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Gene Expression to Genetical Genomics

Hexadecyltrimethylammonium Bromide (CTAB)-based Protocol to Isolate High-Quality RNA in Adequate Quantities for Gene Expression Analyses in Developing Seeds of Lentils (*Lens culinaris* Medik.)

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ABSTRACT: RNA isolation from polysaccharide- and phenolics-rich plant tissues such as developing lentil (*Lens culinaris* Medik.) seeds is challenging. High-quality RNA is needed in adequate quantities for transcriptome analysis to study seed quality traits. To date, a suitable method to isolate high-quality and -quantity RNA from lentil seeds has not been reported. The objective of this study was to develop a simple and reproducible method to isolate high-quantity and -quality RNA from developing lentil seeds for gene expression analysis. Methods based on Trizol™ reagents and phenol:guanidine gave low yields of RNA. A method based on hexadecyltrimethylammonium bromide followed by a lithium chloride precipitation yielded RNA in high quantity (210–260 µg from 200 mg of seeds) and quality ($A_{260/280}$ ratio of about 2.2). Isolated RNA was used to study the expression of the *granule-bound starch synthase I* (*GbssI*) during lentil seed development by RNA gel blot and quantitative real-time polymerase chain reaction. The expression pattern of the *GbssI* was similar to that reported for pea *GbssI* gene.

KEYWORDS: RNA isolation protocol, developing seeds, lentils, CTAB, gene expression

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Introduction

Lentil (*Lens culinaris* Medik.) is a cool season legume crop producing seeds with high nutritional value, widely consumed in the Mediterranean and developing countries. Lentil seeds have high concentration of protein rich in essential amino acids like lysine and threonine which are deficient in cereals. Lentil seeds are also a good source of carbohydrates, minerals, and micronutrients. Carbohydrates are the major storage components in lentil seeds and contribute to their beneficial effects on human health.[1] In recent years, there is an increase in global demand for lentils with desirable seed quality. Thus, seed quality improvement has become a major focus of research to reduce some of the anti-nutritional constituents such as raffinose family oligosaccharides (RFO) and tannins. Although RFO cause stomach discomfort,[2] tannins bind mineral ions thereby reducing their bio-availability to humans.[3]

High-throughput transcriptomic studies involving deep sequencing have contributed valuable genomic resources for lentil.[4] RNA extracted from mixed tissues which included young and mature leaf tissues, stem, flowers, and immature and mature pods was used to construct a cDNA library for transcriptome sequencing, which identified over 84,000 unigenes and enabled validation of sets of eSSR primer pairs for polymorphisms among cultivated genotypes as well as among non-domesticated genotypes.[4] This transcriptomic resource set,[4] while valuable, is challenging to mine for seed-specific transcripts, especially low-abundant transcripts.

For large-scale transcriptome profiling for lentil seed quality traits, it is imperative to establish high-quality RNA isolation procedures from various seed developmental stages. To date, high-quality and -quantity RNA isolation methods from lentil seeds have not been reported. Although this requirement may seem trivial due to the availability of numerous RNA isolation protocols from a number of different species, several of the published protocols could not be used to extract high-quality RNA in adequate quantities from developing seeds of lentil. Most RNA isolation methods reported in the literature employ commercial kits, which yield good-quality RNA with low quantities adequate for applications such as quantitative real-time polymerase chain reaction (QPCR) and high-throughput transcriptome analyses such as microarrays. For cereal and leguminous seeds rich in polysaccharides, commercial kits with minor modifications have been successful to isolate RNA. However, a major limitation of commercial kits is insufficient amounts of RNA for applications such as RNA gel blot analyses. In addition, resource limitations for many laboratories may prevent purchase of these commercial kits for routine use.

A number of RNA isolation methods, both conventional hot acidic phenol:guanidine[5] or formulation thereof like Trizol™ reagent[6,7] have been used for starch-rich seed tissues. The conventional methods are cost-effective and yield higher amounts of RNA than commercial kits, although down-stream applications can sometimes be inhibited due to carry-over of isolation reagents such as phenol, guanidine, and chloroform. The objective of this study was to develop a simple, reproducible, high-yielding and high-quality RNA isolation protocol from developing seeds of lentil. Published methods from our laboratory for developing wheat grains based on hot phenol:guanidine[5] and Trizol[6] followed by a column cleanup were not applicable to developing lentil seeds. Hexadecyltrimethylammonium bromide (CTAB) method, generally used for DNA isolation,[8,9] followed by a lithium chloride RNA precipitation step was developed to isolate high-quality RNA in adequate quantities from developing lentil seeds. The optimized protocol also included polyvinylpyrolidone (PVP), which effectively removed polyphenolic compounds during nucleic acid isolation.[8,9] CTAB-based RNA isolation methods have been used for a number of plant tissues, including polysaccharide-rich loquat,[10] lipid-rich seeds,[11] and polyphenols- and polysaccharide-rich mangrove plants.[12] However, with any new study, methods need to be re-evaluated prior to selecting the most efficient method.

Materials and Methods

Plant material and RNA integrity determination. *Lens culinaris* cv. CDC Redberry[13] seeds were germinated and grown in growth chambers with 18/6 hours photoperiod (250 µmol m^{-2} $seconds^{-1}$ Photosynthetic Photon Flux Density (PPFD)) and 21/15°C day/night temperatures. Plants were fertilized every two weeks with N:P:K; 20:20:20 (Plant Products Co. Ltd., Brampton, Ontario, Canada). At anthesis, flowers were tagged and the developing seeds were collected at two-day intervals from 8 to 32 days after flowering (DAF), frozen in liquid N_2 and stored at –80°C for total RNA isolation. For seedling tissues, seeds were germinated for three days in sterile petri dishes containing half-strength Hoagland's solution.[14] Germinated seeds were grown hydroponically in sterile Magenta boxes containing glass beads (1–5 mm diameter) and half-strength Hoagland's solution for two weeks. Leaves, stems, and roots were collected separately and stored at –80°C for RNA isolation.

Total RNA concentration was determined using a spectrophotometer at absorbance $A_{260\ nm}$ and RNA purity at ratio of absorbance $A_{260\ nm/280\ nm}$. RNA integrity was determined on a Bioanalyzer 2100 (Agilent Technologies Canada, Inc., Mississauga, Ontario, Canada) or by electrophoresis in HAE buffer (0.2 M Na HEPES, 50 mM sodium acetate, 10 mM EDTA, 0.7% (v/v) formaldehyde) and formaldehyde agarose gels (0.2 M Na HEPES, 50 mM sodium acetate, 10 mM EDTA, 0.7% formaldehyde (v/v), 1% agarose (w/v), 0.1 µg/mL ethidium bromide) and visualized under UV light.

Trizol reagent RNA isolation method. Total RNA was isolated from lentil leaves, stems, and roots using the Trizol reagent (Life Technologies, Inc., Burlington, Ontario, Canada) and column cleanup by the PureLink RNA kit (Life Technologies, Inc., Burlington, Ontario, Canada) as optimized for total RNA isolation from wheat leaves.[15] Total RNA isolation from lentil seeds was then tested using this method. An isopropanol precipitation step prior to column cleanup was also performed with another set of samples. Briefly, lentil seeds (200 mg) were ground in liquid N_2 in a –20°C pre-cooled RNase-free mortar. The ground seeds were transferred to an RNase-free container at room temperature containing 2 mL Trizol reagent. The slurry was mixed well and transferred to a 15 mL tube. The tube was vortexed for 10 seconds, incubated at room temperature for 5 minutes, and centrifuged at 15,000 × g for 10 minutes at 2°C. The supernatant was transferred to a new RNase-free tube, 1 mL of chloroform was added and the tube was vortexed for 15 seconds. The slurry was centrifuged at 15,000 × g for 10 minutes at 2°C. The colorless aqueous phase was transferred to a new RNase-free tube and re-extracted with 1 mL of chloroform. The supernatant was transferred to a new RNase-free tube and 1 mL of isopropanol was added, mixed, incubated at room temperature for 10 minutes, and centrifuged at 1000 × g at 2°C for 5 minutes. The aqueous phase was discarded. The RNA pellet was washed with 1 mL of 75% (v/v) ethyl alcohol, briefly air-dried and resuspended in 50 µL of DEPC-treated water.

Phenol:guanidine RNA isolation method. A second isolation method tested was the hot phenol:guanidine method optimized for total RNA isolation from wheat seeds.[5] Briefly, lentil seeds (200 mg) were ground as described earlier. The ground seeds were transferred to an RNase-free container containing 2 mL of hot 65°C phenol:guanidine buffer (50%

(v/v) acidic phenol, pH 4.3; 30% (w/v) guanidine–HCl; 5% (w/v) sodium dodecyl sulfate). The slurry was mixed well, transferred to a 15 mL tube and centrifuged at 15,000 × g for 10 minutes to pellet down cell debris and starchy components. The supernatant was transferred to a new RNase-free tube and equal volume of chloroform was added to the tube and vortexed for 15 seconds. The mixture was centrifuged at 15,000 × g for 10 minutes at room temperature. The aqueous phase was transferred to a new RNase-free tube and re-extracted with equal volumes of chloroform until the interphase was clear. The supernatant was transferred to a new RNase-free tube and 1 mL isopropanol was added and incubated at –80°C for 10 minutes and centrifuged at 1000 × g for 5 minutes. The supernatant was discarded. The RNA pellet was washed in 1 mL of 75% (v/v) ethyl alcohol, briefly air-dried, and resuspended in 50 µL of DEPC-treated water.

Lysis™ buffer RNA isolation method. This method employed the Lysis™ buffer available with the PureLink RNA Mini Kit as per the manufacturer's protocol. Briefly, ground lentil seeds (200 mg) were transferred to an RNase-free container at room temperature containing 4 mL Lysis buffer and 40 µL of β-mercaptoethanol. The slurry was mixed well and transferred to a 15 mL tube and incubated at room temperature for 2–3 minutes. The mixture was centrifuged at 15,000 × g for 10 minutes. The supernatant was transferred to a 15 mL tube and 0.5 volume of 100% ethyl alcohol was added and mixed well by vortexing. The sample was added to a spin column and all subsequent procedures were as per the manufacturer's instructions. The RNA was eluted from the column with a final volume of 50 µL RNase-free water.

A modification of the procedure described above included mixing the ground seeds with the Lysis buffer, followed by addition of phenol:guandine and chloroform extraction and subsequent precipitation of RNA with isopropanol as described earlier.

Hexadecyltrimethylammonium bromide-lithium chloride RNA isolation method. Another method tested involved the CTAB buffer. Ground lentil seeds (200 mg) were transferred to an RNase-free container containing 2 mL of CTAB buffer (4% (w/v) CTAB; 1% (w/v) PVP; 20 mM EDTA and 1.4 M NaCl) maintained at 65°C and containing 20 µL β-mercaptoethanol. The slurry was mixed well, transferred to a 15 mL tube and incubated at 65°C for 15 minutes with intermittent shaking. The mixture was centrifuged at 15,000 × g for 10 minutes at room temperature. The supernatant was transferred to a 15 ml tube, equal volume of chloroform was added and the tube was vortexed for a few seconds. The mixture was centrifuged at 15,000 × g for 15 minutes at room temperature. The chloroform extraction was repeated until the inter-phase was clear. The supernatant was transferred to a new RNase-free tube and $LiCl_2$ was added to final concentration of 4 M. The mixture was incubated at –20°C for 30 minutes and centrifuged at 15,000 × g for 15 minutes at 2°C. The supernatant was discarded and RNA pellet was washed with 1 mL of 75% (v/v) ethyl alcohol, briefly air-dried and resuspended in 50 µL of DEPC-treated water.

Validation of isolated RNA for gene expression analyses. To test the CTAB-isolated RNA for gene expression analyses, RNA gel blot was performed according to standard procedures.[16] Total RNA (25 µg) was loaded on a 1% (w/v) agarose gel containing 0.7% formaldehyde and electrophoresed in HAE buffer for 1.5–2 hours at 100 V. Ribosomal RNA present on the gel was visualized with ethidium bromide to check for equal loading of RNA samples. Transfer of total RNA from the gel to a Hybond–N+ membrane (GE Healthcare, Quebec, Canada) was done using a positive pressure blot, PosiBlot™ apparatus (Agilent Technologies Canada, Inc., Mississauga, Ontario, Canada) for 1 hour at 75 mm Hg pressure. RNA was cross-linked to the membrane in a UV cross-linker (UVP Inc., Upland, CA). Transferred RNA on the membrane was hybridized in Church hybridization buffer (0.5 M Na_2HPO_4, 7% SDS (w/v), and 1 mM EDTA) at 65°C with a 175 bp probe corresponding to the *granule-bound starch synthase I* (*GbssI*) labeled with [α^{32}-P] dCTP. The membrane was washed two times in 1 × SSPE (150 mM NaCl; 10mM NaH_2PO_4. H_2O; 1 mM EDTA; 0.1% SDS (w/v)) for 10 minutes each, and then in 0.1 × SSPE, 0.1% SDS (w/v) until background activity was negligible. Membrane was exposed to Biomax MR film (Kodak) at –80°C for 24 hours to several days depending on observed signal intensities.

For QPCR analyses, 5 µg of DNase-treated total RNA was reverse transcribed using 200 U Superscript III (Life Technologies, Inc., Burlington, Ontario, Canada). The 25 µL reaction volume of PCR consisted of 5 µL of a 1/15 dilution of the cDNA, forward (5′-AGGGTTCATTGGCAGGCTAGAAGA-3′) and reverse (5′-TGCTATCCCGATTGCTTTGCCA-3′) primers for the *GbssI* at 300 nM concentrations and 1 × Maxima™ SYBR Green I QPCR Master Mix (Fisher Scientific Company, Ottawa, Ontario, Canada). Real-time PCR amplification was performed in a Mx3000P real-time PCR machine (Agilent Technologies Canada, Inc., Mississauga, Ontario, Canada) under the following conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 1 minute. The forward (5′-AGTGGTTGAGGCAAGGGATGAGTT-3′) and reverse (5-TGGCGGAGTGAATGAAGACCAAGT-3′) primers corresponding to the lentil *adenosyl ribosylation factor* (*ARF*) gene was amplified for use as reference gene. Threshold values (C_t) were used to calculate relative expression by the $2^{-\Delta\Delta Ct}$ method[17] using the 32 DAF sample as a calibrator. A dissociation step was also performed at the conclusion of the amplification to ensure that a single specific product was amplified for each gene. Real-time PCR experiments were repeated twice with two technical replicates for each sample. Analysis of variance (ANOVA) was performed using the Minitab software (Version 16) (Minitab, Inc., State College, PA, USA).

Results and Discussion

General considerations for total RNA isolation from lentil seeds. Total RNA isolation from developing seeds of field crops has always been challenging due to the complexity of seed composition and seed sizes at various stages of development. In cereals, for example, starch constitutes about two-thirds of the grain dry weight and the interference of starch during RNA isolation leads to reduction of RNA yield and quality. Nonetheless, RNA isolation methods have been developed for successful use in transcriptome profiling studies of developing cereal grains.[5,18,19] However, in pulse crops such as lentils, similar and further challenges are encountered. Besides starch, which constitutes 35–65% dry matter in lentil seeds, oligosaccharide constituents such as raffinose, stachyose, verbascose, and α-galactosides account for 6–18% seed dry weight as reviewed in Ref.[20,] and phenolic compounds such as phenolic acids (0.25–0.47%) and tannins (0.02–1.0%) reviewed in Ref.[21] also interfere with RNA isolation. Furthermore, lentil seeds at early stages of development are very small (Fig. 1). Considering all these factors, we first tested the Trizol method followed by column cleanup, since this is the most widely used method. We have successfully used the Trizol/column cleanup method for RNA isolation from wheat leaves[15] and developing wheat grains[6] for QPCR analyses. When tested with 100 mg leaf, stem, and root tissues of lentil, yield of RNA was high using this method, about 50, 25, and 65 µg, respectively. The quality of RNA was also good (Fig. 2A).

Recalcitrance of lentil seeds to Trizol method for RNA isolation. Since the Trizol RNA isolation method produced high-quality and -quantity RNA from leaf, stem, and root tissues of lentil seedlings, we proceeded with this method for RNA isolation from 22 DAF lentil seeds. This stage was selected as it was in the mid-developmental stage and considered ideal for RNA optimization experiments. However, using the Trizol method, RNA yield was generally low, ranging from 2 to 2.7 µg from 200 mg of seed tissues (Table 1). Most often, RNA was also degraded. One factor considered for improving the Trizol isolation method was to increase the Trizol reagent to 4 mL to dilute and more effectively remove contaminants during the chloroform extraction step, prior to column cleanup. While the $A_{260/280}$ reading was 2.1, a value acceptable for RNA, the yield was still low at 2.1 µg (Table 1). Cloudiness of the supernatant upon addition of equal volume of 70% ethyl alcohol was also observed. Manufacturer's instructions suggest that cloudiness generally disappears upon vortexing, but it still persisted after mixing vigorously. It is suspected that the ethyl alcohol may have precipitated some of the RNA causing reduced RNA yield. A further modification included isopropanol precipitation after Trizol and chloroform extractions. RNA yield was in the range of 73–140 µg, with a low $A_{260/280}$ reading of 1.6 (Table 1) from

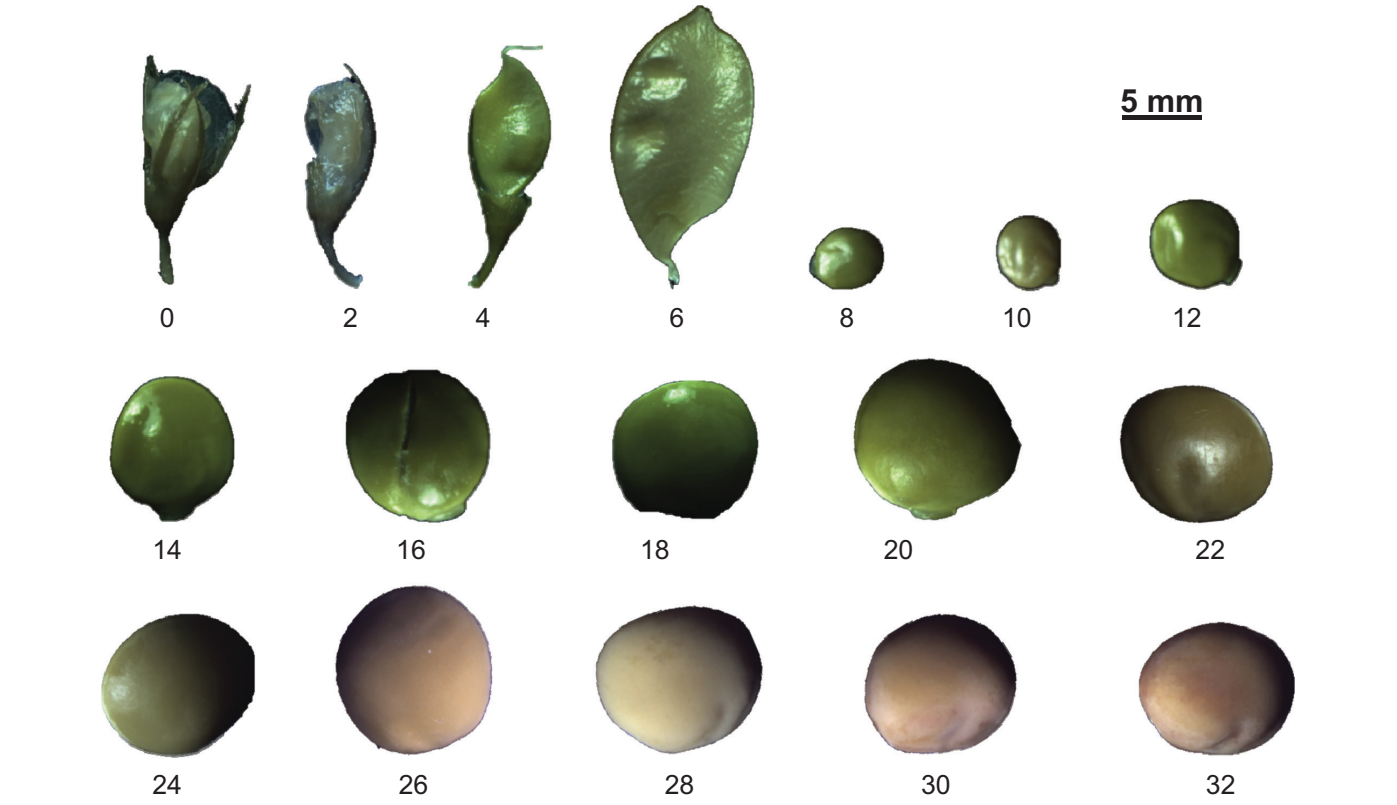

Figure 1. Sizes of lentil seeds at various stages of development from 0–32 days after flowering (DAF). Day 0 is equivalent to the stage at which pollination has just occurred. DAF 2 to 6 are shown with the developing seeds still within the pods.

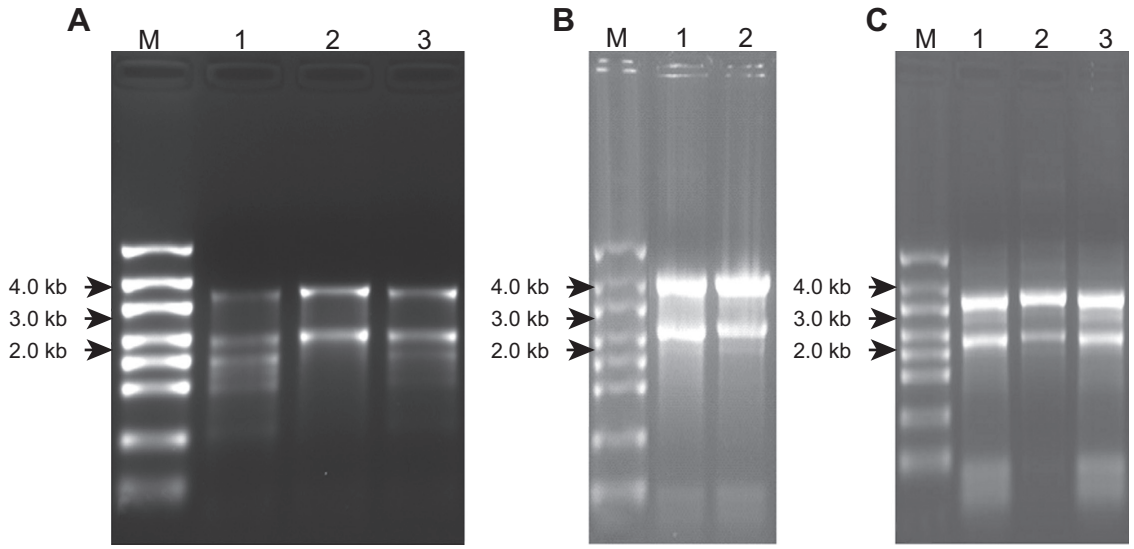

Figure 2. Gel electrophoresis of RNA samples resolved on formaldehyde agarose gels (**A**) isolated using the Trizol™ method from different tissues of lentil seedlings: Lane 1 – Leaf; Lane 2 – Root; Lane 3 – Stem; (**B**) CTAB-extracted RNA: Lane 1 – before Trizol Clean-up; Lane 2 – after Trizol clean-up; (**C**) Modifications of the CTAB extraction: Lane 1 – CTAB-$LiCl_2$ with column clean up; Lane 2 – Incubation of ground seeds in CTAB + βmercapto ethanol buffer for 30 min at –20°C; Lane 3 – Incubation of ground seeds in CTAB + βmercapto ethanol buffer for one hour at room temperature. M-RNA ladder.

400 mg of seeds, indicating protein contamination. Successful use of Trizol reagent for RNA isolation from developing cotyledons of legumes such as *Phaseolus vulgaris*[22] has been reported. It was therefore conceivable that the complexity of the lentil seed composition may have reduced the efficacy of the Trizol reagent isolation method, and subsequent modifications of the original method were tested.

Unsuitability of in-house phenol:guanidine method. Since the Trizol method did not show promising results, a hot phenol:guanidine method previously optimized for RNA

Table 1. RNA isolation methods and modifications tested with lentil seeds.

EXTRACTION METHOD	SEED WEIGHT (mg)	A260/280 RATIO	TOTAL RNA YIELD (µg)
Trizol reagent			
Trizol (2 mL) + PureLink RNA Mini Kit	200	1.7	2.7
Trizol (4 mL) + PureLink RNA Mini Kit	200	2.1	2.1
Trizol + isopropanol precipitation	400	1.6	73–140
Phenol:guanidine			
Phenol-guanidine	200	2.6	2.0
Phenol-guanidine pre-spin	200	1.5	17.5
Phenol-guanidine with $LiCl_2$ precipitation	200	1.7	20
Lysis buffer			
Lysis buffer + β-ME + column	200	2.1	19.45
Lysis buffer + β-ME + isopropanol precipitation	200	1.6	10
Lysis + β-ME + phenol-guanidine + isopropanol precipitation	200	1.8	220
Lysis buffer + Trizol	200	1.8	16–21.7
CTAB			
CTAB + β-ME + 4 M $LiCl_2$ precipitation	200	2.2	140
CTAB + β-ME + $LiCl_2$ precipitation for 30 min at –20°C	200	2.2	260
CTAB + β-ME + 1 hour at room temperature	200	2.2	210
CTAB + $LiCl_2$ before clean up	200	2.2	247.5
CTAB after Trizol clean up	200	2.2	180

Abbreviation: β-ME, β-mercapto ethanol.

isolation from developing wheat grains[5] was tested. Generally, degraded RNA (data not shown) was observed. When RNA was not degraded, 200 mg lentil seed meal yielded only about 2 µg RNA with $A_{260/280}$ ratio of 2.6 (Table 1). With the hot phenol:guanidine isolation procedure, the slurry with the ground seeds became gelatinous. This could be due to the starchy components of the seeds, as reported in a previous study.[23] This would explain the low RNA yield because it could become trapped within this gelatinous mass and would also account for its subsequent degradation. To prevent interference of the starch during RNA isolation when the ground seeds were mixed with the phenol:guanidine and incubated at 65°C, a pre-spin for 10 minutes at 15,000 × g was performed. The supernatant was transferred to a new tube and extracted with equal volume of chloroform followed by column cleanup. This modification improved the yield of RNA; however, its quality was still poor as reflected by the $A_{260/280}$ ratio of 1.5 (Table 1). Another modification was lithium chloride precipitation of RNA, followed by resuspension in RNase-free water and column cleanup. The yield was still low (20 µg/200 mg seed meal) with $A_{260/280}$ ratio of 1.7 (Table 1). The phenol:guanidine method was found to be unsuitable for RNA isolation from lentil seeds.

Commercial Lysis buffer method improves RNA isolated from lentil seeds. Since the two previous methods did not yield good-quality RNA in adequate quantities, Lysis buffer supplied with the PureLink RNA Mini Kit as per the manufacturer's instructions was tested for lentil seed tissue lysis and extract RNA. The resultant RNA showed $A_{260/280}$ ratio of 2.1 and yields of 19.5 µg (Table 1). RNA quality check on the 2100 Bioanalyzer also indicated high-quality RNA (Fig. 3A). Although this kit-based method was easier

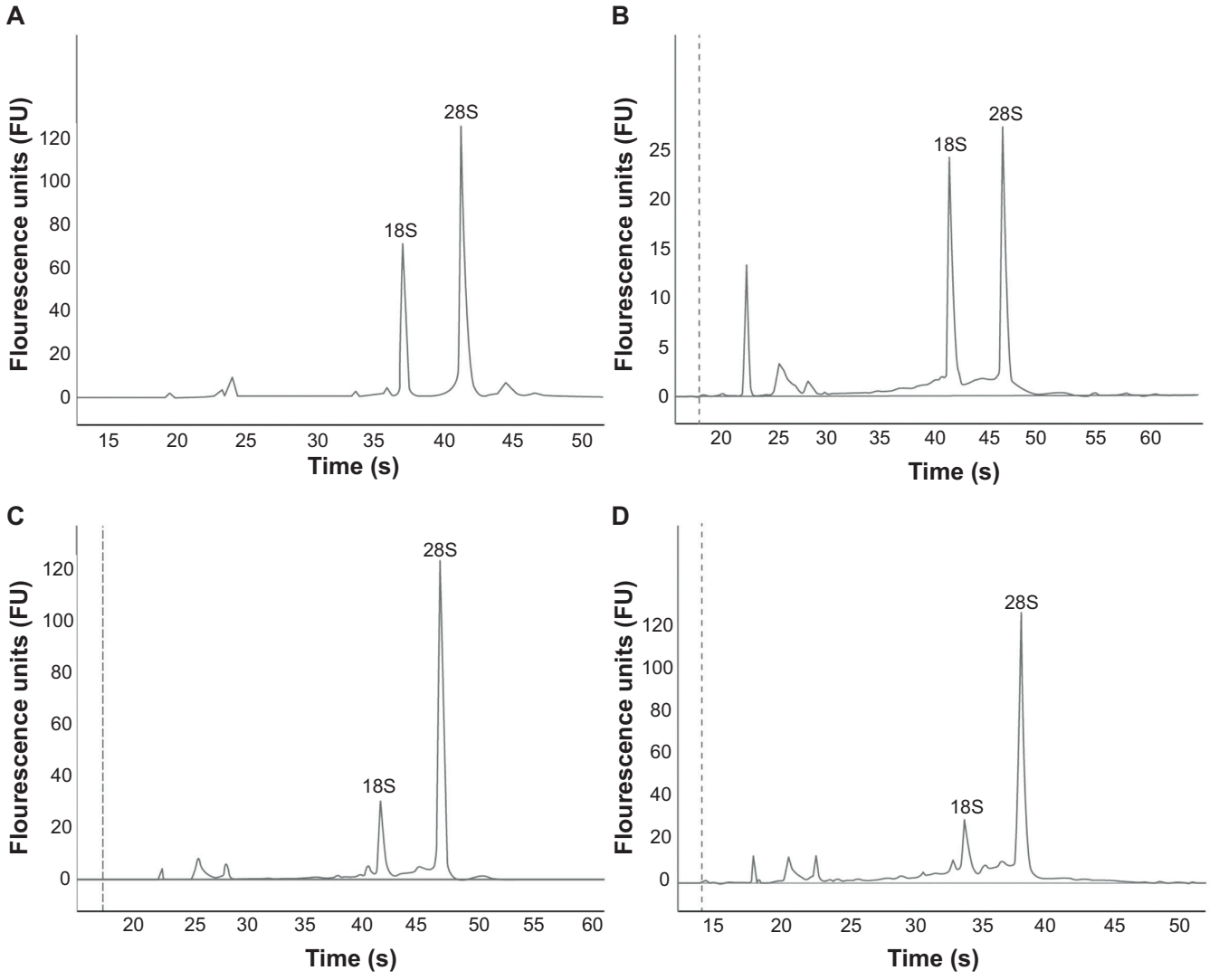

Figure 3. Electropherogram of microfluidic electrophoresis of RNA isolated from lentils seeds. (**A**). Lysis Buffer + β-mercapto ethanol method, followed by column clean-up using the PureLink RNA mini kit; (**B**). Lysis Buffer + β-mercapto ethanol method combined with phenol:guanidine and column-clean-up. (**C**). CTAB method, followed by $LiCl_2$ precipitation and pellet re-suspended in RNase-free water; (**D**). CTAB method, followed by $LiCl_2$ precipitation which was re-extracted after Trizol reagent treatment.

and faster than our previous methods tested, RNA yield was still inadequate for applications such as RNA gel blots. The increase in yield may be attributed to the Lysis buffer (which contains an unspecified concentration of guanidine isothiocyanate), to more effectively prevent RNase activity, thereby yielding good-quality RNA. It is therefore surprising that our in-house phenol:guanidine mix did not yield good-quality RNA with improved yield. In fact, acidic phenol in addition to preventing RNase activity allows partitioning of RNA into the aqueous phase to preferentially maximize RNA isolation.[24] It is possible that the gelatinous mass observed in the hot phenol:guanidine isolation procedure used earlier was due to the phenol forming a complex with the carbohydrates and simultaneously trapping most of the RNA. This was confirmed when we combined the Lysis buffer with the phenol–guanidine mix, as discussed below.

The Lysis buffer with an isopropanol precipitation step, followed by resuspension in RNase-free water and column cleanup did not increase the yield or quality of RNA (10 µg) (Table 1, Fig. 3B). The next procedure included mixing lentil seed meal with the Lysis buffer followed by addition of hot phenol:guanidine, chloroform extraction of RNA, and its precipitation with isopropanol. While the RNA yield was much higher at 220 µg (Table 1), RNA tended to co-precipitate with starch and other polysaccharides upon incubation on ice. Heating the samples dissolved the pellet, but it led to unpredictable amounts of RNA for gel loading, unless the samples were quantified prior to each use. This is not practical for routine RNA gel blot analyses. For RNA of some seed samples, mostly at the later stages of maturity, dissolution of the pelleted gelatinous mass was also difficult even after incubation at 65°C for 30 minutes. Freeze-thaw cycles of the samples at −80°C further exacerbated the dissolution of the pellet. A similar problem was encountered with the use of the Lysis buffer with Trizol, with RNA yield being much low at 16–22 µg (Table 1).

Combined CTAB–$LiCl_2$ method consistently yields high-quality and -quantity RNA. It is evident that conventional methods initially tested for RNA isolation from lentil seeds were more challenging than anticipated. As mentioned earlier, the lentil seed contains relatively high concentrations of carbohydrates and phenolic compounds. One of the well-documented methods for nucleic acid isolation from plant tissues is the CTAB method, for protocols and references therein see Refs.[25,26] Originally developed for isolation of high molecular weight DNA from plant tissues,[27] the CTAB buffer was further modified to include PVP to efficiently bind phenolic compounds to prevent their interference with DNA isolation.[8,9] Similarly, PVP was included in the RNA isolation buffer to remove phenolic compounds.[28]

The CTAB buffer for RNA isolation was the same buffer that has been previously successfully used for large-scale DNA isolation from cereal leaf tissues[29] and was based on the modified method of Doyle and Doyle.[9] The lentil seed meal was mixed with 4% CTAB buffer supplemented with β-mercaptoethanol to isolate RNA which was then extracted with chloroform and finally precipitated with 4 M $LiCl_2$. The RNA yield from 200 mg of seed sample was 140–260 µg with $A_{260/280}$ readings of about 2.2 (Table 1). RNA quality as determined on the 2100 Bioanalyzer was high (Figs. 3C, 3D). $LiCl_2$ selectively precipitates RNA, albeit slowly.[30] However, DNA can also co-precipitate (Fig. 2B) and this can be circumvented by a Trizol cleanup procedure of the RNA sample (Fig. 2B). The RNA sample is mixed with an equal volume of Trizol, followed by chloroform extraction and reprecipitation with isopropanol. Although the yield of RNA decreased from 248 to 180 µg with this step (Table 1), the yield was adequate for RNA gel blot analyses. A 30-minute incubation at –20°C precipitated similar amount of RNA compared to the overnight precipitation initially performed (Table 1) and the RNA was of good quality (Fig. 2C). Similarly, the duration that the ground samples could be left in CTAB buffer + β-mercaptoethanol at room temperature also showed that an hour incubation did not affect RNA yield (Table 1) or quality (Fig. 2C). This was done to ascertain that if multiple samples are being processed for RNA isolation that the initial few samples ground and mixed with CTAB buffer + β-mercaptoethanol did not start to degrade. The optimized protocol (Fig. 4) was used for high-quality RNA isolation from developing lentil seeds (Fig. 5).

CTAB-based RNA isolation protocol is suitable for gene expression analyses. A combined CTAB-lithium chloride method described above was used to isolate RNA from developing lentil seeds of 12, 16, 20, 22, 24, and 28 DAF, and used in an RNA gel blot analysis to study the expression of *GbssI* during seed development (Fig. 6A). Gel blot revealed differential expression of the *GbssI* gene during seed development, showing maximum transcript abundance at 24 DAF (Fig. 6A). For QPCR analysis, the expression of *GbssI* was assessed using cDNA synthesized from RNA extracted from 12 to 32 DAF seeds. Peak expression of the *GbssI* was observed at 24 DAF (Fig. 6B), similar to the observed transcript abundance at 24 DAF on the RNA gel blot (Fig. 6A). The expression patterns corroborate accumulation patterns of *GbssI* in developing seeds of pea, where accumulation of *GbssI* was observed to increase during seed development and decreased toward seed maturity.[31]

Conclusions

Four methods were tested for RNA isolation from lentil seeds. The most commonly used Trizol reagent and hot phenol:guanidine methods did not yield satisfactory RNA quality or quantity. Similarly, the Lysis buffer available with the RNA PureLink Mini kit, while showing improvements, did not yield RNA of adequate quantity. Despite several modifications of the first three protocols, isolation of good-quality and high-yield RNA from the lentil seeds could not be obtained. Subsequently, the CTAB-$LiCl_2$ protocol was found to be the

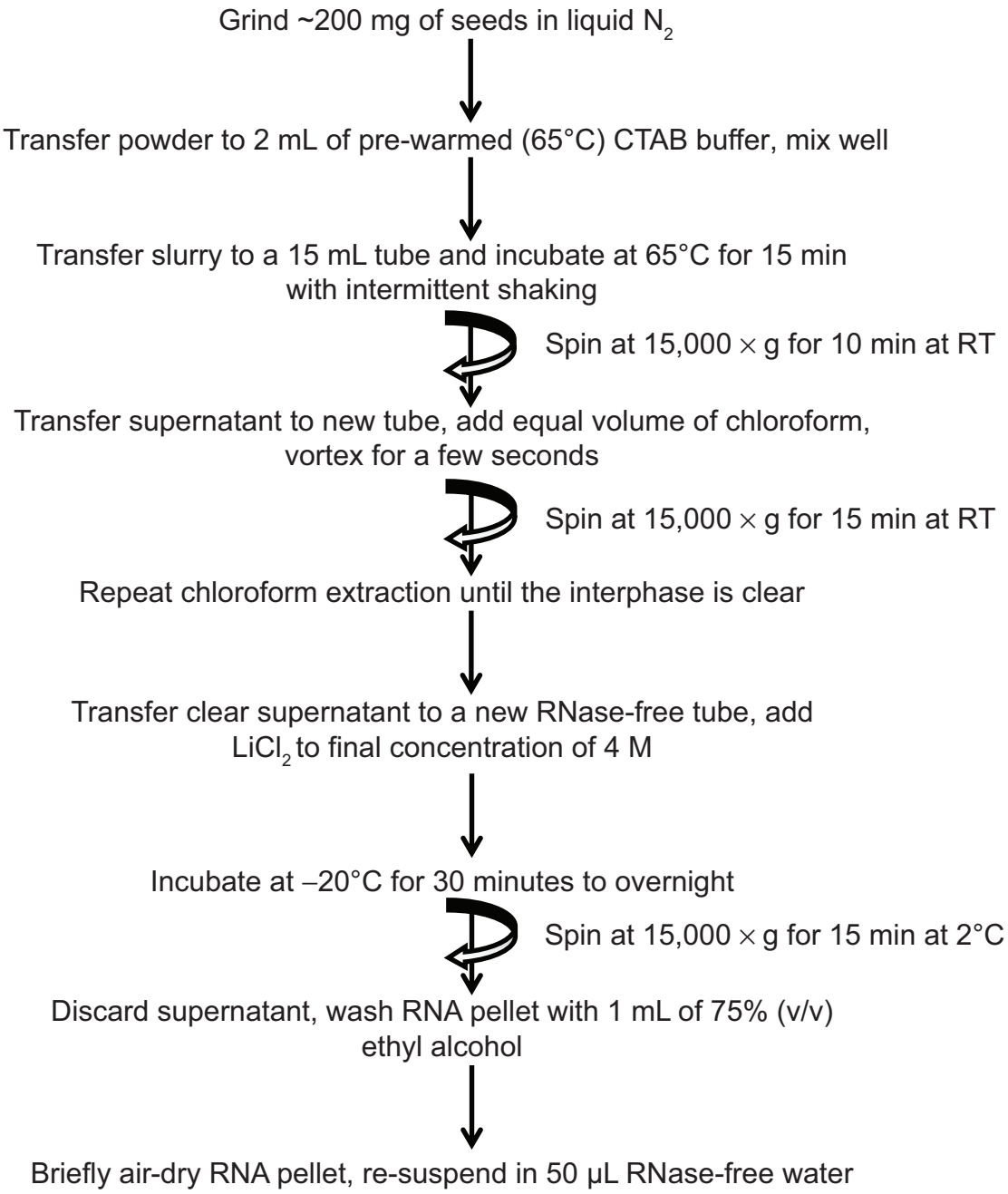

Figure 4. Flow-chart of optimized RNA isolation protocol from lentil seeds using the CTAB-$LiCl_2$ method.

most efficient for RNA isolation from developing lentil seeds. The developed isolation protocol is cost-effective and rapid. Furthermore, the CTAB buffer is the same buffer used for DNA isolation and therefore serves as a dual purpose nucleic acids isolation buffer. The method presented in this study is believed to be more efficient compared to some of the methods mentioned earlier[10–12] because of the high CTAB concentration used, inclusion of PVP, and increased $LiCl_2$ molarity to 4 M for precipitation. Besides validating the RNA for use in gene expression analyses, we have successfully used it to generate a cDNA library from developing lentil seeds, which is being used to isolate and characterize full-length cDNA clones of genes influencing grain quality trait (manuscript in preparation). Contaminating DNA in the isolated RNA samples can be effectively removed by Trizol reagent treatment for less-sensitive applications such as RNA gel blot analyses and by DNase treatment for more sensitive applications such as QPCR analyses. We have successfully used this protocol for RNA isolation from developing wheat grains, with RNA yields ranging from 90 to 115 µg with $A_{260/280}$ ratio of 2.2 from 100 mg of ground 6, 18 and 40 DPA kernels (Chibbar, personal communication). We foresee that the optimized RNA isolation protocol developed in this study will be valuable for RNA isolation from seeds of other pulse crops.

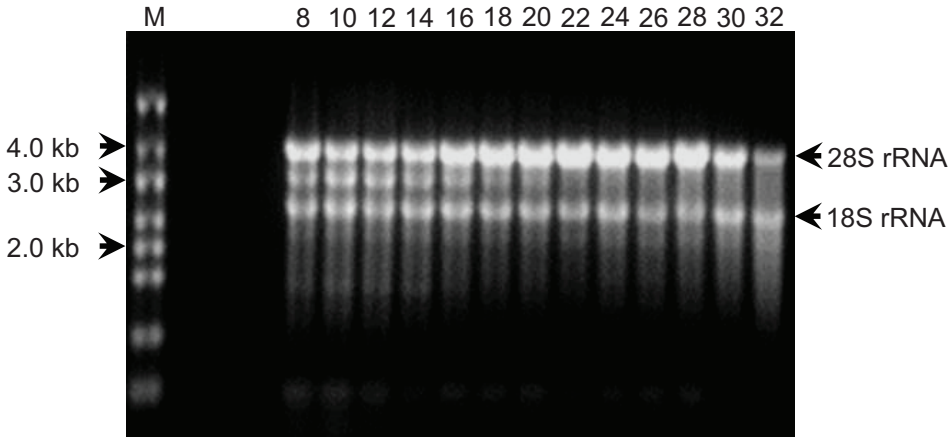

Figure 5. CTAB-extracted RNA from developing lentil seeds (8–32 DAF) resolved on formaldehyde agarose gel. M-RNA ladder.

Author Contributions

UK designed and conducted the experiments, analyzed, interpreted data, and drafted the manuscript. SG conceived and designed experiments, interpreted data, and revised the manuscript. RNC oversaw the project, revised, and approved the manuscript. All authors reviewed and approved of the final manuscript.

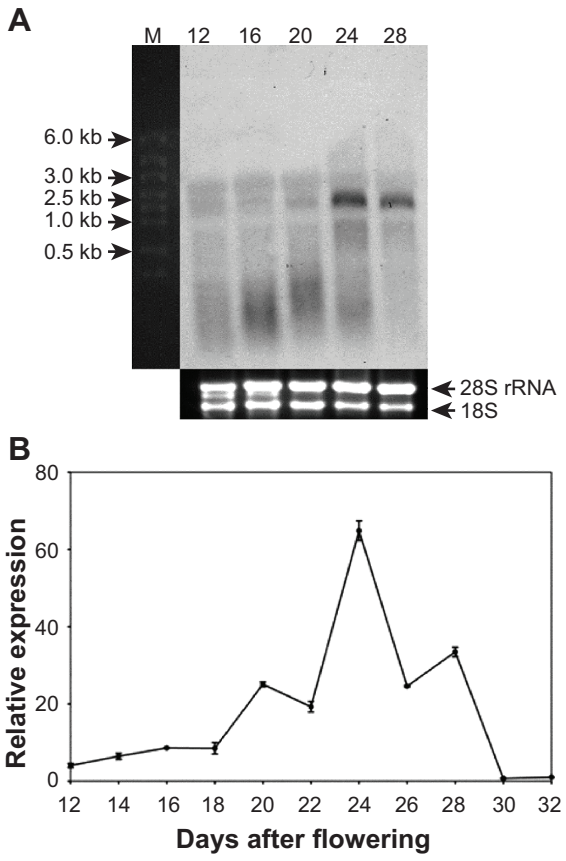

Figure 6. (**A**) RNA gel blot for *Gbssl* expression from developing seeds of lentil from 12,16, 20, 24 and 28 days after flowering. (**B**) Relative expression of *Gbssl* determined by QPCR from developing seeds of lentil from 10 to 32 days after flowering. Error bars indicate standard error of the means.

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