A universal method for high-quality RNA extraction from plant tissues rich in starch, proteins and fiber

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Using existing protocols, RNA extracted from seeds rich in starch often results in poor quality RNA, making it inappropriate for downstream applications. Though some methods are proposed for extracting RNA from plant tissue rich in starch and other polysaccharides, they invariably yield less and poor quality RNA. In order to obtain high yield and quality RNA from seeds and other plant tissues including roots a modified SDS-LiCl method was compared with existing methods, including TRIZOL kit (Invitrogen), Plant RNeasy mini kit (Qiagen), Furtado (2014) method, and CTAB-LiCl method. Modifications in the extraction buffer and solutions used for RNA precipitation resulted in a robust method for extracting RNA in seeds and roots, where extracting quality RNA is challenging. The modified SDS-LiCl method revealed intense RNA bands through gel electrophoresis and a nanodrop spectrophotometer detected ratios of ≥ 2 and 1.8 for A260/A230 and A260/A280, respectively. The absence of starch co-precipitation during RNA extraction resulted in enhanced yield and quality of RNA with RIN values of 7–9, quantified using a bioanalyzer. The high-quality RNA obtained was demonstrated to be suitable for downstream applications, such as cDNA synthesis, gene amplification, and RT-qPCR. The method was also effective in extracting RNA from seeds of other cereals including field-grown sorghum and corn. The modified SDS-LiCl method is a robust and highly reproducible RNA extraction method for plant tissues rich in starch and other secondary metabolites. The modified SDS-LiCl method successfully extracted high yield and quality RNA from mature, developing, and germinated seeds, leaves, and roots exposed to different abiotic stresses.

Modern biotechnological advances in functional genomics and the access to whole-genome sequences are invaluable tools for crop improvement. Identifying the molecular factors affecting crop growth and productivity under harsh environmental conditions and evaluating their functional responses will help decode pathways enhancing stress-tolerance in crops. Deciphering these molecular factors and stress tolerance mechanisms demands high-quality RNA, which is a pre-requisite for various techniques in molecular biology, including gene expression quantification by RT-PCR, RT-qPCR, RNA-seq, northern blot analysis, construction of cDNA libraries and PCR amplification-based gene cloning.

Seeds are complex storage organs, composed of embryo and endosperm covered by a hard seed coat. The endosperm, which occupies the major proportion of the seed, contains significantly high quantity of starch followed by other polysaccharides (cellulose [dietary fiber], arabinoxylan, β-glucan and fructans), proteins, lipids, and secondary metabolites. Most of these components in seeds have structural similarities with nucleic acids and hence co-precipitate along with RNA, thus challenging the extraction of high yield and quality RNA1–5. Several RNA isolation methods have been reported to yield quality RNA from wheat and other seeds rich in starch, polysaccharides, and other secondary metabolites1,2,6. A few limitations of existing RNA extraction methods mentioned above include—(i) need more than one extraction buffers1,2, (ii) methods developed are restricted to extracting RNA only from seeds, (iii) require expensive TRIZOL and/or commercial kits for extraction6 and comparatively cumbersome1,2. Hence, there is a need to develop a rapid and universal RNA extraction method using basic laboratory chemicals that can be applicable to a wide range of tissues containing interfering substances that alter the quality of extracted RNA.

RNA isolation methods with guanidine isothiocyanate based extraction buffers fail to extract quality RNA from seeds rich in starch, as guanidine isothiocyanate induces solidification of starch1. Solidification of starch...
enhances the co-precipitation of starch/polysaccharides along with RNA due to their structural similarities, for example presence of ribose sugar. The large polysaccharide fractions in the seed can physically trap RNA and be entrained during centrifugation and gets discarded during the phase separation, thus leading to low yield. Additionally, small polysaccharide particles can be partitioned into the aqueous phase during phase separation and co-precipitate along with RNA, which further reduces RNA yield and renders it less suitable for downstream applications. Therefore, to overcome the starch solidification encountered by existing methods, additional purification steps or alternative methods are required. In addition, extraction of high-quality RNA from plant tissues subjected to different environmental stresses such as heat, drought and cold stresses can be difficult due to increased accumulation of high molecular weight polysaccharides and secondary metabolites that interfere with RNA isolation. Secondary metabolites in the oxidized form irreversibly bind to the nucleic acids and act as inhibitors for downstream applications or degrade the RNA. Isolating high-quality RNA free from proteins, polyphenols, and polysaccharide contaminants from samples exposed to abiotic stresses is essential for downstream applications. Moreover, the quality and quantity of RNA extracted using existing RNA extraction methods and commercial kits differ depending on the plant species, genotype and tissue type. Hence, it is evident that a universal RNA extraction method that can be used across different plant parts, including seeds, which contain high levels of starch and secondary metabolites and roots would be of great value to the crop science community.

The major objective of the present study was to develop a robust and reliable RNA extraction method to obtain high-quality RNA from different plant tissues, including roots and seeds exposed to different abiotic stresses. To achieve this objective, five RNA extraction methods, i.e. TRIZOL kit (Invitrogen), Plant RNeasy mini kit (Qiagen), Furtado method, CTAB-LiCl method, and our modified SDS-LiCl method, were used to compare the quantity and quality of RNA extracted from mature wheat seeds collected from field-grown plants exposed to ambient, and high night temperatures (for details on crop management and stress imposition see Hein et al.). The extracted RNA was then used to assess the quality through RT-qPCR analysis. In addition, RNA from different tissues of wheat plants including mature, developing and germinated seeds, leaf, and roots, exposed to cold, freezing, and high night temperature stresses were isolated using the modified SDS-LiCl method. The robustness of our modified SDS-LiCl method was further tested on developing sorghum seeds and developing and mature maize seeds, obtained from field grown plants. Given the variability of RNA isolation methods, the ability to achieve comparative analysis of genomes and transcriptomes across crops and tissue types continues to be a challenge for researchers. Here we describe a universal method that can be applied in seed tissues containing high starch and other plant tissues including roots, which contain high fiber and secondary metabolites. Our intent in presenting this modified method is not to oppose other methods that work well for specific crops and tissues, but rather to present a universal method that is more robust, rapid and works equally well across different field crops and plant tissues.

Materials and methods
Two winter wheat (Triticum aestivum L.) genotypes Tascosa and Tx86A5606 with contrasting responses to high night temperature (HNT) stress were used to isolate RNA from different plant tissue and growth conditions. RNA was isolated from physiologically mature wheat seeds collected from field grown plants, flag leaves from mature wheat plants grown in a controlled environment chamber, and germinated seeds, obtained from field grown plants. The field experiment was carried out at Kansas State University, Agronomy research farm at Manhattan, Kansas. High night temperature (HNT) stress was imposed by placing custom built field-based heat tents on already established wheat plants from 10 days after 50% flowering till maturity. The heat tents maintained temperatures < 1 °C higher compared to ambient conditions during the day. Higher night-time (19:00 h to 6:00 h) temperature was achieved using a custom built semi-automated heating system operating independently in each tent during grain filling (For additional details on the construction, operation, and temperature imposition see Hein et al.). Physiologically mature seeds were collected from two winter wheat genotypes (Tascosa and Tx86A5606) grown under ambient night temperature (control; 22.7 °C) and an elevated night temperature of + 3.2 °C (HNT stress) during the grain filling, frozen in liquid nitrogen, and stored at − 80 °C for RNA extraction.

Tissue sampling from control and high night temperature (HNT) stress exposed plants. Mature seeds. Physiologically mature wheat seeds were collected from field grown plants, 21 days after 50% flowering. The field experiment was carried out at Kansas State University, Agronomy research farm at Manhattan, Kansas. High night temperature (HNT) stress was imposed by placing custom built field-based heat tents on already established wheat plants from 10 days after 50% flowering till maturity. The heat tents maintained temperatures < 1 °C higher compared to ambient conditions during the day. Higher night-time (19:00 h to 6:00 h) temperature was achieved using a custom built semi-automated heating system operating independently in each tent during grain filling (For additional details on the construction, operation, and temperature imposition see Hein et al.). Physiologically mature seeds were collected from two winter wheat genotypes (Tascosa and Tx86A5606) grown under ambient night temperature (control; 22.7 °C) and an elevated night temperature of + 3.2 °C (HNT stress) during the grain filling, frozen in liquid nitrogen, and stored at − 80 °C for RNA extraction.

Developing seeds. Developing wheat seeds were collected from controlled environment chamber grown plants at 14 days after heading/HNT stress exposure, from control and HNT treatments. Two winter wheat genotypes, SY Monument (Tolerant) and KS07077M-1 (Sensitive) contrasting for HNT stress were exposed to control (28/15 °C) and a range of HNT treatments (28/18 °C; 28/23 °C; 28/25 °C and 28/27 °C) using the controlled environment chamber facility, at the Department of Agronomy, Kansas State University (For additional details on the high night-time temperature stress imposition see Impa et al.). HNT stress treatments were imposed immediately after the main spike (first emerged) had completed flowering and maintained until physiological
maturity. Three biological replicates of main spikes from all the five night temperature treatments were collected at 14 days after the stress was initiated, and immediately frozen in liquid nitrogen, and stored at −80 °C for RNA extraction.

**Flag leaves.** Flag leaf samples were collected from the controlled environment chamber grown wheat genotypes, Tascosa and Tx86A5606 at 14 days after heading/HNT stress exposure, from control (26/15 °C) and HNT (26/23 °C) treatments. HNT stress treatments were imposed immediately after the main spike (first emerged) had completed flowering and maintained until physiological maturity (For additional details on the HNT stress imposition see Impa et al.19). Three replicate samples were collected for each genotype and treatment. Samples were immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

**Germinated wheat seeds exposed to cold stress.** Seeds of the two wheat genotypes (Tascosa and Tx86A5606) were germinated in plastic Petri dishes (100 mm by 20 mm). Seeds were sterilized with 70% ethanol for 2 min, followed by 3% sodium hypochlorite for 5 min. Subsequently, seeds were rinsed 2 to 3 times with sterile distilled water before placing them on the germination paper. Ten seeds were placed in each petri dish containing the germination paper moistened with sterile distilled water. The Petri dishes were incubated in an incubator (RevSci, Minnesota, USA) maintained at 15 °C to impose cold stress, and at 30 °C for control treatment21. The germinated seeds were collected five days after germination was initiated and immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction. The sample collection was timed to ensure that each germinating seed had five days of continued cold stress after initiation of germination.

**Wheat roots exposed to freezing stress.** Seeds of Tascosa and Tx86A5606 were surface sterilized with 70% ethanol for 2 min and rinsed twice with sterile distilled water. Subsequently, the seeds were washed with 3% sodium hypochlorite for 5 min and rinsed three times with sterile distilled water. Later, the seeds were dried under laminar airflow and were inoculated into sterile test tubes containing half-strength Murashige and Skoog (MS) media (Phytotechnology laboratories, KS) with 0.6% w/v solidified agar. The test tubes were incubated in a growth chamber maintained at 25 °C with 16/8 h day/night photoperiod. After ten days of incubation, the seedlings were exposed to a freezing temperature of −4 °C in the freezer for 16 h. At the end of the 16 h stress exposure, root samples from control and freezing temperatures were collected into liquid nitrogen and immediately stored at −80 °C for RNA extraction.

**Sorghum and corn seed samples.** The developing seeds of Sorghum hybrid (Pioneer 87P06) and developing and mature seeds of Corn (Pioneer P0805AM) hybrid were collected from the plants grown under field conditions at Kansas State University, Agronomy research farm. The samples harvested in the field were immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

**RNA extractions.** All the solutions used in the RNA extraction procedure were prepared with autoclaved 0.1% DEPC (diethyl pyrocarbonate) treated MilliQ water. All the plastic- and glassware and mortar and pestle were pretreated with DEPC and autoclaved.

**Methods followed to extract total RNA.** *Ambion TRIZOL reagent Kit* (by Life Technologies Invitrogen, CA, USA): Total RNA was extracted according to the manufacturer’s instructions. Extraction buffer used in this method contains guanidium thiocyanate and RNA was precipitated in isopropanol.

*RNeasy Plant Mini Kit* (Qiagen, Valencia, CA, USA): RNA was extracted according to the manufacturer’s instructions. The method uses guanidium thiocyanate based lysis buffer and RNA purification was done using silica-membrane column.

Furtado’s method: The total RNA was isolated based on the combination of both TRIZOL and RNeasy Plant Mini Kit (Qiagen) method. RNA extraction was carried out in the TRIZOL reagent, and the collected aqueous phase was extracted in RLC buffer, followed by steps in RNeasy Plant Mini Kit manufactures method.

*CTAB-LiCl method:* RNA was extracted based on the method described by White et al.3. In this method, ionic detergent cetyltrimethylammonium bromide (CTAB) was used in the extraction buffer, and LiCl was used to precipitate RNA.

New Modified method (SDS-LiCl): This method follows the SDS-Phenol based extraction and RNA precipitation with sodium acetate and LiCl (Fig. 1).

**Materials and equipments’ and their set up, step by step procedure and troubleshooting are provided in supplementary document.** *Qualitative and quantitative analyses of RNA.* The quantity and quality of RNA were assessed by measuring the optical density (OD) at 260 nm and 280 nm by a Nanodrop-2000 Ultraviolet Spectrophotometer (Thermo Fisher, MA, USA). The RNA quality of the sample was measured by monitoring the absorbance at A260/280 and A260/230 ratios (Table 1). The purity and integrity of the total RNA bands were evaluated by loading 200 ng of RNA samples on 1% agarose gel stained with ethidium bromide and pictured under UV light in GEL DOC XR + and CHEMIDOC XRS + imaging Systems containing the IMAGE LAB software (Biorad, CA, USA). Further, the total RNA was analyzed with the Total RNA 6000 Nano Kit (Vers. II), precisely optimized for total RNA analysis with Agilent 2100 Bioanalyzer with RNA 6000 Nano Lab Chip (Agilent Technologies, CA, USA). The RIN (RNA integrity number) values were calculated using the Eukaryote Total RNA Nano Assay2. The electropherograms were analyzed using Agilent 2100 Expert B.02.06 SI648 software composed of data collection, presentation, and interpretation functions.
cDNA synthesis, PCR gene amplification and RT-qPCR. The RNA extracted from developing and mature seed samples were subjected to DNase 1 treatment (Thermo Scientific, Waltham, MA, USA) to eliminate remaining traces of genomic DNA (gDNA). The first strand of cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. For polymerase chain reaction (PCR) amplification of 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) gene product (1229 bp), ~ 100 ng of cDNA samples of total RNA extracted from the mature wheat seed tissue using five different RNA extraction protocols were added to a 25 µl PCR reaction mix containing GoTaq G2 Green Master Mix (Promega, Madison, WI, USA) and 0.4 µM of each gene-specific primer (EPSPSF- AAG TCG CTMTCC AAY CGR ATC CT and EPSPSR- GGG AAG GTCTTNCGG GTG CA). The amplification was carried out in a thermal cycler (T100 Thermal Cycler, Bio-Rad Inc, Hercules, CA, USA) with PCR conditions as follows: 5 min denaturing at 95 ºC; 32 cycles of 30 s denaturation at 95 ºC, 30 s annealing at 55 ºC, 45 s elongation at 72 ºC and a final extension for 10 min at 72 ºC.

For RT-qPCR analysis, the following reference and gene-specific primers were used to measure the corresponding genes (Fwd/Rev): Actin (Forward- GCC ATG TAC GTC GCA ATT CA, Reverse- AGT CGA GAA CGA TAC CAG TAG TAC GA) and Beta-amylase (BMY) (Forward- CTAGCCAACTATGTCAGTCTAGT, Reverse- ACT GTGGGATGGGATGTTGACGA). Quantitative PCR/Real-Time PCR (RT-qPCR) was performed to determine the quality of the RNA extracted from the seed samples using the BMY (Beta Amylase) gene. For all the RT-qPCR analysis three technical replicates were used for each of the three biological replicates and RT-qPCR
for ribosomal RNAs (Fig. 4 and Supplementary Figs. S1–S5). RIN values determined on Bioanalyzer (Agilent mal RNAs (rRNAs), whereas other methods exhibited either many small peaks and/or no protuberant peaks using modified SDS-LiCl method with the electropherograms showing distinct, prominent peaks for riboso-
tein contaminants, organic solvents, and other secondary products compared to other RNA isolation methods. samples extracted using modified SDS-LiCl method. This demonstrates the RNA extracted to be free from pro-
ting at 260 nm (Fig. 3) and ratios of A260/A280 and A260/A230 being close to or above 2 (Table 1) for all the RNA
degraded (Fig. 2e). However, other methods produced faint bands of RNA, with higher impurities in the gel-well RNA bands were intact and intense, demonstrating that the total RNA extracted by modified SDS-LiCl was not
degraded (Fig. 2e). However, other methods produced faint bands of RNA, with higher impurities in the gel-well and a smeary background during the run (Fig. 2a–d). Absorbance spectra of RNA with a broad peak pattern at 260 nm (Fig. 3) and ratios of A260/A280 and A260/A230 being close to or above 2 (Table 1) for all the RNA samples extracted using modified SDS-LiCl method. This demonstrates the RNA extracted to be free from protein contaminants, organic solvents, and other secondary products compared to other RNA isolation methods.

### Results and discussion

**SDS-LiCl method extracted higher yield and quality RNA from seeds.** Previous methods using guanidine isothiocyanate based RNA extraction buffer results in starch solidification and co-precipitation of RNA with starch, reducing the quantity and quality of RNA. Modified SDS-LiCl method described here provides a robust method to obtain high-yield and high-quality RNA from wheat seeds containing high starch and other polysaccharides, and other tissues such as roots that are rich in fiber. In addition, this method has the potential to extract high yield and quality RNA from developing and mature seeds exposed to different abiotic stresses. Analysis of total RNA from mature wheat seed on agarose gel electrophoresis revealed that ribosomal RNA bands were intact and intense, demonstrating that the total RNA extracted by modified SDS-LiCl was not degraded (Fig. 2e). However, other methods produced faint bands of RNA, with higher impurities in the gel-well and a smeary background during the run (Fig. 2a–d).

Evaluation of total RNA quality using Agilent bioanalyzer 2100 revealed superior quality of RNA extracted using modified SDS-LiCl method with the electropherograms showing distinct, prominent peaks for ribosomal RNAs (rRNAs), whereas other methods exhibited either many small peaks and/or no protuberant peaks for ribosomal RNAs (Fig. 4 and Supplementary Figs. S1–S5). RIN values determined on Bioanalyzer (Agilent 2100) using Expert software for total RNA extracted using modified SDS-LiCl method ranged from 7.6 to 9.1 (Table 2). The capillary gel electrophoresis virtual gel images (Agilent 2100 Expert B.02.06 SI648) revealed the intact rRNA bands of high quality RNA isolated by modified SDS-LiCl method compared to RNA extracted using other methods, except CTAB-LiCl method, which was also effective in extracting high quality RNA (Supplementary Figs. S1–S5). Total RNA isolated with modified SDS-LiCl method produced significantly higher RNA yield (ranging from 19.41 to 37.24 μg/100 mg FW) compared to other methods including CTAB-LiCl method, which was on average 80% lower compared to modified SDS-LiCl method (Table 2). Similarly, lower RNA yield from wheat seeds using CTAB-LiCl has been reported. The CTAB, as a cationic surfactant, favorably interacts with the anionic nucleic acids (DNA and RNA), including proteins and other biopolymers (polysaccha-
ride and secondary metabolites). Therefore, due to the higher affinity of CTAB with both nucleic acids and other biopolymers, leads to reduced yield of RNA. On the other hand, anionic surfactant SDS does not interact with nucleic acids (anionic) but highly reactive with proteins and other polysaccharides and forms aggregates. Thus, SDS is more effective in separating RNA from other biopolymers during RNA extraction. Moreover, CTAB-LiCl method has a lengthy procedure and is time consuming compared to the modified SDS-LiCl method. Higher RNA yield with the modified SDS-LiCl extraction method suggests the absence of starch interference during RNA precipitation.

| Sample                  | Ambion TRIZOL | RNeasy plant mini kit | Furtado¹ | CTAB-LiCl method | Modified SDS-LiCl method |
|-------------------------|---------------|-----------------------|----------|------------------|--------------------------|
| A260/A280 ratio         |               |                       |          |                  |                          |
| Tx control              | 1.70 ± 0.02   | 3.14 ± 2.70           | 1.68 ± 0.06 | 2.00 ± 0.09       | 2.22 ± 0.08              |
| Tx HNT stress           | 1.76 ± 0.05   | 4.96 ± 1.0            | 1.86 ± 0.18 | 2.10 ± 0.06       | 2.15 ± 0.09              |
| Tascosa control         | 1.82 ± 0.10   | 3.60 ± 3.70           | 1.64 ± 0.18 | 2.14 ± 0.06       | 2.20 ± 0.01              |
| Tascosa HNT stress      | 1.88 ± 0.08   | 3.20 ± 2.46           | 1.71 ± 0.10 | 2.08 ± 0.07       | 2.18 ± 0.02              |
| A260/A230 ratio         |               |                       |          |                  |                          |
| Tx control              | 0.39 ± 0.20   | 0.02 ± 0.03           | 0.17 ± 0.11 | 2.09 ± 0.22       | 2.32 ± 0.02              |
| Tx HNT stress           | 0.78 ± 0.82   | 0.02 ± 0.02           | 0.38 ± 0.02 | 1.88 ± 0.48       | 2.26 ± 0.05              |
| Tascosa control         | 0.51 ± 0.14   | 0.03 ± 0.02           | 0.63 ± 0.15 | 1.98 ± 0.25       | 2.33 ± 0.06              |
| Tascosa HNT stress      | 0.83 ± 0.28   | 0.03 ± 0.02           | 0.69 ± 0.27 | 2.19 ± 0.08       | 2.31 ± 0.08              |

Table 1. Comparative evaluation of A260/A280 and A260/A230 ratios of RNA extracted from mature wheat seeds using different RNA isolation methods, using Nanodrop ND-1000 spectrophotometer for assessing the RNA quality. Control samples were collected from plants grown under ambient temperature, while high night temperature (HNT) stress samples were collected from plants grown under custom built heat tents with +3.2 °C higher night temperature than ambient temperature (22.7 °C) during grain filling (Hein et al.¹⁸). Values presented are the mean ± standard deviation of three technical replicate samples. Tx-Tx86A5606.

was performed with a reaction mixture containing 8 μl of SYBR Green master mixture (Applied Biosystems SYBR Green, Carlsbad, CA, USA), 2 μl each of forward and reverse primers (5 μM), and 20 ng cDNA (2 μl) to make the final reaction volume of 14 μl. RT-qPCR (CFX96-Touch Real-Time PCR Detection System, Bio-Rad Inc, Hercules, CA, USA) was performed at 95 °C for 5 min, and 40 cycles of 95 °C for 30 s and 58 °C for 30 s. Relative gene expression was quantified using the 2−ΔΔCT method as described in Livak and Schmittgen²², and BMY gene expression was normalized using Actin as a reference gene. A melt curve profile was incorporated in the thermal cycling method to check the specificity (with no gDNA contamination, no primer dimers, and no non-specific product) of the RT-qPCR reaction.

Relative gene expression was quantified using the 2−ΔΔCT method as described in Livak and Schmittgen²², and...
Components that make SDS-LiCl effective in extracting quality RNA from seeds and other tissue.

Even though the extraction buffer used in the SDS-LiCl method contains NaCl, PVP, Tris/HCl, EDTA, β-mercaptoethanol, and SDS (see supplementary document), similar to existing methods, we optimized the concentrations of these chemicals for seeds and other tissues including roots. The chemical composition confirmed that 2.5 M NaCl, 2.5% PVP, 100 mM Tris, 25 mM EDTA, 2.5% β-mercaptoethanol, and 2% SDS, helps obtain high quality and yield of RNA without the interference of starch and other impurities. The enhanced yield and quality of RNA achieved in the modified SDS-LiCl method compared to CTAB-LiCl is due to the addition of SDS, and higher concentration of other chemicals (PVP and NaCl) in the extraction buffer. In agreement with previous studies, an increased concentration of NaCl, PVP, β-mercaptopoethanol and SDS in the extraction buffer of the modified SDS-LiCl method, not only promotes cell lysis but also prevents RNAse and polyphenols in the isolated RNA1,2,5,8,30–32. In addition, post-grinding addition of SDS directly to the homogenate suppressed excessive foaming and facilitated proper lysis of seed samples and prevented starch solidification1,2, 33. Additionally, in the modified SDS-LiCl method, phase purification using acidic phenol (pH 4.3) saturated with citrate buffer helped to improve the RNA quality by separating RNA from bulk starch residues compared to CTAB-LiCl method. Efficient separation of bulk starch residues and degraded proteins during the centrifugation of RNA containing aqueous phase using acid phenol saturated with 0.1 M citrate buffer (pH 4.3) supports earlier findings2.

A combination of 2 M Lithium chloride and 1/10 volumes of 3 M sodium acetate (pH 4.8) possibly facilitated better RNA precipitation. This is in agreement with previous studies wherein lithium chloride and sodium acetate were used to promote precipitation and resulted in high RNA yield2,3,34. Adding both lithium chloride and sodium acetate in the precipitation step enhanced the yield, whereas in CTAB-LiCl method, there was no phenol separation and LiCl alone was used for precipitation. Overall, the RNA obtained from modified SDS-LiCl method from field-grown wheat seeds under both control and HNT stress had high yield and quality, demonstrating a marked improvement compared to all other existing methods.

High quality RNA extracted using SDS-LiCl method suitable for downstream applications.

PCR amplification of EPSPS gene full length CDS with 1.2 kb product size and a prominent band provides support that cDNA obtained from RNA using the modified SDS-LiCl method is high quality compared to other methods. In addition, it indicates that RNA integrity is sufficient to amplify large size gene fragments (Supplementary Fig. S6). The RT-qPCR results of total RNA quantification show a tendency to obtain lower Cq values for the cDNA from RNA samples extracted by the modified SDS-LiCl method compared to other four different methods (Supplementary Table S1). When comparing the reproducibility expressed in terms of SEM values, the best results were obtained for samples extracted by the modified SDS-LiCl method. Comparatively,
using other methods, high Cq values and SEM were obtained suggesting that RNA extracted using modified SDS-LiCl method resulted in high quality cDNA with lesser interference of contaminants.

Quantitative real-time PCR (RT-qPCR) was performed with the cDNA synthesized from RNA isolated from mature seeds using the modified SDS-LiCl method. Amplification of all the samples with straight amplification curves and Ct values within the range permitted to perform meaningful analysis of expression data (Fig. 5, see Impa et al.20 for published expression data on developing seeds using the modified SDS-LiCl method). The expression analysis of 21 genes encoding enzymes involved in the starch metabolism in developing wheat seeds exposed to a range of increasing night temperatures was carried out using the RNA extracted by the modified SDS-LiCl method (See Impa et al.20 for published expression data). A single peak in the melt curve analysis and lower standard errors between the Ct values for a gene indicated the specificity of the primers to bind to the cDNA synthesized from the RNA, with no interference of PCR inhibitors (Fig. 5b).

Modified SDS-LiCl method equally effective on other tissues exposed to different abiotic stresses. Further, the modified SDS-LiCl method was used successfully to extract high-quality RNA from germinated wheat seeds, flag leaves, and root tissues that were exposed to cold, freezing and HNT stress. Agarose gel electrophoretic separation and Nanodrop UV spectrophotometer measurements of total RNA from different...
tissues of wheat genotypes exposed to different abiotic stresses were confirmed for RNA quality with A260/280 and A260/A230 ratios of ≥ 2 and a similar RNA yield to that of control samples (Fig. 6; Table 3; Supplementary Figs. S7–S9). Analysis of RNA samples by Bioanalyzer Agilent 2100 further confirmed high-quality RNA with prominent peaks of rRNA in electropherograms with a higher RIN values (> 7) for various wheat plant tissues RNA isolated by the modified SDS-LiCl method (Fig. 7; Supplementary Figs. S10–S12). Our method reliably provided ample yield and high quality RNA from developing wheat seeds that were exposed to a range of increasing night temperatures (15–27 °C) under controlled environment chambers (Supplementary Figs. S13–S18; Supplementary Table S2; also see Impa et al.20 for additional gene expression data). Previous studies have reported decreased concentration of RNA from the samples subjected to abiotic stress12–14,35. Similarly, in our study we observed less RNA concentration in the developing grain samples of K807077M-1 subjected to HNT stress compared to control using the SDS-LiCl method (Supplementary Table S2). However, a similar trend was not observed with the RNA samples extracted using the SDS-LiCl method that were subjected to HNT or other abiotic stresses. Also, RNA extracted using other methods (Ambion TRIZOL, RNeasy Plant Mini Kit (Qiagen), Furtado6) did not differ between control and HNT stress samples due to interference of starch. Similarly, an independent study recorded no difference in the RNA yield of wheat leaf tissue subjected to freezing stress13.

Previous studies have reported that reduced concentration of RNA and interference of secondary metabolites in RNA extraction depends on the severity of stress and tissue age12 also the type of tissue13. Hence, the lack of

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**Figure 4.** Electropherograms of total RNA of mature wheat seeds extracted using different RNA isolation methods with the Eukaryote Total RNA Nano Assay on the Agilent 2100 Bioanalyzer. X-axis units in nt (Nucleotides); Y-axis units in FU (Fluorescence Units). Electropherograms of RNA ladder (a), Ambion TRIZOL (b), RNeasy Plant Mini Kit (Qiagen) (c), Furtado6 method (d), CTAB-LiCl method (e), and modified SDS-LiCl method (f). RIN: RNA integrity number.
Table 2. Comparative quantification of mature wheat seed RNA yield evaluated by Nanodrop ND-1000UV spectrophotometer and RNA integrity numbers analyzed by Bioanalyzer (Agilent 2100) for assessing the quantity and quality of RNA extracted using different isolation methods. Control samples were collected from plants grown under ambient temperature, while high night temperature (HNT) stress samples were collected from plants grown under custom built heat tents with +3.2 °C higher night temperature than ambient night temperature (22.7 °C) during grain filling (Hein et al.18). Tx-Tx86A5606. Values presented are mean ± standard deviation of three technical replicate samples. NA – Not available; these samples had low quality RNA and hence RIN values were not obtained from the bioanalyzer.

| Sample          | Ambion TRIZOL RNA Yield (μg/100 mg FW) | RNeasy plant mini kit RNA Yield (μg/100 mg FW) | Furtado® method RNA Yield (μg/100 mg FW) | CTAB-LiCl method RNA Yield (μg/100 mg FW) | Modified SDS-LiCl method RNA Yield (μg/100 mg FW) | RIN (RNA integrity number) values |
|-----------------|---------------------------------------|-----------------------------------------------|----------------------------------------|------------------------------------------|-------------------------------------------------|----------------------------------|
|Tx Control       | 21.12 ± 13.54                         | 0.55 ± 0.23                                   | 0.84 ± 0.10                            | 6.74 ± 1.17                              | 28.99 ± 2.12                                    | 5.50                              |
|Tx HNT stress    | 23.09 ± 15.58                         | 0.44 ± 0.17                                   | 3.95 ± 0.17                            | 4.94 ± 1.05                              | 19.41 ± 1.23                                    | 2.60                              |
|Tascosa Control  | 28.22 ± 21.53                         | 0.62 ± 0.39                                   | 1.51 ± 0.91                            | 6.09 ± 1.73                              | 31.22 ± 1.24                                    | 6.50                              |
|Tascosa HNT stress | 17.04 ± 7.04                          | 0.56 ± 0.24                                   | 1.24 ± 0.81                            | 5.46 ± 1.40                              | 37.24 ± 1.29                                    | 2.70                              |

Figure 5. Quantitative real-time PCR (RT-qPCR) assay using the RNA extracted from modified SDS-LiCl method. (a) Amplification curves shows the Ct values of PCR amplification fragments of two genes (Actin and β-amylase) from cDNA synthesized using RNA extracted from the modified SDS-LiCl method and no reverse transcriptase (RT) controls. (b) RT-qPCR melt curves of the Actin and β-amylase genes. (c) Comparative expression of β-amylase gene relative to Actin in wheat seeds collected from plants grown under control and high night temperature (HNT) stress. Tx-Tx86A5606.
a significant impact on RNA concentration in this study could be due to the short duration of exposure under different stresses or due to moderate HNT stress levels.

Modified SDS-LiCl method also yielded high quality RNA from other cereals seeds (mature and developing maize seeds and developing sorghum seeds) rich in starch, protein, and polyphenols (Supplementary Figs. S19, S20). RNA extraction protocols and commercial kits are limited to specific species and/or tissue, whereas the

Table 3. Nano-spectrophotometric analysis of RNA yield, purity and Integrity Numbers (RIN) using Bioanalyzer (Agilent 2100) for the total RNA isolated using the modified SDS-LiCl method from germinated wheat seeds, flag leaf and root tissue exposed to different abiotic stresses. Yield and quality were assessed by absorption spectra and ratios of A260/A280 and A260/A230. Tx- Tx86A5606. *Values represent the mean ± SD from at least three technical samples.

|                         | RNA yield (μg/100 mg FW) | A260/A280 ratio | A260/A230 ratio | RIN |
|-------------------------|--------------------------|-----------------|-----------------|-----|
| Germinated seeds        |                          |                 |                 |     |
| Tx control (30 °C)      | 33.92 ± 6.96             | 2.08 ± 0.07     | 2.18 ± 0.17     | 7.10|
| Tx cold stress (15 °C)  | 37.95 ± 6.80             | 2.08 ± 0.08     | 2.32 ± 0.03     | 8.70|
| Tascosa control (30 °C) | 29.77 ± 3.88             | 2.17 ± 0.01     | 2.31 ± 0.03     | 8.30|
| Tascosa cold stress (15 °C) | 36.69 ± 5.84         | 2.09 ± 0.08     | 2.17 ± 0.11     | 8.40|
| Flag leaf               |                          |                 |                 |     |
| Tx control (26/15 °C)   | 19.53 ± 5.05             | 2.16 ± 0.03     | 2.13 ± 0.01     | 6.90|
| Tx HNT stress (26/23 °C)| 25.17 ± 3.67             | 2.15 ± 0.04     | 2.31 ± 0.01     | 7.00|
| Tascosa control (26/15 °C) | 48.40 ± 5.30         | 2.18 ± 0.01     | 2.27 ± 0.11     | 7.60|
| Tascosa HNT stress (26/23 °C) | 30.66 ± 3.71        | 2.14 ± 0.04     | 2.28 ± 0.02     | 6.90|
| Root                    |                          |                 |                 |     |
| Tx control (25 °C)      | 15.00 ± 3.71             | 2.12 ± 0.01     | 2.26 ± 0.01     | 8.90|
| Tx freezing stress (−4 °C) | 16.79 ± 2.12           | 2.12 ± 0.05     | 2.20 ± 0.07     | 8.10|
| Tascosa control (25 °C) | 48.76 ± 6.96             | 2.20 ± 0.05     | 2.15 ± 0.11     | 7.60|
| Tascosa freezing stress (−4 °C) | 54.41 ± 7.86        | 2.11 ± 0.02     | 2.23 ± 0.09     | 8.80|

Figure 6. Agarose gel electrophoresis of total RNA extracted using the modified SDS-LiCl extraction method from germinated wheat seeds (a), flag leaf (b) and root (c) tissues exposed to different abiotic stresses. RNA extracted from two genotypes of wheat seedlings germinated under control (30 °C) and cold stress conditions (15 °C) (a), Lane 1: Tx- control, Lane 2–3: Tx- cold stress, Lane 4: Tascosa Control, Lane 5–6 Tascosa Cold stress. RNA isolated from the flag leaves of two wheat genotypes grown under control (26 °C/15 °C) and HNT stress (26 °C/23 °C) (b), Lane 1–2: Tx Control, Lane 3–4: Tascosa Control, Lane 5–6: Tx HNT stress, Lane 7–8: Tascosa- HNT stress. RNA extracted from the roots of wheat genotypes grown under control (25 °C) and freezing stress (− 4 °C) (c), Lane 1–2: Tx Control, Lane 3–4: Tx Freezing stress, Lane 5–6: Tascosa Control, Lane 7–8: Tascosa Freezing stress. Tx-Tx86A5606. The images of agarose gel electrophoresis used in this figure for the various tissue types were cropped from the original source gel image provided in the supplementary source Fig. 3. The cropped images were auto corrected for the same brightness/contrasts using PowerPoint image tools.
modified SDS-LiCl method was suitable for extracting high quality RNA from various crop tissues differing in developmental stages and exposed to different abiotic stresses under controlled environment and field conditions.

In summary, the modified protocol was exclusively developed for extraction of RNA from cereal seed tissue containing high starch, as it is one of the major hurdles challenging functional studies involving developing or mature seeds. This protocol is cost-effective compared to commercially available kits and has been demonstrated to be successful in obtaining quality RNA from mature wheat grains, while TRIZOL, CTAB, and other kits generally failed. The robustness of the modified SDS-LiCl method helped to extract significantly higher yield and quality of RNA from different wheat plant tissues, including, mature, developing and germinated seeds, leaves and roots, exposed to various abiotic stresses, whereas previously published protocols have limited the protocol to either seed tissues of wheat or other cereals1,2,5,24 or only leaf tissues3. The protocol standardized here has been modified to be a rapid yet reliable, using basic laboratory chemicals that are easily accessible by researchers, whereas previously methods needed more chemicals in the extraction buffer, or expensive reagents kits1,2,5. Besides, the versatility of the universal protocol is demonstrated by extracting high-quality RNA from other cereal crop seeds. A comparative assessment including the advantages of the SDS-LiCl method compared to previous protocols is presented in Table 4.

**Conclusion**

In conclusion, we have optimized a robust method for extracting high yield and quality RNA from wheat seeds, free from starch and other contaminates. The obtained RNA quality was appropriate for downstream applications demonstrating the effectiveness of the protocol with seeds, that contain high starch and polysaccharides. The modified SDS-LiCl method extracted significantly higher yield and quality of RNA from different wheat tissues, including mature, developing and germinated seeds, leaves and roots exposed to different abiotic stresses. Besides, the versatility of the protocol was further strengthened with high quality RNA extracted from field grown maize and sorghum seeds.

**Data availability**

All related data not presented in the manuscript are made available in the supplementary documents.
| Type of extraction buffer | Li and Trick<sup>1</sup> | Wang et al.<sup>2</sup> | Singh et al.<sup>3</sup> | Ambion TRIZOL | RNeasy plant mini kit (Qiagen) | Furtado<sup>6</sup> | CTAB-LiCl | SDS-LiCl method |
|---------------------------|------------------------|-----------------------|------------------------|----------------|-------------------------------|----------------|----------------|----------------|
| SDS + LiCl + GS           | SDS + GITC             | Tris + GHC            | GITC                   | GHC            | GITC + GHC                     | CTAB           | SDS            |
| No. of chemicals in the Ext.Buf | 9                     | 6                     | 9                      | 1 (Mix)        | 1 (Mix)                       | 2 (Mix)        | 7              | 6              |
| Requirement of commercial kit reagents | No                   | Yes                   | No                     | Yes            | Yes                           | Yes            | No             | No             |
| Precipitation chemicals   | IPA + NaCl             | IPA + SA + Ethanol    | SA + Ethanol           | IPA            | Ethanol                       | Ethanol        | LiCl           | LiCl + SA      |
| Tissue types              | Seed                   | Seed                  | Seed                   | Wide range but not suitable for seed | Wide range but not suitable for seed | Mature and developing seed | Leaf, shoot, fruit and seed | Mature, developing, and germinated seeds, root and leaf |
| Tested plant species      | Wheat, Rice and Maize  | Wheat, Rice, Sorghum and Maize | Wheat, Barley and Maize | Wide range of species | Wide range of species | Wheat | Wheat and 15 other plant species | Wheat, Sorghum and Maize |
| Time (From extraction until dry pellet) | 90 min               | 160 min               | 128 min               | 60 min         | 30 min                        | 14 h           | 36 min         | 62 min         |

Table 4. Comparing SDS-LiCl method with previously published RNA extraction protocols. SDS sodium dodecyl sulfate, GITC guanidine isothiocyanate, CTAB cetyltrimethylammonium bromide, GS guaninium sulfate, GHC guanidine hydrochloride, SA sodium acetate, IPA isopropanol, Ext.Buf. extraction buffer, Mix mixture of chemicals provided in one reagent by the company.

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Author contributions

A.R.V, I.M.S, S.V.K.J designed and implemented the experiments; A.R.V carried out the experiments; C.J.D. provided technical advice; A.R.V, I.M.S., C.J.D., S.V.K.J interpreted the data and drafted the manuscript; All authors contributed in editing and finalizing the manuscript.

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Competing interests

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Additional information

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