African Journal of Biotechnology Vol. 10(68), pp. 15430-15437, 2 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1425
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

A mini-scale hot borate method for the isolation of total RNA from a large number of cotton tissue samples

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Accepted 19 September, 2011

Genetic genomics in cotton requires isolating RNA from a large number of plant tissue samples, in a mapping population. However, traditional methods for the extraction of RNA from cotton tissues is time consuming and not suitable for processing many samples at the same time. Here, we present a mini-scale protocol for quick isolation of total RNA from cotton ovaries with high yield and quality suitable for gene expression studies. By modifying and scaling down a hot borate extraction method, a quick and easy method was developed to extract total RNA from ovaries of three genotypes ranging in ages from -1 to 10 days post anthesis (dpa) with an average yield of 600 µg/gfw (gram fresh weight) and ten dpa ovaries of 64 genotypes. The method was also successfully tested using cotton leaves and anthers. The quality and quantity of total RNA were suitable for reverse transcription-polymerase chain reaction (RT-PCR) and cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis.

Key words: Cotton, ovaries, RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR), cDNA-amplified fragment length polymorphism (cDNA-AFLP).

INTRODUCTION

High-quality total RNA from cotton (*Gossypium hirsutum* L.) tissues is the prerequisite for gene expression studies and cloning. The presence of high levels of polyphenols, terpenoids, and other secondary metabolites, makes the extraction of total RNA difficult and time consuming (Schneiderbauer et al., 1991). Wu et al. (2002) reported that commonly used total RNA extraction procedures such as guanidine-HCl–based methods (Logemann et al., 1987; Dolferus et al., 1994) and a commercial kit (Qiagen RNeasy Plant Mini kit) produced very low yields of total RNA. During our studies on cotton fiber initiation and development using cDNA-amplified fragment length polymorphism (cDNA-AFLP) and microarray analyses, various total RNA isolation methods were tried for isolating total RNA from -1 to 26 days post-anthesis (dpa) cotton ovaries. TRIZOL reagent (Invitrogen) was also tested several times for the extraction of total RNA from ovaries of various ages, but the precipitated RNA always contained complex carbohydrates. Even after adding 0.25 ml isopropanol and 0.25 ml of a high salt precipitation solution (0.8 M sodium acetate/sodium citrate and 1.2 M NaCl) per ml TRIzol reagent homogenate the problem remained.

The hot borate method (Wan and Wilkins, 1994; Wilkins and Smart, 1996) specifically optimized for cotton leaves has been commonly used and it has produced total RNA with good quality and high yield (Wu et al., 2002; Ji et al., 2003). However, this method resulted in partial degradation of RNA from ovules of 0 dpa stage (Wu et al., 2002). Furthermore, it is not easy to process more than eight samples at the same time with this method, and the round-bottom of a 50 ml Oak Ridge

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Abbreviations: cDNA-AFLP, cDNA-amplified fragment length polymorphism; **dpa,** days post anthesis; **RT-PCR,** reverse transcription-polymerase chain reaction.

centrifuge tube results in a dispersed pellet and decrease in total RNA recovered. Thus, traditional methods for the extraction of RNA from cotton tissues are time consuming and not suitable for processing many samples at the same time. However, genetic and functional genomics in plants including cotton requires, isolating the RNA from a large number of plant tissue samples or a mapping population. The objective of this study was to overcome these problems encountered in RNA extraction and develop a quick as well as a simple method by scaling down the hot borate method. The modification uses 1.5 ml tubes only and homogenization of tissue powder is done using 2.5 mm zirconia/silica beads. Furthermore, a large number of cotton tissue samples were first ground in liquid nitrogen in a mortar and pestle and stored until RNA isolation. This procedure resulted in both high yield and quality of total RNA. We were able to process 48 samples in three days with the mini-prep method, as compared with only eight samples using the traditional large scale method (Wan and Wilkins, 1994). The total RNA isolated was suitable for RT-PCR, cDNA-AFLP and other analyses to study genome-wide transcript profiling. This procedure is routinely used to isolate total RNA in our laboratory for microarray analysis, cDNA pyro-sequencing studies and construction of microRNA as well as cDNA-AFLP libraries (Zhang et al., 2007; Curtiss et al., 2011; Rodriguez-Uribe et al., 2011; Pang, 2009; Pang et al., 2011). This quick method is especially suitable for molecular biology laboratories that are not well equipped or where commercial RNA isolation kits are unavailable in developing countries.

MATERIALS AND METHODS

Cotton tissues were all collected from field-grown plants. Cotton ovaries were collected from the developmental stages of -1, 0, 4, 8 and 10 dpa in three cotton cultivars; Acala 1517-99 (*Gossypium hirsutum* L.), Pima Phy 76 (*Gossypium barbadense* L.), and SG 747 (*G. hirsutum* L.). Anthers and leaves from the three cultivars were also harvested for RNA extraction. 64 lines were selected from a backcrossing inbred line (BIL) population for RNA extraction from ovaries (developing bolls) at 10 dpa. All ovaries and other tissues were frozen in dry ice or liquid nitrogen in the field and then transferred to a -80°C refrigerator in the laboratory without thawing for storage, until they could be processed. Additionally, ovules, anthers and/or leaves from Pima S-1 and 57-4 (both *G. barbadense*) and their F_1 hybrid and anthers from 8518, 8518R and D8R (all *G. hirsutum*), and young leaves from *G. herbacium africanum* (A_1) and *G. raimondii* (D_5) were harvested and flash frozen in liquid nitrogen.

Preparation of glassware and tubes

To inactivate RNases, glassware metal spatulas, mortars and pestles were wrapped in aluminum foil and baked overnight at 250°C oven. One mortar and pestle, and one spatula were prepared for each sample. Plastic-ware was thoroughly rinsed in 0.1 M NaOH, 1 mM EDTA as well as RNase-free ddH_2O rinsing and autoclaving. Alternatively, chloroform-resistant plastic-ware was rinsed with chloroform to inactivate RNases.

Solutions and reagents for RNA isolation

Water for all dilutions and pertinent solutions was prepared with 0.1% diethyl pyrocarbonate (DEPC). DEPC-ddH_2O was used to prepare the Tris-HCl buffer by adding Tris-base (molecular biology grade, RNase and DNase free) and the pH was adjusted to a desired value with DEPC-treated HCl.

Hot borate extraction buffer: for 100 ml solution (Wan and Wilkins, 1994; Wilkins and Smart, 1996), 0.2 M sodium borate dehydrate (Borax), 7.62 g; 30 mM ethylene glycol bis (beta-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 1.14 g; 1% sodium dodecyl sulfate (SDS), 1 g; and 1% sodium deoxycholate (1 g) were used and pH was adjusted to 9.0 with 5 M NaOH. After mixing the ingredients thoroughly, it was made to 100 ml with preheated ddH_2O, then, 50 µl DEPC was added and mixed thoroughly. After pipetting into 20 ml aliquots and autoclaving for 30 min, it was stored at -20°C until use. The following was added to prepare 20 ml of the extraction buffer; 10 mM dithiothreitol (DTT), 0.0308 g; 1% Nonidet-40 (NP 40), 0.2 ml; and 2% polyvinylpyrrolidone 40,000 (PVP 40), 0.4 g which were mixed until dissolved. Other solutions and reagents used for RNA isolation included: 10 mg/ml proteinase K; 2 M KCl (filtered, DEPC treated and autoclaved); 8 M LiCl (filtered, DEPC treated and autoclaved); 2 M KOAc, (pH 5.5, filtered, DEPC treated and autoclaved); 3 M NaOAc, (pH 4.5, filtered, DEPC treated and autoclaved); TE [10 mM Tris (pH 8.0), 0.1 mM EDTA (pH 8.0)], DEPC treated and autoclaved; 0.1% DEPC-ddH_2O: 1 ml DEPC was added to 1 L ddH_2O, treated at 37°C overnight, and then autoclaved; Ethanol; Qiagene MinElute/RNeasy column.

RNA isolation protocol

1. Cotton tissues stored at a -80°C freezer were ground in liquid nitrogen into powder using a mortar and pestle and transferred to the original tube for storage at -80°C.
2. Water bath was preheated to 80°C and an incubator to 42°C. The extraction buffer (with PVP, DTT and NP-40) was heated to 80°C to equilibrate the buffer. 850 µl extraction buffer was added to a 1.5 ml tube with two or three 2.5-mm zirconia/silica beads.
3. About 200 mg of ground cotton tissue sample were added to the tube, homogenized for 1 min, and then 50 µl proteinase K solution was added, and incubated at 42°C for 1½ h.
4. 68 µl 2 M KCl was added and incubated on ice for 1 to 3 h.
5. It was centrifuged for 20 min at 4°C and supernatant fraction was transferred to a new tube, and the volume determined.
6. 1/3 volume 8 M LiCl (about 320 µl) was added to make a final concentration of 2 M (should not exceed this), vortexed immediately and incubated on ice overnight or for more than 3 h.
7. It was centrifuged for 20 min at 9,000 *g* and 4°C, and supernatant was discarded.
8. Pellet was washed in ice-cold 2 M LiCl at least twice if supernatant remained colored.
9. Pellet was re-suspended in 300 µl DEPC-ddH_2O by vortexing.
10. It was centrifuged for 10 min at 12,000 *g* and 4°C to remove un-dissolvable material, and supernatant fraction was transferred to a new tube.
11. 30 µl ice-cold 2 M KOAc (pH 5.5) was added and incubated on ice for 15 min.

12. it was centrifuged for 10 min at 12,000 *g* and 4°C to remove un-dissolvable material.
13. Supernatant was transferred to a new tube and precipitated with 990 µl ice-cold 100% ethanol overnight at -20°C or for few hours at -80°C.
14. Pellet RNA was centrifuged for 30 min at 12,000 *g* and 4°C, pellet was washed with ice-cold 70% EtOH, centrifuged for 10 min at 12,000 *g* and 4°C and Speedvac dried for 5 to 10 min, until pellet was clear.
15. Pellet was re-suspended in 50 µl DEPC-ddH_2O, RNA was precipitated with 5 µl, 3M NaOAc and 165 µl cold EtOH for a minimum of 2 h at -20°C.
16. It was centrifuged for 20 min at 12,000 *g* and 4°C; washed with 70% EtOH, and then pellet was dried.
17. It was re-dissolved in 50 µl DEPC-ddH_2O.
18. Concentration was determined using a DU 530 UV/Vis spectrophotometer (Beckman Coulter).
19. Total RNA samples were treated with Qiagen RNase-free DNase to remove any genomic DNA contamination, followed by cleaning with an RNeasy MinElute Cleanup kit (Qiagen). Each total RNA sample was then adjusted to 0.5 µg/µl.

RNA isolation using a standard hot borate method

To compare with the mini-prep hot borate method, RNA from various tissues (2 to 4 g) such as ovules, anthers and leaves from Pima S-1 and 57-4 (both *G. barbadense*), anthers from 8518, 8518R and D8R (all *G. hirsutum*), and young leaves from *G. herbacium africanum* (A_1) and *G. raimondii* (D_5) were extracted based on the golden standard hot borate method (Wan and Wilkins, 1994; Wilkins and Smart, 1996).

RNA gel analysis

To check the integrity of the RNA, 7 µg total RNA were loaded on a 1.2% agarose gel containing 2.2 M formaldehyde and resolved by electrophoresis. After electrophoresis, the gel was stained in ethidium bromide (Sambrook et al., 1989) and the integrity of RNA was judged by the presence and intensity of rRNA bands.

First and second strand cDNA synthesis

For each sample, 5 µg total RNA was used in a doubled reaction volume for the first strand cDNA synthesis using ProtoScript® First Strand cDNA Synthesis kit (BioLab®). For RT-PCR analyses, the reaction mixture was diluted to 100 µl with ddH_2O and 2 µl was used for each RT-PCR reaction. Histone-3 amplified by primers (forward primer, 5'AGAGGTCGAGTCTTCGGACA3'; and reverse primer, 5'GCTTGATCTTCTTGGGCTTG3') was used as an internal criterion to verify that equal amounts of total RNA were used in the reactions, as indicated by equal intensity bands of 221 bp amplified for each sample.

The second strand cDNA synthesis was as follows: 40 µl of total first strand cDNA reaction was mixed with 6.0 µl 10X *Escherichia coli* DNA ligase buffer, 2.0 µl dNTPs, 2.0 µl *E. coli* DNA pol I (10 U/µl), 2.0 µl RNaseH, 0.5 µl *E. coli* DNA ligase and 7.5 µl ddH_2O. The final reaction mixture of 60 µl was incubated for 2 h at 16°C. After the reaction, the second strand cDNA reaction was subjected to purification with a MinElute Reaction Cleanup kit (Qiagen). The purified second stranded cDNA mixture was diluted to 100 ng/µl.

RT-PCR analysis

Ovary RNA samples from Acala 1517-99 and SG 747 were used to perform RT-PCR using 25 cycles. Five pairs of primers were designed from the following five genes: GhTublin (GenBank accession: AF009565), forward primer-5'CATGGCTTGYTGTTTGATGTAYCG3', reverse primer-5'CTCACGAGCCTCAGAGAAYTCTCC(Y=C/T)3'; GhV-ATPase (AF009568), forward primer- 5'GAAACTGCTAAACTTTTAAG3', reverse primer- 5'CACCTAGTTTCATCCTCC3'; GhTUA4 (AF106570), forward primer- 5'GGCCACCATCAAGACCA3', reverse primer- 5'CGAGGTATTCAACCCTGA3'; GhTUA1 (AF106567), forward primer- 5'TGCCACCATCAAGACCA3', reverse primer- 5'AGCCGAACTACTCGTCA3'; GhSus1 (U73588), forward primer- 5'CTGGGATAAGATCTCCCAG3', reverse primer-5'AAGTCACCATATTTAACTGG3'.

cDNA-AFLP analysis

64 individual lines from a backcross inbred line (BIL) population were selected for genome-wide transcript profiling, based on cDNA-AFLP. For each sample, 500 ng of purified double stranded cDNAs were used for the detection of cDNA-AFLP (Vos et al., 1995). The adapters for restriction enzymes *Eco*RI/*Mse*I, ligase and primers used in the PCR for pre-selective and selective amplifications followed Vos et al. (1995), with minor modifications (Lu et al., 2008). After selective amplification, the PCR reactions were separated on 5% non-denatured polyacrylamide gel using a Sequi-Gen GT Nucleic Acid Electrophoresis Cell (Bio-Rad) followed by silver staining (SILVER SEQUENCE™ Sequencing System, Promega).

RESULTS AND DISCUSSION

RNA yield

Compared with the grinding method reported by Wu et al. (2002), we found that, collecting and grinding cotton tissues for all plant samples in liquid nitrogen in one unit of time was more convenient for the downstream work of RNA. A portion of the ground tissue powder can be used for RNA isolation, while the remaining powder can be stored for future use in RNA isolation if needed and/or for other purposes such as DNA and/or protein extraction. However, Wu et al. (2002) reported that both grinding tissues in liquid nitrogen and grinding preserved tissues in RNALater with extraction buffer, produced similar quality RNA, but the RNA yield from the latter (1,000 mg/gfw-) was two times higher. Table 1 shows the total RNA yield for 15 samples from the three cultivars at five different stages of boll development. The total RNA yields from different samples were highly consistent, ranging from 440 to 770 µg/gfw ground ovary tissues and averaged as 608 µg/gfw; 50% higher than that reported by Wu et al. (2002) using the same grinding method. An exception was Acala 1517-99 at -1 dpa, where considerable complex carbohydrate co-precipitated with total RNA due to unknown reasons, resulting in very low RNA concentration (77.5 µg/gfw).

Table 1. Total RNA yield (µg/gfw) for different genotypes and different ovary development stages extracted using the mini-scale method.

Genotype	-1 dpa	0 dpa	5 dpa	8 dpa	10 dpa	Mean	Std
1517-99							
RNA yield (mg/gfw)	78	705	740	463	440	485	266
A260/A230	nt	2.22	nt	2.04	nt	2.13	0.13
Phy 76							
RNA yield (mg/gfw)	505	770	888	670	565	680	154
A260/A230	nt	1.82	1.72	nt	nt	1.77	0.07
SG 747							
RNA yield (mg/gfw)	720	710	753	668	455	661	119
A260/A230	nt	2.22	2.17	2.05	2.08	2.13	0.08

gfw, grams fresh weight-, 200 mg per tissue sample; dpa: days post-anthesis; Std: standard deviation; A260/A230, ratio of spectrometer readings under wavelength 260 and 230 nm, respectively; nt, not tested.

Table 2. RNA quality and quantity from ovule tissues extracted using the large scale method (Wan and Wilkins, 1994).

Parameter	Pima S-1(0 dpa)	57-4(0 dpa)	Pima S-1(1 dpa)	57-4(1 dpa)	Mean	Std
Tissue weight (g)	2.05	2.08	2.10	1.80		
RNA yield (mg/gfw)	1010	1050	800	620	870	200
A260/A230	2.13	2.14	2.23	2.21	2.18	0.05

A260/A230, ratio of spectrometer readings under wavelength 260 and 230 nm, respectively; gfw, grams fresh weight; dpa, days post-anthesis; Std, standard deviation, respectively.

Table 3. RNA quality and quantity from anther tissues (-1 dpa) extracted using the large scale method (Wan and Wilkins, 1994).

Parameter	Pima S-1	57-4	8518	8518R	D8R	Mean	Std
Tissue weight (g)	4.70	3.70	2.70	4.20	4.20		
RNA yield (mg/gfw)	410	380	200	300	360	330	80
A260/A230	2.19	2.21	2.28	2.24	2.22	2.23	0.03

gfw, grams fresh weight; dpa: days post-anthesis; Std, standard deviation; A260/A230, ratio of spectrometer readings under wavelength 260 and 230 nm, respectively.

As a comparison, the results obtained from the standard hot borate method (Wan and Wilkins, 1994; Wilkins and Smart, 1996) are shown in Tables 2, 3 and 4. The RNA yield from the ovule tissues was highest among the three tissues ranging from 600 to 1,000 µg/gfw with an average of 870 mg µg/gfw (Table 2). Young leaf and anther tissues produced much lower but roughly similar RNA yields (330 vs. 350 µg/gfw). The range for anther tissue was from 200 to 400 µg/gfw (Table 3), while the range for the leaf tissue was 100 to 450 µg/gfw (Table 4). It should be pointed out that, when the standard large scale hot borate method was developed (Wan and Wilkins, 1994; Wilkins and Smart, 1996), the highest RNA yields (1200 to 1500 µg/gfw) from cotton anthers and high RNA yields (600 to1200 µg/gfw) from leaves, petals, ovlules and embyos were reported, while root tissues produced lower RNA yields (300 to 600 µg/gfw). However, using the same method but with small amount of tissues (200 to 400 mg) from leaves, cotyledons and ovules, lower RNA yields (82 to 500 µg/gfw) were obtained (www.eeob.iastate.edu/faculty/WendelJ/rnaextraction.htm). It appears that cotton total RNA yields differed in tissues, samples and laboraotories/persons.

Table 4. RNA quality and quantity from young leaf tissues extracted using the large scale method (Wan and Wilkins, 1994).

Parameter	Pima S-1	57-4	F_1	A_1	D_5	Mean	Std
Tissue weight (g)	4.00	4.00	4.00	4.00	4.00		
RNA yield (mg/gfw)	340	360	460	120	460	350	140
A260/A230	1.91	1.84	1.97	0.86	2.01	1.72	0.48

gfw: Grams fresh weight; dpa: days post-anthesis; Std: standard deviation; A260/A230: ratio of spectrometer readings under wavelength 260 and 230 nm, respectively.

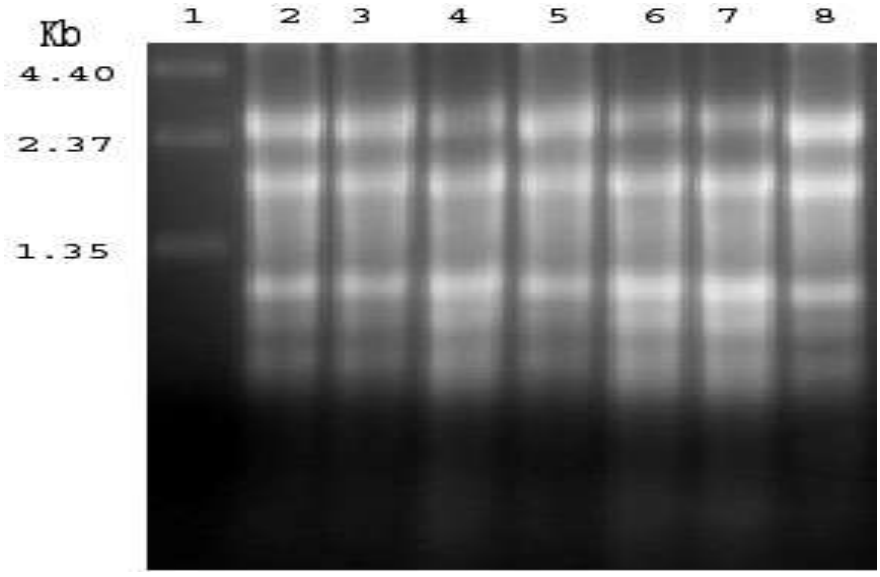

Figure. 1. Electrophoretic analysis of total RNA isolated from developing ovaries at 10 dpa separated on a formaldehyde denaturing agarose gel (1.2%) stained with ethidium bromide. Lane 1, 0.24 to 9.5 kb RNA ladder (GIBCO); lane 2, 1519-99 0 dpa ; lane 3, 1517-99 5 dpa; lane 4, 1517-99 10 dpa; lane 5, Phy76 8 dpa; lane 6, Phy76 10 dpa; lane 7, SG 747 8 dpa; lane 8, SG747 10 dpa). 28S and 18S RNA, together with other RNAs of smaller molecular weights were visible.

RNA quality

Absorption at 230 nm reflects contaminations from carbohydrates, peptides, phenols, or other aromatic compounds. The ratio of A260/A230 should be 2.0 or above for pure RNA samples. However, the ratio in cotton RNA samples usually ranged from 1.7 to 1.9 (Wilkins and Smart, 1996). Using the large scale method, the five leaf tissue samples (Pima S-1, 57-4 and their F_1 hybrid, A_1, and D_5) gave the lowest ratio with an average of 1.72 (ranging from 1.84 to 2.01 except for one sample with a ratio of 0.86) and standard deviation (STD) of 0.48 because of higher concentration of pigments, gossypol and other polypheonol compounds. However, the ovule (average: 2.18 and std: 0.05) and anther tissues (average: 2.23 and std: 0.03) showed a higher ratio (Table 2 and 3), using the same large scale RNA extraction method (Wan and Wilkins, 1994; Wilkins and Smart, 1996). The ratio for the anther tissue (Loper, 1986) was slightly but insignificantly higher than the ovule tissue. Using the modified mini-prep hot borate method, it appears that developing bolls (ovaries) from Pima (*G. barbadense*), Phy 76 yielded total RNA with lower but acceptable A260/A230 ratios (average: 1.77 and std: 0.07) due to higher gossypol content in this species (Lee 1973), while RNA from the two upland cotton (*G. hirsutum*) genotypes (Acala 1517-99 and SG 747) had similar A260/A230 ratios (average: 2.13) and standard deviations (0.08 to 0.13) (Table 1). Thus, the improved quick RNA extraction method gave high and acceptable RNA quality regardless of cotton tissues, similar to the large scale hot borate method. As shown in Figure 1, in addition to the two major bands of ribosomal RNA (28S and 18S),

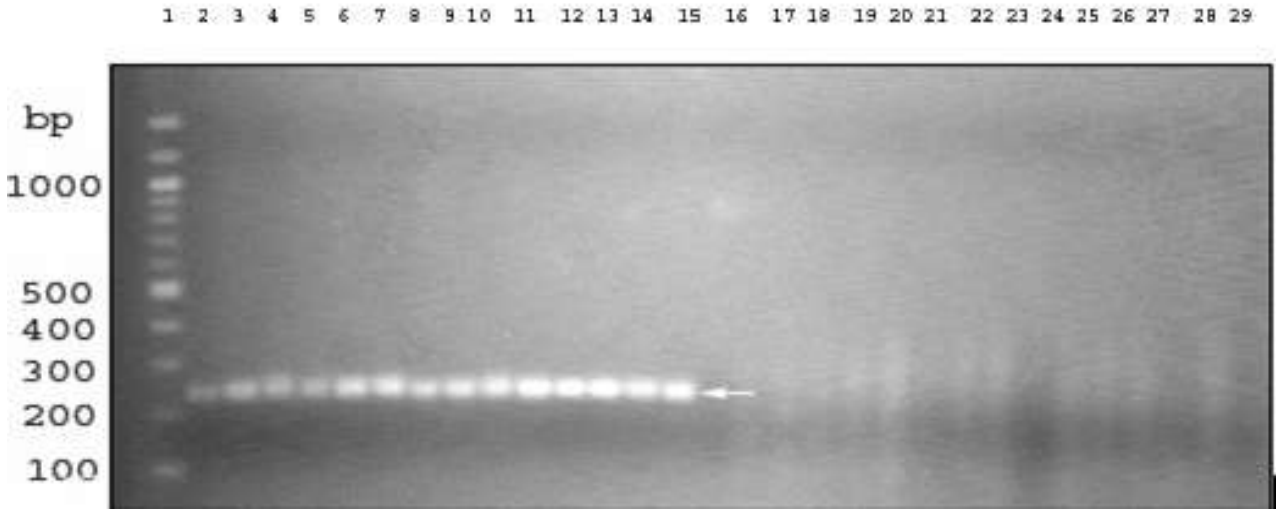

Figure 2. Electrophoretic analysis of RT-PCR products amplified from first strand cDNA and purified total RNA as templates. The primers (forward primer, 5'AGAGGTCGAGTCTTCGGACA 3' and reverse primer, 5'GCTTGATCTTCTTGGGCTTG 3') used were designed from histone-3 gene sequence. Lane 1, DNA ladder; lanes 2 to 15, PCR products (221 bp in size) amplified from first strand cDNA made from 14 ovary RNA samples (in the following order: 0, 5, 8 and 10 dpa ovaries from Acala 1517-99; -1, 0, 5, 8 and 10 dpa ovaries from SG 747 and -1, 0, 5, 8 and 10 dpa ovaries from Pima Phy 76); lanes 16 to 29, PCR results amplified from the same RNA samples (in the same order as above), indicating no genomic contaminations in the purified total RNA.

other RNAs of smaller molecular weights were also distinguishable. We also used the total RNA extracted by this method to successfully clone microRNA (Pang, 2009; Pang et al., 2011). However, most commercial kits for total RNA extraction would not allow for isolation of RNA of small size (below 200bp). Wu et al. (2002) reported that, adding proteinase K during homogenization process reduced RNA degradation and increased RNA yield. Our results are consistent with their findings. We routinely used the improved method in isolating RNA from other cotton tissues such as leaves and anthers and the results were always satisfactory. However, Wu et al. (2002) encountered difficulties in isolating high quality RNA from 7 dpa fibers, using their method due to high content of polysaccharides in the fiber samples. To remove contaminants including polysaccharides from nucleic acids, a cetyltrimethyl ammonium bromide (CTAB)-based cleanup procedure can be implemented as proposed by Zhang and Stewart (2000). The cleanup procedure was also successful in cleaning cotton RNA (Zhang, unpublished), without the need of using commercial columns. If this mini-prep CTAB-based DNA/RNA cleanup procedure is adopted for cotton RNA extraction, at least 100 samples can be processed in two days. Our experience indicates that when young and small tissues are used for DNA isolation, grinding in liquid nitrogen is unnecessary when beads are used for in-tube maceration by high speed shaking. This procedure may be applied to RNA extraction, thus saving time in grinding samples using liquid nitrogen.

RT-PCR, cDNA-AFLP and other molecular analysis

For the fact that, the concentration of total RNA from -1 dpa ovaries of Acala 1517-99 was low, only 14 samples were used in RT-PCR. As shown in Figure 2, the 14 samples (far left) are RT-PCR products amplified by histone-3 primers. The negative controls (14 samples to the right in Figure 2) had the same PCR conditions but the RNA samples were not subjected to reverse transcription before PCR. To further confirm the RT-PCR results, five cloned genes implicated in fiber development were selected. Figure 3, shows the differential expressions of the five genes at different ovary development stages in the two cultivars tested. The RNA was also further used for quantitative RT-PCR analysis and showed satisfactory results (Pang 2009; Pang et al., 2011; Rodriguez-Uribe et al., 2009, 2011; Curtiss 2010; Curtiss et al., 2011).

Figure 4, shows a part of a polyacrylamide gel, for a set of cDNA–AFLP. The amplification bands were clear and distinguishable. Using ovary total RNA from Pima Phy 76, SG 747 and Acala 1517-99 extracted by the modified method, cDNA libraries were constructed based on AFLP analysis for comparative gene sequence analysis and a

Figure 3. Electrophoretic analysis of RT-PCR products amplified for primers designed for genes. Histone-3 was used as an internal standard. The ovary samples were Pima Phy 76 (lanes 1 to 4) and SG 747 at 0, 4, 8 and 10 dpa (lanes 5 to 8)

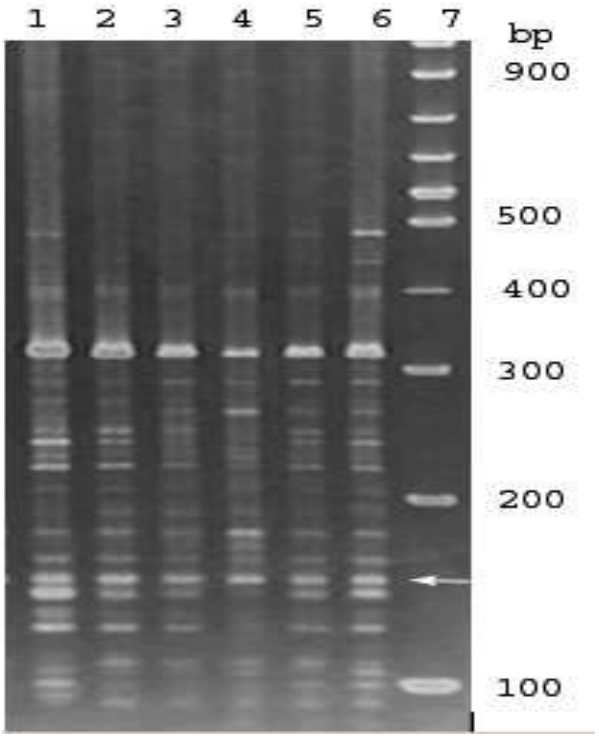

Figure 4. A partial gel image of cDNA-AFLP products separated on polyacrylamide gel electrophoresis. Lanes 1 to 6, cDNA-AFLP from 10 BIL lines; Lane 7, 100bp DNA ladder.

number of single nucleotide polymorphisms (SNP) were also detected (Zhang et al., 2007). Using ovary RNA extracted by the mini-scale hot borate method, we performed genome-wide gene expression studies on 64 backcross inbred lines, developed from a cross between Upland cotton and Pima cotton and more than 140 qualitative expression markers were identified, cloned and sequenced (Pang, 2009). The RNA isolated was of

high quality and was also successfully used for other downstream RNA work including cDNA pyrosequencing using 454 Life Sciences sequencing, cDNA microarray and microRNA isolation and cloning (Curtiss et al., 2011; Pang, 2009; Rodriguez-Uribe et al., 2009, 2011; Suzuki et al., 2010). The results suggested that the protocols were consistent from purification of total RNA, first strand cDNA synthesis, to second strand cDNA synthesis and library construction.

It should be pointed out that the standard macro-prep hot borate method (Wan and Wilkins, 1994; Wilkins and Smart, 1996) has been widely adopted for RNA extraction from cotton and many other plant species including our work when only a few tissue samples are involved (Zhang et al., 2008). It usually takes three working days to isolate RNA from eight tissue samples. When we were later faced with a number of genotypes in genetic genomics research which normally does not require a large amount of RNA but more tissue and stage specific, the hot borate method was scaled down and modified to accommodate the need. Several changes are made in this mini-prep method including, (1) always use 1.5 ml tubes and do not use 50 ml Oakridge tubes; (2) do not use a homogenizer but beads in 1.5 ml tubes for homogenization; (3) omit a filtering step using miracloth; (4) reduce steps and time in the procedure. These modifications make the RNA extraction more convenient, easier and faster. Since the modifications were made for the purposes of convenience and time-saving and no other chemicals were added or eliminated, it is not surprising that the mini-prep method, produced similar RNA yield and quality to the large scale method, as expected. Due to this reason, no strict side-by-side comparison using same tissues but differing in extraction methods was performed in this study. The mini-prep hot borate method described here has been used in our laboratory to extract RNA from other cotton tissues such as leaves, anthers and flowers, and has proven to be an efficient, fast, simple and reliable protocol. This mini-prep hot borate method is also applicable to other recalcitrant plants rich in polysaccharides and polyphenols, such as cotton and especially suitable for laboratories where RNA extraction kits are not accessible in many developing countries.

ACKNOWLEDGMENT

The work was supported by the New Mexico Agricultural Experiment Station.

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