optional: dissolve RNA in 0.5–1 mL RNase-free water, add 1/30 vol 3 M sodium acetate (pH 5.2) and 0.1 vol 100% ethanol, mix, and keep on ice for 30 min. Pellet the polysaccharides by centrifugation at 4°C for 30 min. To the supernatant, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M and 3 vol 100% ethanol. Precipitate RNA at -20°C to -80°C for at least 30 min and collect by centrifugation for 30 min at 4°C.
17. Rinse the final RNA pellets twice with 70% ethanol, air-dry, and dissolve in 50–100 μL RNase-free water or 10 mM Tris, pH 7.5, and 10 mM EDTA. Store at -80°C.

Reagents (B)

Extraction Buffer:
1% tri-isopropynaphtalene sulphonic acid (w/v)
6% p-aminosalicylic acid (sodium salt) (w/v)
100 mM Tris, pH 8.0
50 mM EGTA, pH 8.0
100 mM NaCl
1% (w/v) SDS
0.5% (w/v) sodium-deoxycholate
0.5% (w/v) Nonidet™ P-40 (Tergitol™; Sigma, St. Louis, MO, USA)
0.5% (w/v) PVP (Mw, 40 k)
1%–0.5% (w/v) PVPP (insoluble, add last)
5 mM thiourea
1% (v/v) β-mercaptoethanol (add immediately before use)

All solutions were DEPC-treated or prepared in DEPC-treated water. Glassware, mortars, and pestles were baked at 180°C overnight.

Acid Phenol:
Add 65 mL 0.25 M sodium acetate, pH 4.5, with 19% glycerol to 100 g molecular-grade phenol (Sigma).
RNA. To remove polyphenols, we included both soluble [polyvinyl-pyrrolidone (PVP); molecular weight ($M_r$), 40 k] and insoluble [polyvinyl-poly- pyrrolidone (PVPP)] polymers in the extraction buffer. To further prevent polyphenols from complexing with RNA, we avoided performing phenol extractions until the samples were substantially purified with chloroform-isoamyl alcohol extractions (10). Arabidopsis seeds are also rich in starch and soluble sugars, and the seed coat contains a high concentration of polysaccharide mucilage (11). We used the protocol originally introduced by Manning (9) and modified by Chung et al. (4) to selectively precipitate polysaccharides using 2-butoxyethanol (ethylene glycol monobutyl ether) and a low concentration of acidic sodium-acetate (80 mM, pH 4.5). We found it necessary to repeat the 2-butoxyethanol precipitation twice for the seed samples and, in some cases, to include an additional precipitation step using ethanol (1). Precipitation with LiCl, which is recommended for polysaccharide removal, was ineffective in purifying Arabidopsis extracts. The acid phenol-chloroform extraction was used to minimize genomic DNA contamination (3).

Using the procedure described here, the RNA yields from developing Arabidopsis seeds were 20–80 μg/100 mg fresh weight, depending on the age of the seed. The average $A_{260/280}$ ratio from 15 separate extractions was 2.04 ± 0.10. The $A_{260/230}$ ratio, a measure of polysaccharide contamination, was consistently high (2.55 ± 0.77). However, despite this apparent purity (and high integrity as judged by denaturing agarose gel electrophoresis) of the RNA samples, some interfering compounds remained. Although the viscosity of the samples was significantly reduced by this procedure, when compared to our initial attempts, reverse transcription still gave inconsistent yields. We purified the RNA samples further with a silica-based absorption method (RNeasy® Mini Kit; Qiagen, Valencia, CA, USA). Only about 30 μg RNA were applied to each column because of the remaining sample viscosity, and the columns were incubated for 10 min with 30–50 μL RNase-free water during the final elution step. In some instances, RNeasy treatment was performed twice. This procedure has the added benefit of slightly enriching the preparations in poly(A)$^+$ RNA because RNAs smaller than 200 nucleotides are excluded.

In an earlier study (5), we labeled probes by direct incorporation of Cy3- or Cy5-labeled dCTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA) into cDNA during the reverse transcription of 1 μg poly(A)$^+$ RNA from developing Arabidopsis seeds (isolated from about 100 μg total
Figure 2. The effect of BSA prehybridization and pre-warming on the array quality. cDNA was synthesized from 3 μg Arabidopsis seed total RNA using 3DNA reverse transcription primers (Cy3 and Cy5) and 200 U SUPERSCRIPT enzyme (20 μL total reaction volume). Probe mixtures were applied on cDNA microarrays printed on aldehyde substrates. The arrays were hybridized overnight at 65°C underneath a coverslip and washed for 10 min each in 2× standard saline citrate (SSC), 0.2% (w/v) SDS (55°C), followed by 2× SSC (25°C), and finally in 0.2x SSC (25°C). The arrays were dried by brief centrifugation (500 rpm for 3 min, about 500x g) and scanned with ScanArray® 5000 (GSI Lumonics, Watertown, MA, USA). The false-color images represent signals from Cy3 fluor. Identical RNA samples were used for the hybridizations, and the same section of the array is shown in both figures. (A) The probe mixture contained 0.3 μL anti-fade reagent (antioxidant from the 3DNA Kit that was applied to prevent the dyes from bleaching during scanning). The probe was incubated at 55°C for 20 min and cooled to room temperature before applying it to the array. (B) The array was prehybridized in 50 mL buffer containing 5x SSC, 0.1% (w/v) SDS, and 1% (v/v) BSA for 30 min at 65°C, washed with water, and dried by centrifugation. The anti-fade reagent was omitted from the probe mixture, and the probe was applied on a pre-warmed substrate (55°C) while it was still warm.

Figure 1. Comparison of hybridization efficiency and cross-hybridization thresholds between direct and indirect fluorescence probe labeling techniques. This comparison is based on the Arabidopsis FAD2 gene that codes for oleate desaturase and is moderately expressed in all tissues. No closely related genes to FAD2 are known in Arabidopsis. The fluorescence intensities of three different fragments of FAD2 are plotted for eight independent, developing Arabidopsis seed probes prepared either from 1 μg poly(A)+ RNA using first-strand reverse transcription in the presence of fluorescent Cy3 or Cy5 nucleotides (referred to as direct labeling; solid symbols and solid lines, data from Reference 5) or 3 μg total RNA using the 3DNA system (referred to as indirect labeling; open symbols and dotted lines). The 3 FAD2 fragments are displayed on the x-axis in the following order: 356-bp fragment with 100% identity to the 3′ coding region of FAD2, and two synthetic fragments with 90% and 80% identity covering the same 356-bp fragment as the 100% fragment. The two synthetic fragments contain evenly spaced mismatches and were synthesized by PCR (5). The fragment with 90% identity gave about 50% weaker signals than the 100% identity fragment, demonstrating that the cross-hybridization threshold was between 80% and 90% similarity.
RNA). The reason for the high amount of starting material was the low incorporation efficiency of the fluorescent nucleotides. Before the probes were used for hybridizations, the labeling was confirmed by running an aliquot of the probe on a gel, followed by the analysis of the fluorescent signal from the separated products using a laser scanner. For our time-course experiments in which RNA yields were much less than 100 μg, we used an indirect probe labeling method, when it was not possible to determine the quality of the probe before hybridization. Therefore, each RNA sample was tested with reverse transcription in the presence of 33P-labeled cDNA (NEN Life Science Products, Boston, MA, USA). The radiolabeled cDNA products were separated on a 1% agarose gel, transferred to a nylon membrane, and visualized using autoradiography. Only samples that produced sufficient cDNA were used for probe synthesis. The same ratios of RNA sample and reverse transcriptase were used for the test reaction and later for the probe synthesis.

For time-course studies of developing Arabidopsis seeds, we chose a detection system that requires much less RNA than the conventional method. The 3DNA® technology (Genisphere, Montvale, NJ, USA) is based on a large DNA complex that binds several fluorescent molecules. RNA is reverse transcribed using oligo-dT primers that contain either Cy3 or Cy5 dye-specific capture sequence. The cDNA is then combined with a Cy3 or Cy5 detection reagent that recognizes the sequence incorporated to cDNA molecules. Figure 1 shows a comparison of the results of probes prepared with either direct fluor incorporation or the 3DNA method. Probes prepared from 3 μg total RNA using the 3DNA method produced signal intensities that were comparable to probes synthesized from 1 μg poly(A) + RNA with the direct incorporation, confirming that the detection limit was maintained around two copies of mRNA per cell (5). In addition, the cross-hybridization threshold remained between 80% and 90% identity. We also compared the seed/leaf expression ratios of 3000 genes from experiments using probes prepared with the two methods and found that they corresponded well. The average Pearson correlation coefficient for the ratios was 0.87 (data not shown).

We introduced some modifications to the hybridization protocols to increase the quality of the probe and minimize interfering background. The probes were synthesized from 3 μg total RNA from developing Arabidopsis seed using the primers included in the 3DNA Submicro Expression Array Detection Kits (Cy3 and Cy5 labels). We used the SUPERSCRIPT II™ enzyme (Invitrogen, Carlsbad, CA, USA) with its own reaction buffer and DTT because this enzyme was substantially less inhibited by remaining RNA contaminants than other reverse transcriptases. After the cDNA synthesis and template RNA degradation, Cy3 and Cy5 reactions were combined and concentrated according to the manufacturer’s recommendations. The cDNA pellet was dried for 10–15 min at 55°C–65°C and thoroughly dissolved in 30 μL GlassHyb™ hybridization buffer (Clontech Laboratories, Palo Alto, CA, USA). Finally, 2.5 μL Cy3 and Cy5 3DNA-capture reagents and 0.6 μg human Cot-1 DNA (Invitrogen) were added, and the probe mixture was incubated at 55°C for 20 min. The cDNA microarrays were prepared from an Arabidopsis seed-specific cDNA library (5) by spotting 6000 DNA elements on aldehyde-coated glass substrates (SuperAldehyde™; TeleChem International, Sunnyvale, CA, USA). The substrates were postprocessed according to the manufacturer’s recommendations, including the sodium borohydride treatment used to reduce the remaining free-aldehyde groups. We found that a prehybridization of the substrates for 30 min in 50 mL buffer containing 1% BSA at 65°C, as recommended by Hedge et al. (6), substantially decreased nonspecific binding of the probe on the array surface (Figure 2). In addition, the quality of the arrays increased when the anti-fade reagent (antioxidant included in the 3DNA kits) was omitted from the probe mixture, and the 55°C probe was applied on a pre-warmed (55°C) substrate.

REFERENCES


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