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Modified method for combined DNA and RNA isolation from peanut and other oil seeds

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Abstract Isolation of good quality RNA and DNA from seeds is difficult due to high levels of polysaccharides, polyphenols, and lipids that can degrade or co-precipitate with nucleic acids. Standard RNA extraction methods utilizing guanidinium–phenol–chloroform extraction has not shown to be successful. RNA isolation from plant seeds is a prerequisite for many seed specific gene expression studies and DNA is necessary in marker-assisted selection and other genetic studies. We describe a modified method to isolate both RNA and DNA from the same seed tissue and have been successful with several oil seeds including peanut, soybean, sunflower, canola, and oil radish. An additional LiCl precipitation step was added to isolate both RNA and DNA from the same seed tissues. High quality nucleic acids were observed based on A_{260}/A_{280} and A_{260}/A_{230} ratios above 2.0 and distinct bands on gel-electrophoresis. RNA was shown to be suitable for reverse transcriptase polymerase chain reaction based on actin or 60S ribosomal primer amplification and DNA was shown to have a single band on gel-electrophoresis analysis. This result shows that RNA and DNA isolated using this method can be appropriate for molecular studies in peanut and other oil containing seeds.

Keywords RNA · DNA · Isolation · Peanut · Oil seeds

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Abbreviations

PCR	Polymerase chain reaction
RT	Reverse transcriptase
RFLP	Restriction fragment length polymorphism
SSR	Simple sequence repeat
SNP	Single nucleotide polymorphism
MAS	Marker-assisted selection

Introduction

Peanut (*Arachis hypogaea* L.), belonging to legume family, is grown as an annual predominantly in Southeast US and in different parts of the world. Cultivated peanuts are an important oil seed crop worldwide because oil contains 50 % of dry seed weight [6]. Seeds often contain high levels of polyphenols, polysaccharides, and lipids which can bind to nucleic acids resulting in low yield and quality that is not suitable for many molecular studies [2]. Many seed specific molecular studies including reverse transcriptase polymerase chain reaction (RT-PCR), quantitative PCR, and cDNA library construction require the isolation of high quality and sufficient quantity of RNA. A good quality genomic DNA is required for the application of marker-assisted selection (MAS) methods in a plant breeding program, in which the markers could be restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR), and single nucleotide polymorphism (SNP). The isolation of RNA and DNA from the same seed tissue enables the correlation between seed gene-expression and specific genomic DNA sequences. Standard RNA extraction methods utilizing guanidinium salts or Trizol solution have not been successful [7]. The combination of protein, polyphenol, polysaccharide, and

lipid levels will influence the relative yield and quality of nucleic acids isolation. This emphasizes the need to develop new or optimize existing method for nucleic acid isolation for a specific organism, stage of development, or different tissues under specific environmental stress or a disease pressure.

There are several advantages performing DNA extraction on seeds instead of leaf tissue. The DNA extraction from seeds can be conducted during the off-growth season, which will allow MAS to be carried out in more flexible manner to determine the desirable genotype selected before the next breeding cycle. The seeds are a more dependable source for DNA extraction compared to leaf tissue in which the plants must be growing in either field or greenhouse.

The objectives of this research are to modify an existing RNA isolation procedure published by Li and Trick [7] to obtain high quality DNA and RNA from dry peanut seeds and to apply this modified method to extract high quality nucleic acids from other oil seeds such as soybean (*Glycine max*), sunflower (*Helianthus annuus*), canola (*Brassica napus* L.), and oil-seed radish (*Raphanus sativus*).

Materials and methods

Plant materials

Matured dry peanut seeds of 14 genotypes: 'A-104', 'AP-4', 'AT-215', 'AT3085RO', 'C76-16', 'Exp27-1516', 'Exp3-1114', 'Florida-07', 'Flavor Runner 458', 'Georgia Green', 'Georgia-03L', 'Tifrunner', 'VC-2', and 'York' were utilized in this study. Among these genotypes, 'Exp3-1114' and 'VC-2' are Virginia-Type peanut with large seed size; the rest of genotypes are Runner-Type peanut. Oil seeds of soybean ('William82'), sunflower ('Hybrid 894'), canola ('Flint') and common oil-seed radish were also utilized for DNA and RNA extractions.

RNA and DNA isolation

Approximately 1–1.2 g of dried seed of each genotype was utilized in extraction. Seeds were frozen with liquid nitrogen and pulverized by grinding with mortar and pestle until a fine powder. Tissue was placed into 10 mL solution I (100 mM Tris, 150 mM LiCl, 50 mM EDTA, 1.5 % SDS, 2 % PVP-40, 1.5 % β-mercaptoethanol) buffer, pH 7.5, and mixed thoroughly by vortexing or inversion. To the homogenized tissue, a 5 mL acid phenol, pH 4.3 (Fisher Scientific, Pittsburg, PA) was added and tissue was mixed thorough for 5 min, followed by the addition of 5 mL chloroform:isoamyl alcohol (24:1) and mixed for an additional 5 min. The homogenate was centrifuged for 10 min and the upper aqueous layer (around 9 mL) was transferred to a fresh 40 mL tube containing 9 mL of solution II (2 M guanidinium thiocyanate, 0.5 M sodium citrate, 1.5 M ammonium acetate, 1 % *N*-lauroylsarcosine) buffer, pH 5.2, mixed by gentle inversion, and allowed to incubate for 10 min at room temperature. After incubation, a 4.5 mL phenol:chloroform:isoamyl alcohol (25:24:1), pH 6.7 was added, mixed thoroughly for 5 min, centrifuged for 10 min at 13,000×*g*, and the aqueous layer (around 16 mL) was transferred to a new 40 mL tube. To the transferred solution, 9 mL 1.2 M NaCl and 9 mL isopropanol were added, mixed, placed on ice for at least 1 h, and centrifuged for 15 min at 13,000×*g*. The liquid was discarded and the resulting pellet was washed with 2 mL cold 70 % ethanol, dried, and resuspended in 4 mL RNase-free water. A 4 mL 4 M LiCl was added, mixed, placed on ice in the refrigerator for at least 1 h, and centrifuged for 15 min at 4 °C at 13,000×*g*. The supernatant (around 8 mL) was transferred to a new 40 mL tube for DNA isolation and the pellet was retained for RNA isolation. For RNA isolation, the pellet was washed with 2 mL ice-cold 70 % ethanol, dried, and resuspended in 750 µL RNase-free water. For DNA isolation, 8 mL isopropanol was added to the transferred supernatant tube, mixed well, centrifuged for 5 min, discarded the liquid, washed pellet with 2 mL ice-cold 70 % ethanol, dried, and resulting pellet was resuspended in 500 µL 10 mM Tris, pH 7.5. The amount of DNA or RNA was determined using Nanodrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE).

Primer design

Primers were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA) using peanut (*A. hypogaea* L.) ESTs [4, 5]. Actin primers were utilized to analyze peanut RNA samples (Fwd: 5′-CACATGCCATCCTTCGATTG-3′ and Rev: 5′-CCAAGGCAACATATGCAAGCT-3′) to produce a 150 bp PCR product. Control 60S (L19) ribosomal primers were utilized for other oil seeds RNA samples (Fwd: 5′-AGAGGGAAGGTTTGGCTTGAC-3′ and Rev: 5′-CGGGAATTGGCCATGGA-3′) to produce a 60 bp PCR product.

RT and PCR amplification

The quality of RNA was evaluated by reverse transcription followed by PCR. To eliminate genomic DNA contamination prior to cDNA synthesis, total RNA was treated with TURBO Dnase (Ambion, Austin, TX) according manufacture's instructions. One microgram of RNA was annealed with 0.5 µg oligo dT primer (in 14 µL) by heating sample to 65 °C for 5 min and immediately cooled on ice for 2–5 min. cDNAs were produced using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) according to manufacture's instruction and 1 µL of 1:200

Table 1 Relative yield and purity of RNA and DNA isolated from dry peanut and other oil seeds

Species	RNA			DNA		
	Yield (μg)	$A_{260/280}$	$A_{260/230}$	Yield (μg)	$A_{260/280}$	$A_{260/230}$
Peanuts						
A-104	352	2.04	2.04	44	1.80	2.02
AP-4	360	2.03	2.05	20	1.84	2.07
AT-215	348	2.02	2.02	57	1.83	2.13
AT3085RO	351	2.06	2.13	67	1.83	2.30
C76-16	335	2.06	2.10	41	1.83	2.08
Exp27-1516	301	2.00	2.01	37	1.83	2.28
Exp3-1114	281	1.99	1.97	66	1.84	2.33
Florida-07	354	2.02	2.04	45	1.83	2.34
Flavor Runner 458	313	2.00	2.01	36	1.81	1.97
Georgia Green	365	2.01	2.07	33	1.83	2.16
Georgia-03L	343	2.02	2.00	31	1.83	1.99
Tifrunner	321	2.01	1.95	67	1.83	2.29
VC-2	326	2.00	2.05	34	1.85	2.25
York	352	2.03	2.00	41	1.78	1.90
Mean	336	2.02	2.03	44	1.83	2.15
STDEV	24.7	0.022	0.05	14.7	0.02	0.15
Other oil seeds						
Soybean	357	2.06	2.17	121	1.89	1.78
Sunflower	365	2.08	2.17	81	1.84	1.87
Canola	338	2.07	2.17	64	1.84	1.71
Oil-seed radish	304	2.06	2.06	58	1.86	1.53

dilution of cDNA was used as template in PCR reaction. PCR reaction was performed in a 20 μL reaction:1 μL diluted cDNA, 10 μL Green GoTaq enzyme (Promega, Madison, WI), 1 μL Fwd primer (10 μM), 1 μL Rev primer (10 μM), and 7 μL sterile water. PCR conditions were applied: 94 °C for 2 min, 30 cycles of 45 s at 94, 50 °C for 45 s, 72 °C for 1 min, and 1 cycle at 72 °C for 10 min. PCR products and 100 bp DNA molecular weight marker (Promega) were separated on a 1 % TAE-agarose gel and image was captured on a Gel Logic 200 Imaging System (Kodak, Rochester, NY).

Identification of FAD2A alleles using real-time PCR

Selection of peanut genotypes with high oleic trait has been demonstrated by incorporating probe/primer TaqMan real-time assay to identify wild-type SNP (G) and/or mutant (A) at position 448 (448 G>A) [1]. A reaction volume (20 μL) with 10 ng genomic DNA was utilized as template for each sample. Three independent samples were performed for each peanut genotype. Primers and PCR conditions were as described by Barkley [1] with the exception that Cy5 fluorescent label was substituted for VIC in (G) SNP probe. Real-time PCR was performed in an ABI 7500 real-time PCR machine (Applied Biosystems) utilizing Allelic Discrimination module. Data was analyzed and compared for fold changes between wild-type and mutant allele.

Results

RNA and DNA yield

The modified RNA isolation method for dry seed consistently yielded on average 336 μg total RNA and 44 μg genomic DNA per gram for peanut (Table 1), 357 and 121 μg for soybean, 365 and 81 μg for sunflower; 338 and 64 μg for canola, and 304 and 58 μg for oil-seed radish (Table 1). High purity, based on low level of protein and polysaccharide contamination, was indicated by A_{260}/A_{280} ratios ranging from 1.99 to 2.06 and A_{260}/A_{230} ratios ranging from 1.95 to 2.13 for peanut RNA; 2.06 to 2.08 and 2.08 to 2.17 for soybean, sunflower, canola, and oil-seed radish. For DNA, A_{260}/A_{280} and A_{260}/A_{230} ratios range from 1.78 to 1.85 and 1.90 to 2.33 for peanut; 1.84 to 1.89 and 1.53 to 1.87 for soybean, sunflower, canola, and oil-seed radish.

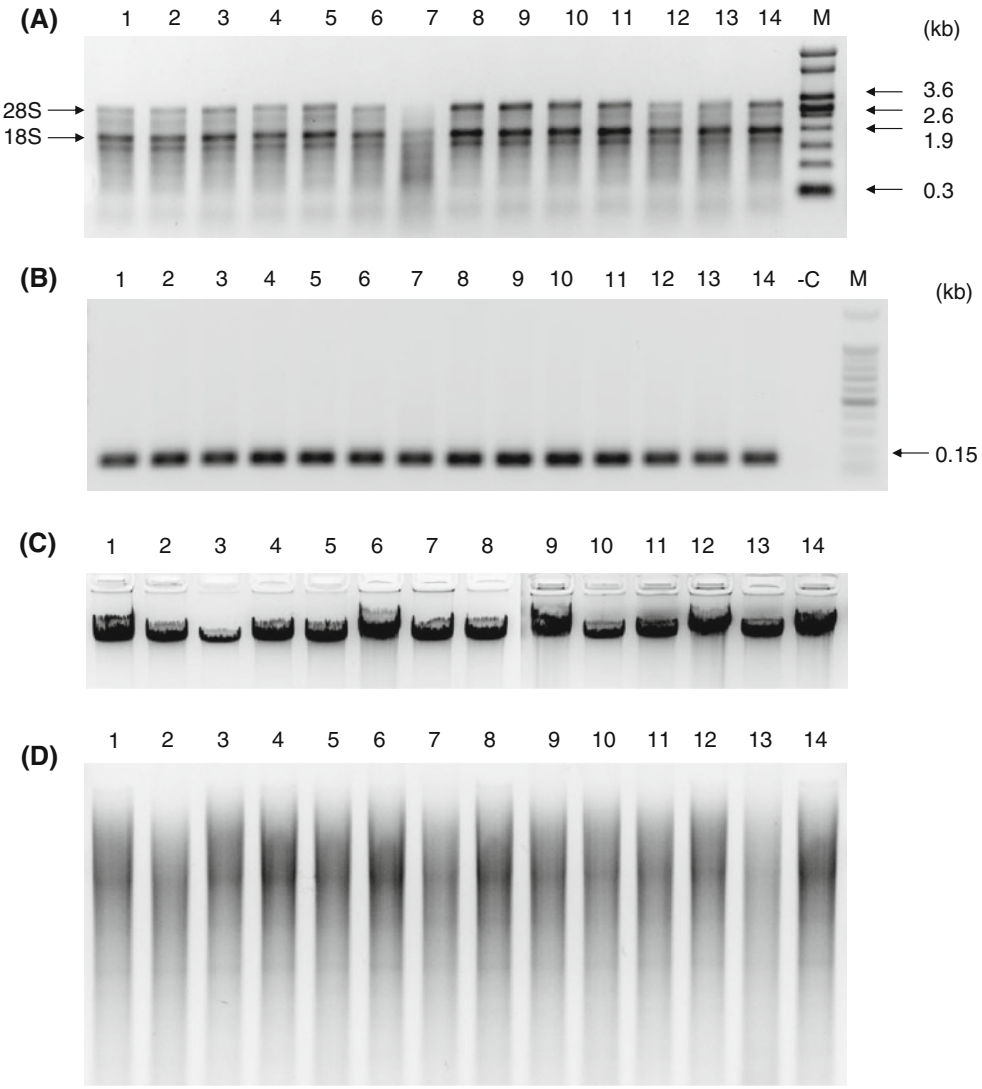

Fig. 1 Analysis of different peanut varieties: (*1*) A-104, (*2*) AP-4, (*3*) AT-215, (*4*) AT3085RO, (*5*) C76-16, (*6*) Exp27-1516, (*7*) Exp3-1114, (*8*) Florida-07, (*9*) Flavor Runner 458, (*10*) Georgia Green (GG), (*11*) Georgia-03L, (*12*) Tifrunner, (*13*) VC-2, and (*14*) York. **a** RNA gel-electrophoresis of peanut varieties (*lanes 1–14*) and RNA molecular weight marker (*M*). **b** RT-PCR analysis of actin in different peanut varieties, no-RT control (*-C*), and 100 bp DNA molecular weight marker (*M*). Gel-electrophoresis analysis of un-digested (**c**) and *Eco*RI digested (**d**) genomic DNA of peanut varieties

Gel-electrophoresis analysis

When total RNA was separated on a 1.2 % denaturing agarose gel stained with ethidium bromide, distinct 28S and 18S ribosomal bands were present for peanut (Fig. 1) as well as for soybean, sunflower, canola, and oil-seed radish (Fig. 2) indicating samples were free of RNases. When uncut and *Eco*RI digested genomic DNA were separated on 0.8 % standard agarose gel, distinct high molecular weight bands were observed for peanut (Fig. 1), soybean, sunflower, canola, and oil-seed radish (Fig. 2) indicating little or no contaminants.

RT-PCR analysis

When peanut RNA was reverse transcribed and amplified with actin specific primers, expected molecular weight band was observed for all peanut samples while no-RT control sample did not produce any PCR band. Soybean, sunflower, canola, and oil-seed radish RNAs were also reverse transcribed and amplified with 60S ribosomal specific primers. Expected molecular weight PCR product was observed for all samples except for canola and no-RT samples which did not produce the PCR band.

Real-time PCR of FAD2A gene in peanut

When genomic DNA from 14 peanut genotypes were utilized as template in real-time PCR analysis, 3 were verified as high oleic to linoleic (O/L) ratio peanut (AT3085RO, Florida-07 and VC-2) with the presence of only the mutant (A) allele (Table 2). Normal O/L peanuts were also verified (AP-4, C76-16, Exp27-1516, Exp3-1114, Flavor Runner 458, Georgia-03L and Tifrunner) with the presence of both A and G allele. York is a high O/L exception with the presence of both alleles; however, the ratio of A to G is

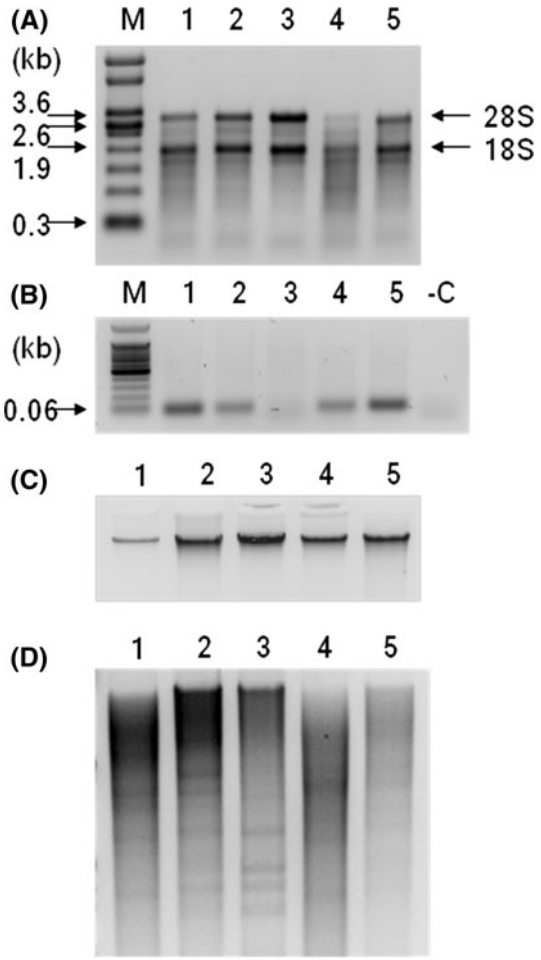

Fig. 2 Analysis of different oil seeds: (*1*) soybean, (*2*) sunflower, (*3*) canola, (*4*) oil-seed radish, and (*5*) peanut (GG). **a** RNA gel-electrophoresis of a molecular weight marker (*M*) and oil seeds (*lanes 1–5*). **b** RT-PCR analysis of a 60S ribosomal subunit in oil seeds (*lanes 1–5*) and no-RT control (*-C*). Gel-electrophoresis analysis of un-digested (**c**) and *Eco*RI digested (**d**) genomic DNA of oil seeds

significantly higher than the other normal O/L peanuts. A-104 O/L is unknown; however, it is predicted to have normal to low O/L since only G allele is present.

Discussion

Attempts at isolating RNA from peanut dry seeds using standard guanidinium–phenol–chloroform extraction methods resulted in low yield and quality not suitable for molecular studies. To optimize RNA isolation from dry peanut seeds, a method developed by Li and Trick [7] was modified to obtain high quality and yield of both DNA and RNA from the same tissue. We have also successful isolated RNA and DNA from mature peanut seed parts, embryos and cotyledons, separately. RNA isolated from embryos only should be enriched for transcripts that highly

Table 2 Real-time PCR results of FAD2A gene in peanut

Genotype	Allele		O/L ratio
	A	G	
A-104	−−−	+++	Unknown
AP-4	7.78 ± 0.419	1	Normal
AT-215	9.17 ± 1.918	1	Normal
AT3085RO	+++	−−−	High
C76-16	12.21 ± 2.464	1	Normal
Exp27-1516	16.64 ± 4.970	1	Normal
Exp3-1114	18.57 ± 2.233	1	Normal
Florida-07	+++	−−−	High
Flavor Runner 458	11.34 ± 1.515	1	Normal
Georgia-03L	11.00 ± 0.784	1	Normal
Georgia Green	11.88 ± 1.422	1	Normal
Tifrunner	9.48 ± 0.539	1	Normal
VC-2	+++	−−−	High
York	116.45 ± 7.088	1	High

Quantitative fold changes representing gene dosage from G to A allele. Samples with either A or G allele only is represented by +++

expressed in that tissue. This method was also applied to other seed crops such as soybean, sunflower, canola, and oil-seed radish. The original procedure by Li and Trick [7] was developed to isolate RNA from starch filled seeds such as wheat, rice, and maize using a small amount of tissue (50–100 mg) while this modification allows a larger amount of tissues (1–1.2 g) to be processed from dry seeds. Seeds, especially oil seeds, often contain proteins, polysaccharides, polyphenols and high level of lipids that can degrade or co-precipitate with nucleic acids and render DNA or RNA not suitable for many molecular studies. In the modified method, 2 % PVP-40 was added as a strong polyphenolic compound binding agent to minimize nucleic acid degradation [8]. Solution II was changed to include 2 M guanidinium thiocyanate (a strong denaturant) instead of 6.5 M guanidinium sulfate (a mild stabilizer) [3] to maximize cell disruption. Similar to the original method, proteins and cell debris are removed by phenol–chloroform extraction and centrifugation. Other significant changes to solution II include the addition of 1.5 M ammonium acetate, pH 5.2 to replace 2 M sodium acetate to facilitate complete removal of residual salt after a 70 % ethanol wash of DNA or RNA pellet and a reduction from 10 to 1 % lauroylsarcosine since this was observed to be sufficient for complete protein solubilization. This modified procedure also included a LiCl step which precipitate RNA as a pellet, leaving DNA in the supernatant which can subsequently precipitated by the addition of isopropanol.

Using this modified method, high quality DNA and RNA were obtain based on high A_{260}/A_{280} and A_{260}/A_{230} ratios with good yield for peanut and other oil seeds

(Table 1). Undigested and *Eco*RI digested genomic DNA showed high quality based on gel analysis for peanut (Fig. 1c, d) and other oil seeds (Fig. 2c, d). RNA from peanut (Fig. 1a) and other oil seeds (Fig. 2a) also showed distinct 28S and 18S ribosomal bands, indicating no RNase contamination, and RNA was suitable for RT-PCR analysis (Figs. 1b, 2b).

This method was optimized by addition of different protectants to maintain nucleic acid integrity as well as maximizing yield. An extra LiCl precipitation step was added to isolation high quality DNA from the same tissue. This method was also successful to other oil seeds such as soybean (*G. max*), sunflower (*H. annuus*), canola (*B. napus* L.), and oil-seed radish (*R. sativus*).

Downstream application such as the identification of high O/L ratio peanut utilizing real-time PCR showed that the isolated genomic DNAs were of high quality. Samples showed consistent result allowing the relative gene dosage quantitation between A and G alleles (Table 2).

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