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OPTIMIZATION OF EXTRACTION AND PURIFICATION OF RNA FROM PLANT BULBS

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RNA extraction involves several main stages, regardless of the method of extraction: homogenization, effective denaturation of proteins from RNA, inactivation of ribonuclease and removal of any DNA, protein, and some residual contamination. Isolation of undamaged intact RNA is challenging when the related tissue contains high levels of polysaccharides and phenols. Several efforts have been made towards the comparison and optimization of extraction and purification methods for RNA from plant tissues. This is dictated by the necessity of obtaining RNA of a good quality and in a sufficient quantity for further molecular analyzes. Plant storage organs (such as bulbs or seeds) rich in polysaccharide and polyphenolic compounds present distinct challenges for total RNA isolation. Such components, considered in this case as contamination, may bind and co-precipitate with nucleic acids and negatively affect later assays. Since standard routine protocols yield unacceptable results in bulbs, we have designed a new method for RNA extraction. We used two modified procedures (based on CTAB and sarkosyl reagents) of RNA extraction from so called "difficult plant material" and compared them to a popular RNA isolation base on the column isolation kit and TriPure reagent. Our modified protocols dealt with problems of both RNA degradation and low yield caused by co-purification with polysaccharides present in plant bulbs. In this study we have shown that improvement of the CTAB and sarkosyl method with a lyophilization step of plant tissues leads to isolation of high quality RNA from difficult material like storage organs of bulbous plants. The main changes in the procedure compared

to the previously described methods concerned the different order of lithium chloride and sodium acetate addition, lithium chloride concentration increase and modification of centrifugation conditions. Gel electrophoresis and spectrophotometer analysis confirmed the high quality and integrity of the obtained RNA. The modified procedures allowed for obtaining a satisfying amount of RNA concentration in the range from 280 to 950 ng/µl depending on the plant species. Thus, the demonstrated RNA isolation methods are efficient and can be used for plant material rich in polysaccharides, such as bulbs.

Key words: RNA isolation; polyphenols; polysaccharides; bulbs; ornamental plants

INTRODUCTION

Successful RNA isolation, in the result pure and not degraded RNA, is the first and critical step in genomic studies. The RNA dependent assays such as cDNA library construction, quantitative RT-PCR, ribonuclease protection assay, micro-arrays, Northern hybridization and RNA mapping require isolated RNA of high quality and purity. The use of low-quality RNA while performing complementary molecular tests may compromise the results of further applications, where most of them are very expensive and time consuming. Therefore, scientists need high-quality RNA for molecular bioassays for various applications. Although RNA is easily and successfully isolated from most cells and tissues, intact RNA extraction from storage organs like bulbs, rhizome or fruits is difficult because of the high level of polyphenols and polysaccharides. Plant storage organs are considered difficult to isolate nucleic acids. In addition to bulbs, seeds are considered as such. Apart from plant materials that contain large amounts of polysaccharides, samples with high levels of RNases, various different kinds of phenolics including tannins, low concentrations of nucleic acids (high water content), and fibrous tissues such as lignin that are difficult to break up and remove often cause problems during RNA isolation. It has been proven that the usage of commercially available kits makes it possible to succeed and obtain a good starting material for research. However, such sets are dedicated mainly for animal tissues and do not meet expectations in the case of RNA isolation from "difficult plant material". The plant cell structure, cell wall presence, and in particular the high content of primary and secondary metabolites may be a problem during RNA extraction and purification. The emerging methods for plant RNA isolation are rather a result of the classical method of modification by scientists in the lab (Rubio-Piña and Pérez, 2011; Morante-Carriel et al., 2014; Silva et al., 2016).

There are several basic methods for RNA extraction and they can be divided into three groups: 1) the column-based techniques that use phenol-free buffers and RNA recovery columns, 2) the combined phenol and column-based techniques in which phenol and chloroform are utilized in order to seperate RNA from other constituents and a column is used for RNA adsorption, 3) the phenol-based techniques that use organic solvents, separation of phase and RNA recovery by precipitation. In conventional methods for RNA isolation usually detergents such as SDS (sodium dodecyl sulphate) or CTAB (cetyltrimethylammonium bromide), denaturing organic solvents (chloroform, phenol), denaturing agents such as GITC (guanidinium isothiocyanate salt) or reducing agents (β-mercaptoethanol or dithiothreitol - DTT) are used. The methods which were developed for animal tissues as well as kits for RNA isolation of numerous biotechnology companies can also be used in the case of plant tissues such as parenchyma, meristems or organs like young roots or leaves. They are all based on the guanidine-phenol-chloroform method and can be successfully applied directly without any modifications. The key reagent in this method developed by Chomczynski and Sacchi (1987), is a mono-phasic solution of phenol and guanidine isothiocyanate, which in the commercial

industry is called TriReagent, TRIzol or TriPure. In this simple method, samples are directly homogenized in the presence of phenol-containing solution and then centrifuged. During centrifugation and after addition of chloroform, three seperate phases are formed in the samples: an upper aqueous phase containing RNA, a middle phase containing denatured proteins and genomic DNA (gDNA) and a lower organic phase. The upper phase with RNA is then recovered and nucleic acid is collected by alcohol precipitation and rehydration. Another RNA extraction procedure is based on the CTAB reagent. This method, originally developed for pine tree tissues (Chang et al., 1993), has been successfully used for RNA extraction from many plant tissues (Zeng and Yang, 2002; Gambino et al., 2008). There are also many modifications of this method. However, in case of tissue rich in polyphenols and polysaccharides, the method is ineffective and the quality and quantity of RNA does not fulfil expectations (Morante-Carriel et al., 2014).

Plant material presented in this report are bulbs of ornamental plant species. They are reproductive organs and also important nutrient storage, therefore studying the development mechanism of plant bulbs is the base to produce high-quality cut flowers. This is quite important due to the economic aspect. In addition, bulbous plants also have an important role in science. Studies carried out on these plants lead to the discovery of new enzymes (Świeżawska et al., 2014, 2015, 2017; Pawełek et al., 2017), as well as compounds that not only broaden knowledge about metabolic pathways but also give information of a wide range of chemical structures and interesting biological properties, showing pronounced antimalarial, anticancer and acetylcholinesterase inhibitory activity (Yoshihara et al., 2005, 2008; Masi et al., 2015). In these experiments, the final result would not have been possible to achieve without obtaining high quality RNA in an adequate amount. However, the received RNA was not purified using one, universal method but developed individually for each tested plant species. The main problem affecting the efficiency of RNA purification was significant concentration of polysaccharides, co-precipitating with nucleic acids or acting as inhibitors of many enzymes (Manning, 1991; Sharma et al., 2002). As a consequence, for several years there was less research on RNA extraction and further experiments on bulbs (Du et al., 2017; Yong et al., 2018), while many studies on roots, stems and flowers succeeded (Balk et al., 1999; Tian et al., 2015; Howlader et al., 2017; Li et al., 2018).

The aim of the current study was to determine the optimal method of RNA isolation from bulbs of ornamental plants. To achieve this goal, we utilized various bulbs, which contain a lot of various balance compounds in five different RNA isolation methods including conventional sets, popular methods and new formulation techniques. In addition, in order to evaluate pre-isolation procedure we analyzed the lyophylization process.

MATERIAL AND METHODS

PLANT MATERIAL

Five different ornamental bulbous plants: *Hippeastrum x hybridum, Hymenocallis x festalis, Narcissus pseudonarcissus* and *Narcissus jonquilla* belonging to *Amaryllidaceae* and *Iris hollandica* from *Iridaceae* were used for the study, as a source of RNA.

The bulbs were thoroughly washed and cut into small pieces and quick-frozen in liquid nitrogen. Half of the material was lyophilized (freeze-dried) for 24 h in a speedvac concentrator (Labconco). All samples were ground into fine powder in a sterile mortar and pestle in liquid nitrogen condition and stored at -80°C until the RNA extraction. For each isolation 100 mg of tissue was used. All RNA extractions from five studied plant species were performed in three biological replicates.

SOLUTIONS AND REAGENTS

(1) TriPure Isolation reagent was purchased from Roche.

(2) GeneMATRIX Universal RNA Purification Kit was obtained from EURx.

(3) RNA extraction buffer and SSTE buffer were prepared according to Chang et al. (1993). Extraction buffer consisted of 2% CTAB, 2% PVP, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaC1, 0.5 g/L spermidine, 2% β-mercaptoethanol (added just before use). SSTE contained 1.0 M NaCI, 0.5% SDS, 10mM TrisHC1 (pH 8.0), l mM EDTA (pH 8.0).

(4) RNA extraction buffer for the modified CTAB based method (Morante-Carriel et al., 2014) contained 300 mM Tris-HCl (pH 8), 25 mM EDTA, 2M NaCl, 2% CTAB, 2% PVPP, 0.05% spermidine, 2% β-mercaptoethanol (added just before use).

(5) RNA extraction buffer for the sarkosyl reagent based method consisted of 0.5% N-lauroylsarcosine sodium salt (sarkosyl), 0.1 M Tris-HCl, 0.2 M NaCl, 15 mM EDTA, 2% PVP-40, 8 µl/ml β-mercaptoethanol (added just before use).

RNA EXTRACTION METHODS

Five various methods of RNA extraction were tested. To extract total RNA from plant material 100 mg of tissue (lyophilized and non-lyophilized) was used. Finally RNA was dissolved in 50 µl of RNase free water.

In the first method TriPure reagent (Roche) was used for RNA isolation and purification, according to the manufacturer's instructions.

In the second method isolation was achieved using a Universal RNA Purification Kit (EURx) according to the manufacturer's instructions.

In the third method RNA extraction was done following Chang et al. (1993) protocol. This method was used as a classical CTAB protocol.

In the fourth method the modified CTAB protocol, based on Rubio-Piña and Pérez (2011) and Morante-Carriel et al. (2014) with our modifications, was tested. In the procedure 2 ml of pre-warmed (65°C) isolation buffer, prepared according to Morante-Carriel et al. (2014) protocol, was added to powdered tissue homogenized by vortexing, and incubated at 65°C (10 min), shaken every 2 min (Fig. 1). The volume of the added isolation buffer was doubled in comparison with the original method of Morante-Carriel et al. (2014), in order to increase the extraction efficiency and dilute the impurities including polysaccharide and polyphenols, avoiding viscous and gelly-like consistency. Then the mixture was extracted with an equal volume of chloroform:isoamyl alcohol (24:1), shaken for 1 minute and centrifuged at 5000 *x* g (10 min, 4°C). The aqueous layer was transferred to a new tube, an equal volume of chloroform:isoamyl alcohol (24:1) was added, vigorously mixed, and centrifuged at 10000 *x* g (10 min, 4°C). The aqueous layer was transferred to a new tube and 0.3 volume of 10 M LiCl was added, mixed and incubated for 30 min at -20°C. This step was essentially according to Rubio-Piña and Pérez (2011) protocol, however, instead of 8 M LiCl we used a higher concentration and shorter (reduced from 4 h to 0.5 h) incubation at -20ºC . Next, the sample was centrifuged (20000 *x* g, 30 min, 4°C), the supernatant was discarded and the pellet was dissolved by adding mqH_2O and 0.1 volume of 3 M sodium acetate. The sample was mixed by a vortex and 2 volumes of 96% ethanol were added. Compared to the Rubio-Piña and Pérez (2011) protocol, the steps of alcohol washing before water and sodium acetate addition were omitted. The sample was centrifuged (20000 *x* g, 20 min, 4°C), the supernatant was decanted and the pellet washed with 70% ethanol. The sample was then centrifuged (10000 *x* g, 5 min, 4°C), the alcohol fraction was removed and the semi-dry RNA pellet was dissolved in 50 µl of RNase-free water.

In the fifth method modification of sarkosyl reagent procedure, based on Accerbi et al. (2010), was used. An isolation buffer was prepared according to the protocol for guanidinium-free RNA isolation with 2% PVP-40 addition. 1 ml of isolation buffer was added to the tissue powder, then each sample was homogenized by vortexing and centrifuged (10 min, 4°C,

16000 *x* g). Compared to Accerbi et al. (2010), an additional centrifugation step was applied (Fig. 2). The clarified supernatant was transferred to a clean tube and an equal volume of phenol was added. The sample was thoroughly mixed for 5 min, then incubated on ice for 10 min and centrifuged (15 min, 4°C, 16000 *x* g). In Accerbi et al. (2010) protocol this centrifugation step does not occur. The aqueous layer was transferred to a clean tube, an equal volume of chloroform:isoamyl (24:1) and 0.1 volume of 3 M sodium acetate was added, the sample was mixed, incubated on ice for 15 min and centrifuged as described previously. The aqueous layer was transferred to a clean tube and an equal volume of phenol:chloroform:isoamyl (25:24:1) was added, the sample was mixed and incubated on ice for 15 min, then centrifuged as previously. This action was applied twice. The aqueous layer was transferred to a clean tube and an equal volume of isopropanol was added, the sample was carefully mixed and incubated for 24 h at -20°C. Subsequently, the sample was centrifuged (10 min, 4°C, 16000 *x* g). The pellet was washed by adding 1 ml of cold 70% ethanol, mixed and centrifuged (10 min, 4°C, 16000 *x* g). Alcohol was removed and the RNA pellet was dissolved in 50 µl of RNase-free water.

RNA EVALUATION

The concentration of isolated RNA was quantified by measuring the absorption at 260 nm, where 1 absorbance unit is equal to 40 µg/ml, at a pH of about 7.5. RNA quality was analyzed based on the 260 nm/280 nm and 260 nm/230 nm absorbance ratios using a Nanodrop spectrophotometer (Thermo Fisher Scientific). For high quality RNA, A_{260}/A_{280} ratio should be in the range of 1.9–2.1. RNA integrity was evaluated by electrophoresis on 1% agarose gel.

All experiments were carried out in three biological repetitions with three technical repetitions for each.

RESULTS

The success in RNA isolation can be assessed through the quantity, purity, and integrity of the recovered RNA. RNA purity was estimated using the absorbance ratio at 260 and 280 nm (A_{260}/A_{280}) and A_{260}/A_{280} ratio of 1.8～2.0 corresponds to a good RNA quality (Sambrook et al., 1989).

EXTRACTION USING ESTABLISHED PROTOCOLS

Three established RNA procedures: TriPure Isolation Reagent (Roche), GeneMATRIX Universal RNA Purification Kit (EURx) and one CTAB method (Chang et al. 1993) were tested.

The RNA isolation was carried out as described in the manufacturer's instructions or literature. The RNA purification using a column-based kit was absolutely ineffective, because columns were clogging up which caused very low yield (Table 1).

The good yield of RNA was obtained using TriPure reagent, however the purity (A_{260}/A_{230} ratio below 1.0) was not sufficient for molecular biology applications (Table 1). Furthermore, for some samples it was impossible to measure RNA concentration due to contamination with polysaccharides, causing very high viscosity and jelly-like consistency of the RNA solution.

Better results were obtained for the standard CTAB method. The RNA integrity analysis revealed that samples extracted from lyophilized tissues were degraded, compared to non-lyophilized fractions (Fig. 3). The observed ratio fluctuated between 1.97 and 2.03 (A_{260}/A_{280}), 1.78 and 2.03 (A_{260}/A_{230}) for non-lyophilized samples and 1.36 and 2.10 (A_{260}/A_{280}), 0.6 and 2.17 (A_{260}/A_{230}) for lyophilized ones. The integrity of the majority of

RNA samples evaluated by gel electrophoresis was acceptable as evidenced by the presence of two bright bands (28S rRNA and 18S rRNA), however in both cases these samples were contaminated with a significant amount of genomic DNA, which appeared as high molecular weight bands in agarose gel (Fig. 3). An additional step with DNase treatment seems to be necessary to remove this contamination.

EXTRACTION USING MODIFIED PROTOCOLS

The two changed protocols: modification of the CTAB method and the method based on N-lauroylsarcosine sodium salt were also tested. By modification and combination of the two previous methods (Rubio-Piña and Pérez, 2011; Morante-Carriel et al., 2014), we developed a new procedure, which met our expectations. In comparison to Morante-Carrielet al. (2014), the main difference concerned the different order of lithium chloride and sodium acetate addition. In turn, compared to Rubio-Piña and Pérez (2011) procedure, an increase in lithium chloride concentration and removal of the alcohol rinsing stage after precipitation with sodium acetate were introduced into the procedure. Moreover, we modified the sarkosyl reagent based protocol (Accerbi et al., 2010) by double centrifugation steps with a stronger centrifugal force.

In contrast to the established protocols, lyophilization prior to isolation led to a significant (twentyfold) increase in the amount of purified RNA without any negative impact on the nucleic acid quality, especially in the modified CTAB method (Table 1). This led to high RNA concentration (600-800 ng/µl) and high RNA purity. The A_{260}/A_{230} ratio (1.93-2.01) and A_{260}/A_{280} ratio ranged from 2.04-2.09 for RNA isolated from lyophilized bulbs of *H. hybridum*, *H. festalis*, *Narcissus* and *I. hollandica* using the modified CTAB method which indicated absence of nucleic acid contamination. Thus, both methods are suitable for RNA isolation from bulbous plants. The RNA was relatively free of protein, polysaccharide and phenolic compounds as indicated by the A_{260}/A_{280} and A_{260}/A_{230} ratios (Table 1), and the intact 28S, 18S and 5S rRNA bands on agarose gel indicated that no RNA degradation occurred during extraction, however a band representing genomic DNA contamination was visible in the case of both RNA extraction methods (Fig. 4).

DISCUSSION

Isolation of high-quality RNA is the first and critical step in many molecular techniques such as cDNA library construction, real-time PCR (RT-qPCR), transcriptome and northern analysis.

Based on our previous molecular experiments carried out on bulbous ornamental plants such as *H. hybridum*, we found that a high content of polysaccharides accumulated in bulbs is the main obstacle to obtain RNA that meets the requirements of traditional methods.

The amount of polysaccharides in bulbs is variable and depends on the seasons and certain physiological stages of development, at which intense photosynthetic activity is present. These polysaccharides are stored and then digested to carbohydrate monomers which are used by the cells (Orthen and Wehrmeyer, 2004). Differences in the level of polysaccharides are also evident in various species of bulbous plants. For example, polysaccharides build almost 30 % of a dry weight of bulbs and roots of *Juno drepanophylla* (Arifkhodzhaev, 1986). In the case of the lily family members, in the tiger lily bulbs 13.7% of dry mass was starch, while *Liliumtestaceum* bulb mass contains nearly 20% of a β-1,4-glucomannan (You et al., 2010).

Although literature provides information about techniques used for RNA isloation from plant material, they do not seem to be suitable for the “difficult plant material”. All bulbs are

rich in polyphenols and polysaccharides, which are the main contamination substances that have to be removed during the RNA isolation process, mainly because of oxidation of polyphenols and physicochemical similarities of polysaccharides and RNA (Dash, 2013; Liu et al., 2018). Very often, this leads to co-precipitation with RNA, which may cause appearance of viscous gelatin-like pellet (Logemann et al., 1987; Gao et al., 2001; Morante-Carriel et al., 2014). Therefore, in some methods of RNA isolations complexes with contaminants are formed, which results in low quality of poly(A)+RNA and also very low yields of RNA are obtained. This is unsuitable for performing a RT-PCR reaction or synthesis of the first strand cDNA (Koonjul et al., 1999).

The goal of the conducted experiments was to analyse and compare five methods to find the most useful and universal one for RNA extraction from bulbs. At the beginning, commonly used column-based RNA isolation kits were assayed. They are dedicated to all tissues and these time-saving and easy sets work well with some plant materials guaranteeing isolation of high quality RNA. GITC-based methods have been widely used to extract RNA from various sources, such as animals, bacteria and plants (Sha et al., 2005; Soncini et al., 2007). However, usage of a GeneMATRIX Universal RNA Purification Kit for RNA extraction from tissues rich in secondary metabolites led to a low yield of RNA. Therefore, modifications of these existing protocols were necessary. It is known that RNA precipitation in the presence of high salt content can increase the quality of tissues rich in carbohydrates or polysaccharides (Li et al., 2006; Zhu et al., 2017). Hence, at the stage of isopropanol addition to the aqueous phase, we also added 0.8 M sodium citrate and 1.2 M sodium chloride. However, although the RNA concentration was high, the A_{260}/A_{230} value and gelly-like consistency of the samples suggested significant polysaccharide contamination. Therefore, this method was not sufficient for plant material rich in secondary metabolites.

One of the ways to improve the technique of obtaining good quality RNA is to use lyophilization. The lyophilization step potentially preserves the molecular integrity of the samples for processing at a later date, eliminates the need for liquid nitrogen during the process and allows to store samples without the need for ultra-low temperatures, which also facilitates the transport of the sample and exchange between collaborators. Despite advantages, an RNA isolation procedure from lyophilized plant tissues has not been extensively reported. It was successful in the case of: tea leaves (Jaiprakash et al., 2003), potato tuber (*Solanum tuberosum* L.), sweet potato root (*Ipomeabatata* L.), turnip root (*Brassica rapa* L.), radish root (*Raphanussativus* L.), ginger rhizome (*Zingiberofficinale* L.) (Kumar et al., 2007) and grapevine buds (García-Baldenegro et al., 2015). In theory, freeze-drying, by inactivation of proteolytic nucleases and enzymes, should limit, delay or reduce the rate of degradation of the cellular component, allowing storage or transport of the samples in the room temperature for a long time (Saha et al., 1997; Jaiprakash et al., 2003). However, it was also reported (Saha et al., 1997) that lyophilization of cotton (*Gossypiumhirsutum* L.) tissues led to complete degradation of RNA in leaf and root tissue.

In our research the modified CTAB, CTAB and sarkosyl techniques proved to be better and high quality RNA was obtained for almost all samples. RNA was not only of high purity and integrity but also of a high yield. Moreover, freeze-drying significantly improved the yield of RNA isolated by the modified CTAB and sarkosyl methods.

Further modifications to our methods were additional centrifugation steps and increased centrifugal speed. Double centrifugation steps with stronger centrifugal force were used. These steps prevented transfer of the trace amounts of insoluble interphase material between the interphase and the tip (Zeng and Yang, 2002). A higher centrifugation speed improved the rejection of the insoluble material and residual chloroform which positively influenced the A_{260}/A_{280} ratio. A higher lithium chloride concentration was used in order to recover more low molecular weight RNA. It is known that longer incubation with a high concentration of

lithium chloride results in an increased amount of RNA impurities, despite a higher RNA yield (Chan et al., 2004). For this reason, we used a shorter incubation time in our procedure.

As another option to improve the RNA isolation and purification procedure, the addition of contaminant absorbents (PVP together with spermidine) into the extraction buffer (Wang et al., 2005; Reid et al., 2006; Sánchez et al., 2016) was proposed. PVP is responsible not only for the removal of phenolic compounds and secondary metabolites from nucleic acid preparations but also prevents the browning effect of polyphenols (Shu et al., 2014). It is worth mentioning that in this case PVPP was preferred to PVP. The soluble PVP may not be compatible with some phenolic extractions and bind to nucleic acids, which further hinders RNA precipitation. On the other hand, PVPP is chemically inert, which supports the effective formation of complexes with polyphenols through a hydrogen bond, allowing them to be separated from RNA. Removal of PVPP-polyphenol complexes along with other contaminants by chloroform extraction proved to be convenient. It allowed forthe subsequent use of phenol / chloroform to isolate RNA, which strongly denatured RNase and removed proteins much more successfully than just chloroform extraction (Wang et al., 2005; Peng et al., 2014).

Spermidine is a polyamine that binds and precipitates with nucleic acids (Sánchez et al., 2016). It is often used in a combination with PVP or PVPP in RNA extraction buffers in order to eliminate DNA. Spermidine in higher concentrations can also be used as an RNase inhibitor (Ouyang et al., 2014). However, in our case, addition of spermidine to the buffer did not remove all of the genomic DNA, as can be seen from Fig. 3 and Fig. 4.

RNA obtained by the modified protocols was of good quality and quantity, higher than in other published reports. Using the modified CTAB method, RNA concentrations were up to 5 times higher, compared to the results obtained for lily bulbs (Li et al., 2011) and up to 6 times higher compared to the isolation from *Cinnamomumtenuipilum* (Zeng and Yang, 2002). The differences between these methods are primarily the changes in the reagent concentrations in the buffer and the order of the steps in the method. This indicates the significance of the applied changes, method modifications and the use of freeze-dried material contributed to the increase in the amount of the obtained RNA.

CONCLUSION

Summarizing, RNA isolation performed with commonly used extraction and purification methods yielded good results, with the exception of tissues rich in polysaccharides and polyphenols. Several studies have shown that RNA isolation must involve a number of important steps before, during, and after the actual RNA purification. Thus, we changed the RNA extraction procedure by introducing several modifications with particular focus on the effect of pre-treatment and getting rid of polysacharides and polyphenolic compounds, which affect the RNA quality. In this study we showed that improvement of the CTAB and sarkosyl methods leads to isolation of high quality RNA from difficult material like storage organs of bulbous plants. Usage of a lyophilized sample with the modified CTAB method, allowed to obtain a good yield of RNA and this process did not cause any negative impact on the nucleic acid quality, which suggests that this step could improve RNA extraction efficiency. The presented method has a universal character and matches the plant material such as bulbs.

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AUTHORS' CONTRIBUTIONS

KJ, AS-J: designing and coordinating the study; MD, BŚ: conducting the experiments; BŚ, MK: data analysis; MD, MK: writing the manuscript; AS-J: revision and critical evaluation of the manuscript

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FIGURES:

Fig. 1. Scheme of RNA isolation using modified CTAB method (the modified steps have been underlined).

Fig. 2. Scheme of RNA isolation using modified sarkosyl method (the modified steps have been underlined).

Fig. 3. Total RNA isolated from different plant species by (**a**) TriPure method, (**b**) GeneMATRIX Universal RNA Purification Kit, c: CTAB method separated on an agarose gel (1%). Three intact RNA bands for 28S, 18S and 5S rRNA and one band for genomic DNA (CTAB) are located on the right side. L: lyophilized tissue, NL: non- lyophilized tissue.

Fig. 4. Total RNA isolated from different plant species by modified (**a**) sarkosyl and (**b**) CTAB method separated on an agarose gel (1%). Three intact RNA bands for 28S, 18S, 5S rRNA and one band for genomic DNA are located on the right side. L: lyophilized tissue, NL: non- lyophilized tissue.

Figure 1

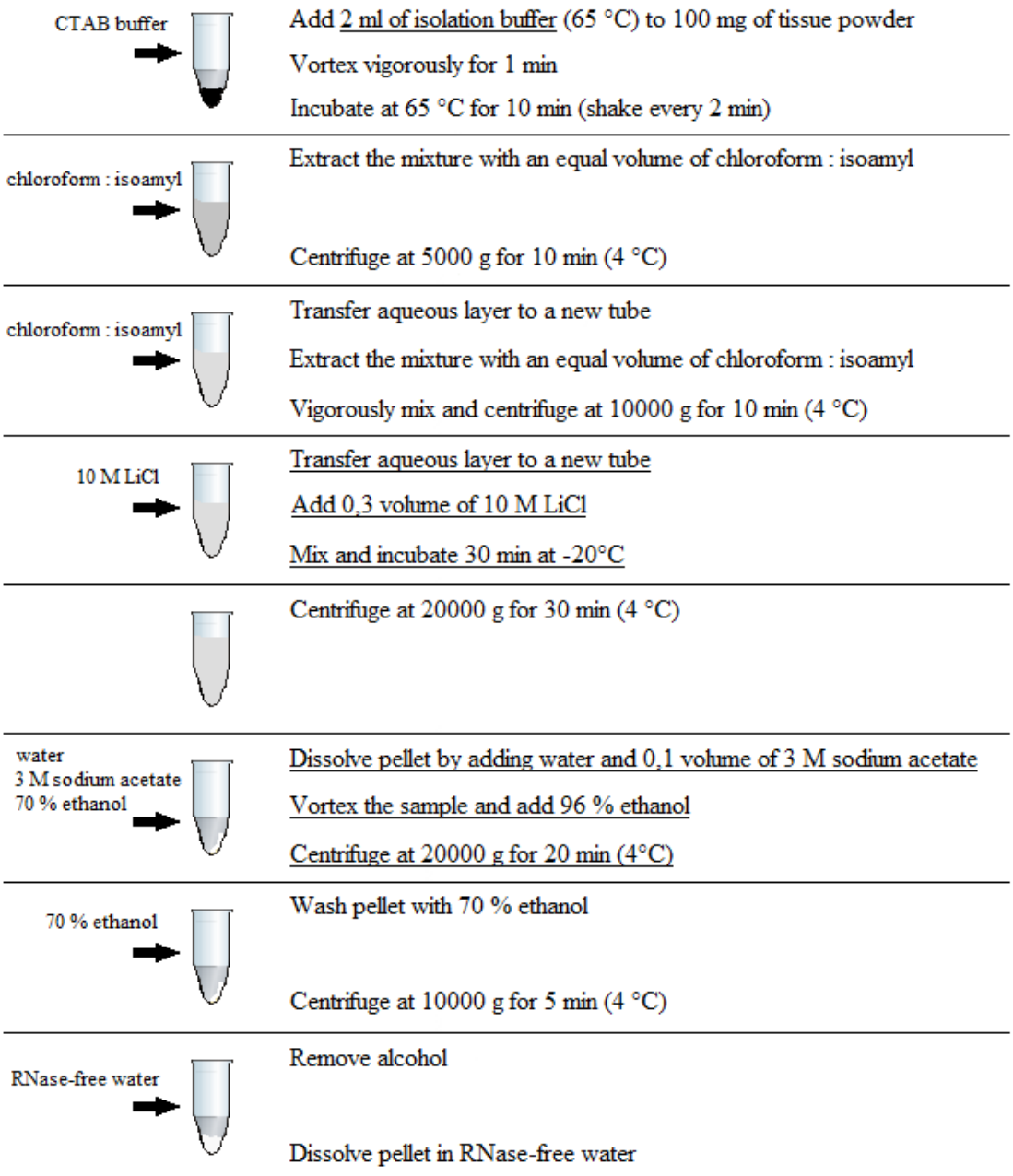
CTAB buffer
Add 2 ml of isolation buffer (65 °C) to 100 mg of tissue powder
Vortex vigorously for 1 min
Incubate at 65 °C for 10 min (shake every 2 min)
chloroform : isoamyl
Extract the mixture with an equal volume of chloroform : isoamyl
Centrifuge at 5000 g for 10 min (4 °C)
chloroform : isoamyl
Transfer aqueous layer to a new tube
Extract the mixture with an equal volume of chloroform : isoamyl
Vigorously mix and centrifuge at 10000 g for 10 min (4 °C)
10 M LiCl
Transfer aqueous layer to a new tube
Add 0,3 volume of 10 M LiCl
Mix and incubate 30 min at -20°C
Centrifuge at 20000 g for 30 min (4 °C)
water
3 M sodium acetate
70 % ethanol
Dissolve pellet by adding water and 0,1 volume of 3 M sodium acetate
Vortex the sample and add 96 % ethanol
Centrifuge at 20000 g for 20 min (4°C)
70 % ethanol
Wash pellet with 70 % ethanol
Centrifuge at 10000 g for 5 min (4 °C)
RNase-free water
Remove alcohol
Dissolve pellet in RNase-free water

Figure 2

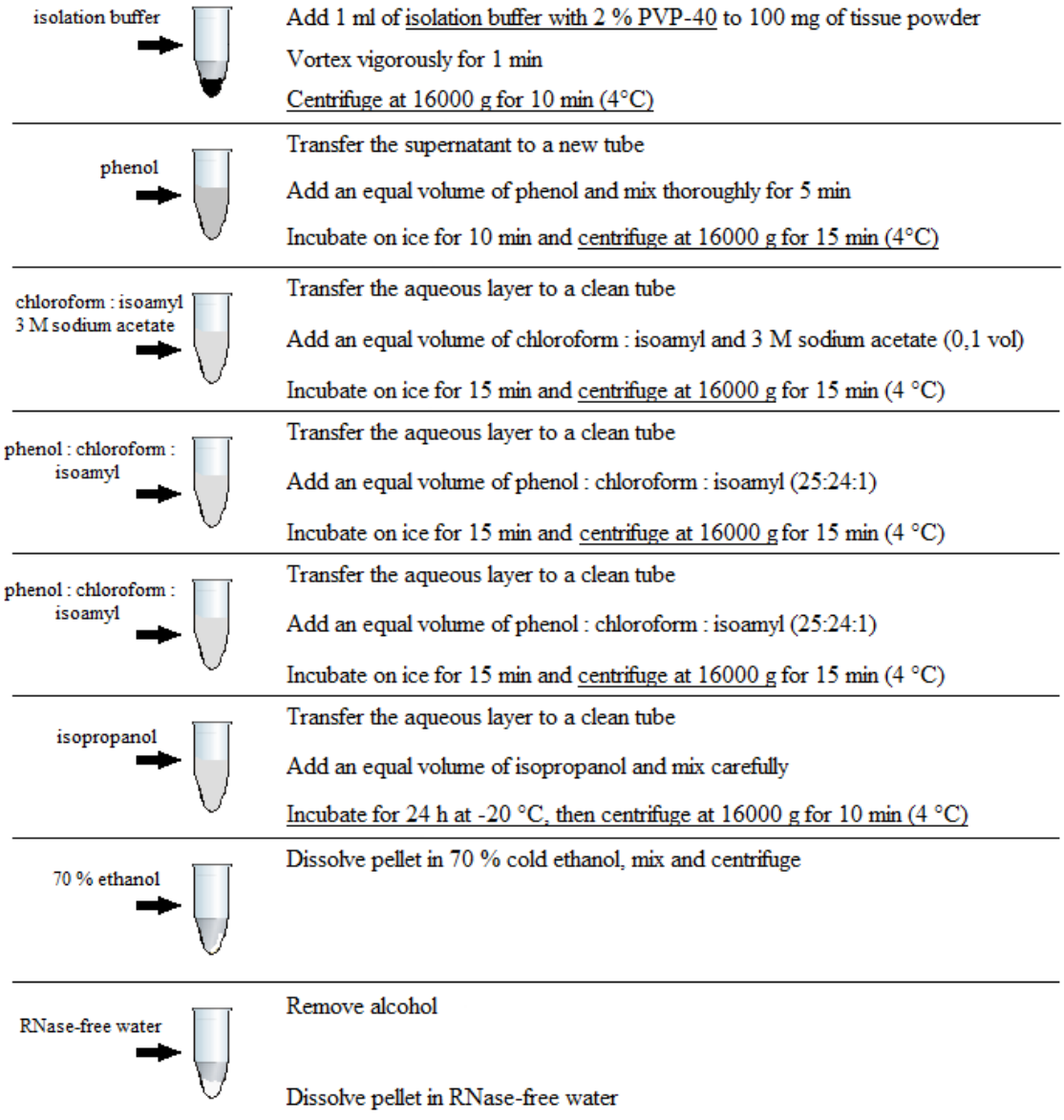
isolation buffer
Add 1 ml of isolation buffer with 2 % PVP-40 to 100 mg of tissue powder
Vortex vigorously for 1 min
Centrifuge at 16000 g for 10 min (4°C)
phenol
Transfer the supernatant to a new tube
Add an equal volume of phenol and mix thoroughly for 5 min
Incubate on ice for 10 min and centrifuge at 16000 g for 15 min (4°C)
chloroform : isoamyl
3 M sodium acetate
Transfer the aqueous layer to a clean tube
Add an equal volume of chloroform : isoamyl and 3 M sodium acetate (0,1 vol)
Incubate on ice for 15 min and centrifuge at 16000 g for 15 min (4 °C)
phenol : chloroform : isoamyl
Transfer the aqueous layer to a clean tube
Add an equal volume of phenol : chloroform : isoamyl (25:24:1)
Incubate on ice for 15 min and centrifuge at 16000 g for 15 min (4 °C)
phenol : chloroform : isoamyl
Transfer the aqueous layer to a clean tube
Add an equal volume of phenol : chloroform : isoamyl (25:24:1)
Incubate on ice for 15 min and centrifuge at 16000 g for 15 min (4 °C)
isopropanol
Transfer the aqueous layer to a clean tube
Add an equal volume of isopropanol and mix carefully
Incubate for 24 h at -20 °C, then centrifuge at 16000 g for 10 min (4 °C)
70 % ethanol
Dissolve pellet in 70 % cold ethanol, mix and centrifuge
RNase-free water
Remove alcohol
Dissolve pellet in RNase-free water

Figure 3

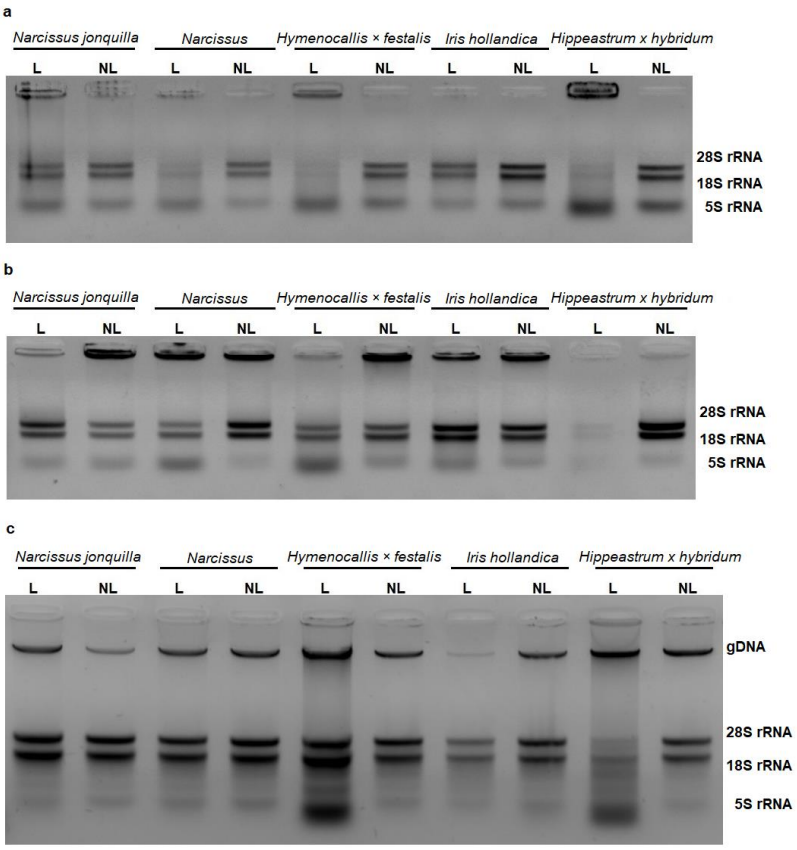

Figure 4