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Isolation of high-quality RNA from plant seeds

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Abstract

The apple (*Malus domestica* Borkh.) is one of the major fruit tree crops, but it hasn't been well-studied as a breeding object for molecular investigations. It is important to develop reliable and rapid methods that allow the preparation of plant material for future research. We introduce a quick and simple method for isolating high-quality RNA from lipid-rich apple seeds (*M. domestica* cv. Golden Delicious). Our method does not employ highly toxic reagents, because we exclude phenol, 2-mercaptoethanol and others. The chemical composition of the extraction buffer is simple and has a minimum level of toxicity. We showed that, in chaotropic conditions (i.e., with lithium chloride-urea), silica (SiO_2) can bind with the lipids and RNA will remain in the solution. The extracted RNA was of high quality and we successfully used it for synthesizing cDNA and RT-PCR. The protocol developed by us can be useful for researchers working with RNA extraction from plant seeds.

Keywords: *Malus domestica,* seeds, RNA, lipids, silica, RT-PCR

Introduction

Modern molecular studies play an important role in investigating the adaptive abilities of plants and their inheritance mechanisms. Extraction of high-quality RNA is a preliminary and necessary step in most genetic studies that aim to determine the level of gene expression and regulation, etc. However, the process of RNA extraction from different tissues of wooden plants is time-consuming. It requires stepwise purification of the initial plant material from high levels of polysaccharides, proteins, lipids, polyphenols and other secondary metabolites (Gambino, Perrone and Gribaudo, 2008; Xu et al., 2009; Paula et al., 2012; Tong et al., 2012; Skipars et al., 2014; Islam and Banu, 2019).

Apple leaves, buds and fruits contain a high quantity of polyphenols and polysaccharides. Mature apple seeds are protein-rich and lipid-rich. Proteins constitute about 40–50 % of the total weight of the seed; oil — about 20 % (Tian, Zhan and Li, 2010). The extracted oil is composed of 80 % fatty acids such as linoleic (more than 50 %), oleic, palmitic, stearic and arachidonic acids (Lu and Foo, 1998; Tian, Zhan and Li, 2010; Radenkovs et al., 2018). Cellulose, fructose, glucose, sucrose, polyphenols and cyanogenic glycoside (amygdalin) are present in small amounts in apple seeds. Phloridzin is a major compound of the polyphenol group that accounts for 70–90 % of the total amount of polyphenols (Tian, Zhan and Li, 2010; Fromm et al., 2012; Xu et al., 2016).

For extraction of RNA from various apple tissues (leaves, shoots, fruits, buds), researchers use either CTAB (cetyltrimethylammonium bromide) buffer or a buffer containing guanidinium isothiocyanate. Guanidinium isothiocyanate is a strong chaotropic agent which leads to denaturation of ribonucleases (Lay-Yee et al., 1990),

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but it has a highly toxic effect. CTAB buffer often contains 2–10 % 2-mercaptoethanol (Gasic, Hernandez and Korban, 2004; Asif et al., 2006; Gambino et al., 2008; Ferrero et al., 2015), which also contains toxic substances that affect the respiratory tract. In some studies on RNA isolation from polysaccharide- and lipid-rich seeds, the extraction solution was heated at 75–80 °C to remove the lipids and phenol-chlorophorm-isoamyl alcohol solution was added to remove the proteins (Birtic and Kranner, 2006; Huang et al., 2012; Lan et al., 2013). Therefore, the RNA isolation protocol in different variations is a very toxic process. In addition, a great variety of commercial kits and reagents for RNA isolation exists, including TRI Reagent® (Sigma, USA), ConcertTM (Invitrogen, USA), TRIZOL® (Invitrogen Life Technologies, USA), RNeasy plant mini kit (Qiagen, Germany) and others; they are not only limited in use but also quite expensive and toxic (Paula et al., 2012; Skipars et al., 2014; Islam and Banu, 2019).

In this work, we have developed a new effective, less toxic and simple RNA extraction method for conducting molecular studies with lipid-rich seeds of apple cultivars and their hybrids.

Methods and materials

Plant material. We collected the plant material in open ground on the territory of the central department of the Experimental Production Facility in Krasnodar Krai, Russia (45.15893 N 38.93253 E) in 2018–2020. The fruits of the apple cultivar (*Malus domestica* Borkh. cv. Golden Delicious) of the winter ripening were harvested from 10–15-year-old plants grown on rootstock M9. Seeds were stored at room temperature for 3–4 months.

Solutions and reagents for extraction. The solutions were prepared with deionized water. During the experiments, sterile disposable plastic materials were used. Glass, mortars and pestles, as well as SiO_2 powder, were heated for 4–6 h at 300 °C.

The RNA extraction buffer was as follows: 0.2 M Tris-HCl (pH 8.5; Helicon, Russia, Am-O497-0.5), 0.5 M lithium chloride (LiCl, Panreac, Spain, CAS-7447-41-8), 1 % sodium dodecyl sulfate (SDS; Helicon, Russia, Am-0227-0.1). The other reagents were chloroform-isoamyl alcohol (ratio 24:1; ECOS-1, Russia, CAS-67-66-3; LenReactiv, Russia, 010131); deproteinizing solution (8M LiCl, 8M urea; LenReactiv, Russia, 120427), 4 mM ethylenediamine tetraacetic acid (EDTA; Helicon, Russia, Am-OB105-0.1)); silicon dioxide (silica, SiO_2, LenReactiv, Russia, 100505); 70 % and 96 % ethanol.

RNA isolation (protocol).

1. Apple seed material (seed skin removed), about 0.4–0.5 g, was ground into a fine powder in liquid nitrogen in a mortar and pestle (pre-cooled in liquid nitrogen).
2. The ground tissue was transferred to extraction buffer in a ratio of 1:4.
3. The sample was mixed vigorously for 10 min and centrifuged at 5500 g for 2 min at 4 °C.
4. The supernatant was transferred to clean 2-ml microfuge tubes (SSIbio 1310-00, USA) and an equal volume of chloroform-isoamyl alcohol was added to the solution.
5. The sample was immediately vortexed for 5 min at room temperature and precipitated at 16000 g for 2 min at 4 °C.
6. The upper aqueous phase was transferred to a new clean tube, and purification was repeated again.
7. Two equal volumes of 96 % ice-cold ethanol were added to the upper aqueous phase and tubes were homogenized for 1 min and incubated for 20 min at –20 °C.
8. The sample was centrifuged at maximum speed (at 16000g) for 25 min at 4 °C.
9. The pellet was dried for 10–15 min and resuspended in 2 ml deionized water.
10. Cold LiCl-urea solution in an equal volume and 0.25g SiO_2 were added to the solution and vortexed for 5 min.
11. The solution was centrifuged at 16000 g for 2 min at 4 °C and the supernatant was kept overnight at 4 °C.
12. The sample was centrifuged at maximum speed for 50 min at 4 °C.
13. The pellet was washed twice with ice-cold 70 % ethanol and centrifuged at 16000 g and 4 °C for 10 min.
14. After air drying for 10–15 min, the precipitate was dissolved in a minimum volume of deionized water (30–40 μl).
15. The solution was treated with DNase I (Bio-Rad, USA) to remove DNA according to the manufacturer's instructions and stored at –20 °C for further use.

Assessment of isolated RNA purity and quantity. The quality and concentration of RNA obtained was determined spectrophotometrically at 230, 260, and 280 nm and by the A_{260}/A_{280} and A_{260}/A_{230} ratios using an Implen NanoPhotometer NP80 spectrophotometer (IMPLEN, USA).

RNA integrity was evaluated electrophoretically on 2.1 % agarose gel in non-denaturing conditions and on Tris-acetate-ethylenediamine tetraacetic acid (TAE) 1.2 % agarose gel in RNA samples denaturing conditions by applying 60 % formamide (Masek et al., 2005). Ethidium bromide in ultraviolet radiation was used to visualize 28S and 18S ribosomal RNA bands.

Real-time PCR analysis. For RT-PCR reverse transcription of 1–2 μg RNA was carried out as described by manufacturer's recommendation using MMLV revertase (Eurogen, Russia) and Oligo (dT) Primer (Eurogen,

Russia). RT-PCR was performed on a Roche Lightcycler 96SW1.1 (Roche, Switzerland) with the commercial qPCRmix-HS SYBR kit (Eurogen, Russia) using the SYBR Green I detection system according to the recommended protocol. The amplification conditions were selected and optimized depending on primer pairs. Primers were taken from the study of Japanese researchers (Saito et al., 2017) for apple gene of elongation factor (EF1) and gene of lipoxygenase (MdLOX1A). The sequences of the primers were as follows: EF1 (F): 5'–TCACATCAACATCGTCA–3; EF1 (R): 5'–TCGAACCTCTCAATCACACG–3'; MdLOX1A (F): 5'–TTCAAGGAAATCGGTGACAG–3'; MdLOX1A (R): 5'–ACTTCAGGCATTGGGAATC–3'.

The 25μl PCR reaction mix contained 5μl of 5 x qPCRmix-HS SYBR (Eurogen, Russia), 0.5 μM each primer and cDNA: 50 ng in the remaining volume of sterile nuclease-free water. The PCR conditions included: initial denaturation step at 95 °C for 2 min, followed by 25 cycles at 95 °C for 20 s, 60 °C for 30 s, 72 °C for 30 s and the melting curve beginning at 95 °C.

The results of PCR were recorded as Ct (threshold cycle) values. The Ct value is defined as the cycle number at which a statistically significant increase in the fluorescence can be first detected.

Efficiency of RNA isolation method. To prove the efficiency of the described protocol we conducted several experiments.

1. The isolation of RNA was made with SiO_2 in LiCl-urea solution and without it to compare the obtained results. To exclude the possibility of RNA binding to SiO_2, we estimated the amount of RNA in the precipitate, obtained by centrifuging LiCl-urea solution and separating the aqueous phase from it. This precipitate was dried at 37 °C for 1–2 hours, then washed twice with cold 70 % ethanol, followed by centrifugation at 16000 g for 10 min at 4 °C. Next, it was dried from ethanol for 10–15 min at room temperature, and then we added 2 ml of water to it. After the centrifugation, the aqueous phase was determined spectrophotometrically for the presence of RNA.

2. We tested two kits — AurumTM Total RNA Mini Kit (Bio-Rad, USA) and Quick-RNA MiniPrep (Zymo Research, USA) — and the CTAB method of RNA extraction from apple seeds. The kit protocols were used following the manufacturers' recommendations. The CTAB method was used according to the protocol reported by Tong Z. et al. (2012) with some modification (Sundyreva et al., 2018).

3. We compared three apple cultivars — Idared, Granny Smith and Orphey — using our method.

4. We used thin layer chromatography on a Sorbfil mechanical applicator (IMID, Russia) to determine lipid contamination at several steps of RNA isolation. Lipids were identified in the extraction solution after two treatments with chloroform-isoamyl alcohol, 96 % ethanol and silica. The samples were applied to Sorbfil PTSX-P-V plates (IMID, Russia). The choice of eluent and derivatization of the plates was substantiated according to the developed protocol of K. Korte and L. Casey (1982). The seed oil content was determined using the nuclear magnetic resonance method (NMR) on an AMB 1006M NMR analyzer (NMR analyzer oil content and moisture AMV-1006M, VNIIMK, Russia; Prudnikov, Vityuk and Zverev, 2003). The fatty acid composition of seed oil was determined using the gas chromatograph Chromatec Crystal 5000 (Chromatec Crystal, Chromatec, Russia) with an automatic dispenser on a capillary column SolGel-Wax (30m*0.25 mm*0.5μl) SGE 054785 (SGE, Australia), in a flow of helium carrier gas, at 25 cm/s, at temperature programming within 185–235 °C, according to the Codex standard for vegetable oils (Codex Alimentarius, 2009).

The data are presented as means and standard errors from three or more experiments carried out in triplicate analytical repetition.

Results and discussion

To isolate high-quality RNA from lipid-rich and protein-rich apple seeds, we modified the extraction protocol and simplified the extraction buffer. The extraction buffer contained a detergent SDS (1 % SDS) and a low concentration of LiCl salt (0.5 M), which allowed the efficient separation of nucleic acids in the aqueous phase (Antonova et al., 2010); whereas the presence of 0.2 M Tris-HCl helped to preserve nucleic acids' integrity (Plotnikov and Bakaldina, 1996). The protein fraction and lipids were removed with an organic solvent — chloroform-isoamyl alcohol solution. Then, we performed nucleic acid precipitation in ice-cold 96 % ethanol. Previous studies showed that RNA binds to silica (Rott and Jelkmann, 2001). That is in contrast with our findings: RNA remained in the LiCl-urea solution, but not on silica particles. To verify these findings, we flushed out RNA from silica but still failed to detect any RNA (Table 1). Therefore, we suggest that SiO_2 binds lipids, not RNA. As noted earlier, apple seed oil consists of 80–90 % fatty acids (Lu and Foo, 1998; Tian, Zhan and Li, 2010; Radenkovs et al., 2018), where linoleic acid accounts for more than 50 %. We examined seeds of the Golden Delicious apple to determine their oil content and fatty acid composition. Our results are comparable with previously reported findings (Lu and Foo, 1998; Tian, Zhan and Li, 2010; Radenkovs et al., 2018). We revealed that the oil content of cv. Golden Delicious seeds is 26.5 % of DW. The fatty acid composition analysis revealed 13 fatty acids in apple seed oil. The main fatty

Table 1. Comparison of RNA extracting procedures and methods for cv. Golden Delicious seeds

RNA extraction procedures and methods	Sample mass, g	RNA concentration, ng/μl	A_{260}/A_{280}	A_{260}/A_{230}
Presented method — Urea-LiCl with SiO_2	0.5	447.70 ± 38.22	2.17	2.22
Presented method — Flushing with SiO_2	0.9	3 ± 0.04	1.84	0.53
Presented method — Urea-LiCl without SiO_2	0.5	171.37 ± 32.9	1.07	1.12
Kit — AurumTM Total RNA Mini Kit	0.06	4.15 ± 0.87	2.23	0.25
Kit — Quick-RNA MiniPrep	0.05	11.32 ± 0.98	2.24	0.29
CTAB-method	0.08	223.78 ± 27.93	2.15	2.3

acids comprised 96 % and were represented by palmitic (6.3 %), oleic (34.6 %) and linoleic (55.1 %) acids. Linoleic acid effectively absorbs ultraviolet light at a wavelength of 230 nm, which caused distortion of the results related to the A_{260}/A_{230} ratio in the experiments when RNA was isolated without silica (Table 1).

To separate RNA from DNA and proteins, the solution was incubated for 18–20 hours with urea and lithium chloride. In these conditions, ribonucleases were inhibited, and purified RNA precipitated. Then, the purified RNA was washed with ice-cold 70 % ethanol to remove LiCl contaminates. Several researches used the solution of 8M LiCl and 8M urea in the extraction buffer to obtain RNA from seeds of woody plants (Tai, Pelletier and Beardmore, 2004). However, together with high values of the A_{260}/A_{280} ratio (2.19), those authors did not provide values of the A_{260}/A_{230} ratio, which are fundamentally important for RNA isolation from lipid-rich apple seeds.

To exclude the possibility of coprecipitation of DNA molecules with RNA, dissolved RNA was treated with DNase. The content of isolated RNA in seeds of the cultivar Golden Delicious was more than ~200–400 ng/μl and the A_{260}/A_{280} and A_{260}/A_{230} ratios were close to 2 and higher than 2, respectively (Table 1 and 2). These values indicate that RNA preparations were free of contaminants including proteins, lipids and polysaccharides.

Surprisingly, RNA extraction using a column-based method with standard commercial kits AurumTM Total RNA Mini Kit and Quick-RNA MiniPrep yielded an extremely small concentration of RNA, not more than 4–11 ng/μl (Table 1). Since the A_{260}/A_{230} ratio was about 0.3, these RNA samples cannot be used for further studies. Good results were applied by using the CTAB method (Table 1). The A_{260}/A_{230} and the A_{260}/A_{280} ratios ranged from 2.15 to 2.3, and RNA concentration was greater than 200 ng/μl. Despite a high quantity of RNA, 10 % 2-mercaptoethanol was used in this method.

The quality and quantity of isolated RNA was confirmed by samples from several winter cultivars of foreign and Russian selection — Idared, Granny Smith and Orphey. The concentration of RNA ranged from 240 to 370 ng/μl, with the A_{260}/A_{280} and A_{260}/A_{230} ratios being 1.78–2.15 and 2.08–2.22, respectively (Table 2). These results indicate that the samples were free from proteins, polysaccharides and lipids.

Table 2. Comparison of RNA content from apple seeds of different cultivars using presented method

Cultivars	Sample mass, g	RNA concentration, ng/μl	A_{260}/A_{280}	A_{260}/A_{230}
Golden Delicious	0.4	266.39 ± 35.5	1.98	2.08
Idared	0.4	241.82 ± 3.70	2.15	2.22
Granny Smith	0.4	370.04 ± 3.92	1.78	2.11
Orphey	0.4	293.18 ± 0.78	1.87	2.19

To verify the purification of the samples from lipids at different steps of RNA extraction, we used the method of thin layer chromatography. We estimated samples taken during three steps: 1) the solution of chloroform-isoamyl alcohol after centrifugation and separation of the aqueous phase (after the first and second purification); 2) the solution of 96 % ethanol after RNA deposition; 3) SiO_2 precipitate after centrifugation in the LiCl-urea solution. We found that lipids were present on the chromatogram at all three steps (Fig. 1). At the first step, there were mainly triglycerides (Rf=0.68) and free fatty acids (Rf=0.59; 1–4, Fig. 1). Therefore, we suggest that the purification with chloroform-isoamyl alcohol makes it possible to remove most of the triglycerides from the solution but is not sufficient to entirely clean the samples from lipids. At the second step, in the concentrated ethanol extract, in addition to fatty acids, there were three fractions of diglycerides (Rf=0.31) and monoglycerides (Rf=0.05). The polar lipids, which stayed at the start during elution, were mainly phospholipids (5–6, Fig. 1). The final step of purification from lipids was carried out by silica (7–8, Fig. 1): silica bound with the lipids, taking them from the solution with remaining RNA.

Electrophoresis of isolated RNA on a 2.1 % agarose gel in non-denaturing conditions showed clear separation of 28S and 18S rRNA, indicating its good quality

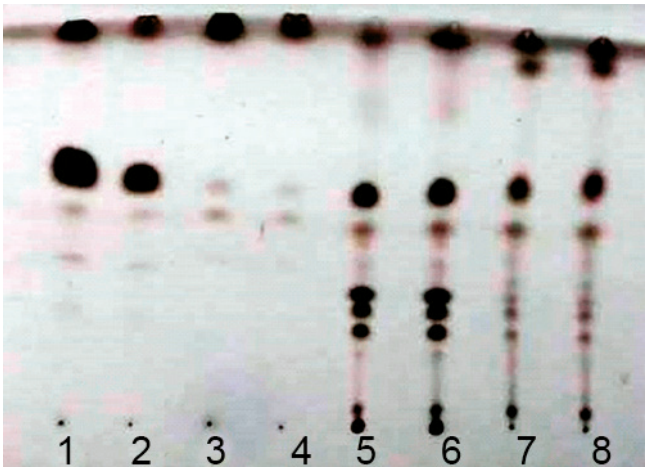

Fig. 1. Chromatogram of lipids obtained from different RNA extraction steps. 1-2 — after first treatment by chloroform-isoamyl alcohol, 3-4 — after second treatment by chloroform-isoamyl alcohol, 5-6 — after treatment by 96 % ethanol; 7-8 — after treatment by silica.

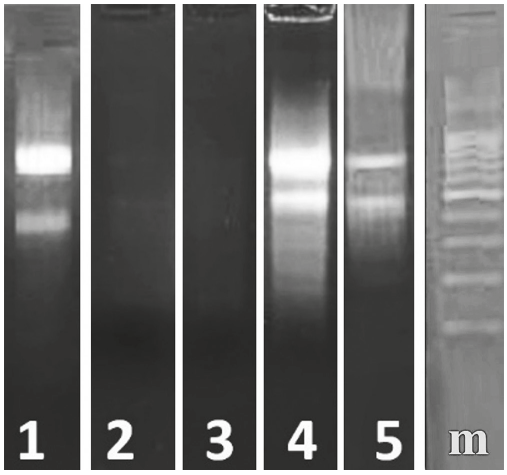

Fig. 2. Agarose gel electrophoresis of total RNA isolated from apple seeds of cv. Golden Delicious using various methods. Lane 1 — presented method; lane 2 — presented method without SiO_2; lane 3 — Quick-RNA MiniPrep; lane 4 — CTAB-method; lane 5 — presented method; lane m — marker (100–1000 bp); using denaturing RNA samples for electrophoresis in TAE agarose gel.

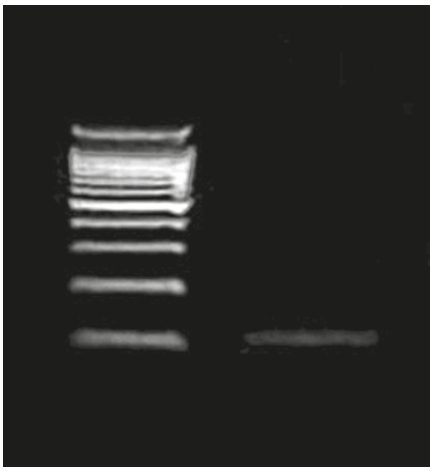

Fig. 3. PCR amplified products separated in agarose gel for the primer EF1 gene using the total RNA isolated by presented method.

(1, Fig. 2). Nevertheless, RNA samples isolated using a kit and urea-LiCl method without SiO_2 were completely unusable (2, 3, Fig. 2). RNA extracted by the CTAB method showed good quality with no DNA, protein or lipid contamination (4, Fig. 2). We also applied denaturation of RNA samples extracted by our method using 60 % formamide in TAE 1.2 % agarose gel, which demonstrated clear rRNA bands (5, Fig. 2).

The RNA extracted by our method was successfully used for reverse transcription into cDNA. Our RT-PCR results revealed that the qPCR cycle threshold (Ct) for EF1 gene was between 18.68–21.32 cycles, in different samples. The melting curve was specific, with a single peak occurring at 83 °C for EF1 gene. Notably, MdLOX1A gene expression was not observed in the studied samples. The most likely explanation is that the expression of the lipoxygenase enzyme gene, which catalyzes the breakdown of fatty acids, does not occur in resting mature seeds of apple tree (Goulao and Oliveira, 2007). A clear PCR product was detected in the agarose gel (Fig. 3).

It should be noted that the concentration of isolated total RNA is comparable to the RNA values obtained with other methods. Moreover, in mature fruits of the apple tree, the values range from 15 to 40 μg/g FW (Lay-Yee et al., 1990; Gasic, Hernandez and Korban, 2004; Asif et al., 2006); in leaves—from 170 to 540 μg/g FW (Moser et al., 2004; Gambino, Perrone and Gribaudo, 2008); in buds—from 650 to 780 μg/g FW (Gasic, Hernandez and Korban, 2004); in seeds — the average value is 140–160 μg/g FW (Dal Cin et al., 2005). After recalculating concentration values of the extracted RNA, our results were slightly lower — from 60 to 100 μg/g FW. Other researchers used phenol-chloroform-isoamyl alcohol solution (25:24:1) in a volume of 10 ml per sample, preheating phenol to 65 °C. Our RNA extraction method excludes phenol. The CTAB method showed good results for comparison with our method too, but we didn't use highl toxic 10 % 2-mercaptoethanol in extraction buffer with preheating.

Conclusion

We propose a new method for RNA isolation from lipid-rich and protein-rich apple seeds using silica. Our results showed that the presented method is reliable: the preparations of isolated RNA were successfully purified from polysaccharides, proteins and lipids. RT-PCR results and agarose gel electrophoresis confirmed RNA integrity. Other advantages of this method include the good quantity of isolated RNA, the simplified composition of the extraction buffer, the availability of reagents, as well as the low level of toxicity due to the absence of highly toxic substances such as guanidine isothiocyanate, 2-mercaptoethanol and phenol. Moreover, our method is low-cost since it does not employ expensive commercial kits for RNA extraction.

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