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Modified CTAB Protocol for The Isolation of High-Quality RNA From Silver And Downy Birch Leaves.

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ABSTRACT

Obtaining high quality RNA preparations is an important initial step in transcriptomic studies. Isolation of RNAs from tissues of woody plants, birch for instance, is hampered by the presence of polyphenols and polysaccharides, which make RNA samples useless in downstream applications. To overcome this obstacle, a modified CTAB protocol was proposed for the RNA isolation from birch leaves. This protocol includes acetone treatment of a plant issue. The protocol is applicable to the two birch species, silver birch and downy birch. It allows obtaining high-quality RNA preparations that feature high purity and high concentration along with low extent of degradation, in a highly reproducible manner.

Keywords: modified CTAB protocol, RNA, birch leaf, transcriptomic studies, acetone treatment.

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INTRODUCTION

Representatives of *Betula* family belong to the tree plants widespread in Eurasia, which are of high economic importance [1-3]. Birch is a source of timber for timber industry, paper industry and for obtaining biofuel. This is why great attention is paid to the selection focused on genetic improvement of birch causing an increased productivity and stress resistance [4, 5], especially using modern molecular genetics methods that allow accelerate selection of tree plants. One of the promising approaches that increase selection efficiency is the transcriptomic study, which allows determining the gene function and predicting its value for genetic improvement of plants [6, 7].

For *Betula platyphylla* transcriptomic studies were carried out that were focused on studying genes associated with polyploidy [2], involved in young shoot development [3], connected to stress-related changes in xylem development [8]; and also genes associated with triterpenoid synthesis carbohydrate metabolism, and biosynthesis of cell wall in plants exposed to nitrogen oxide [9]. In the case of *Betula pendula*, transcriptomic study was carried out of plants with altered leaf pigmentation. Results of this study have expanded our knowledge of biosynthetic and regulatory processes associated with these phenotypic manifestations [10].

As transcriptomic studies are directed towards the dissection of differential gene expression at the level of transcription, the first step that determines success in further work is obtaining high-quality preparations of intact RNA. Isolation of RNA from woody plant tissue is hampered by the presence of polysaccharides, polyphenols and other secondary metabolites in plant cells. Polyphenols are able to bind and co-precipitate with various macromolecules, including nucleic acids [11]. This leads to the competitive displacement of nucleic acids by them. Being strong inhibitors, polyphenols can further significantly reduce efficiency of numerous enzymatic reactions such as PCR and the reverse transcription. Contamination with polysaccharides impedes solubilization of precipitated RNA and hinders determining the RNA concentration using spectrophotometric methods [12].

There are a number of ways to overcome the obstacles associated with isolation of RNA from plant tissues with high levels of polyphenols and polysaccharides: using soluble form of polyvinylpyrrolidone (PVP) followed by ethanol precipitation of RNA [13]; protocol for RNA isolation including three precipitation steps using high concentrations of insoluble form of PVP and pre-warmed extraction buffer containing guanidine thiocyanate and excluding precipitation with LiCl [14]; using polyvinylpolypyrrolidone (PVPP) and combination of the CTAB method and the SDS method [15]; a combination of the CTAB method with using TRIzol and sarkosyl [16]. Authors of these and other works note that though basic methods for RNA isolations using guanidine thiocyanate, CTAB or SDS/phenol exist, successful obtaining high-quality RNA preparation requires modifying these methods for each plant species. Present work is focused on selection and optimization of a method for isolation of total RNA from birch leaf tissue, which would allow obtaining high-quality RNA preparations suitable for subsequent transcriptomic analysis.

MATERIALS AND METHODS

Plant material

Young leaves from the top of *Betula pendula* and *Betula pubescens* plantlets were used in the work. These were collected both under field conditions and under semi-natural conditions.

Solutions and chemicals:

- 100% acetone
- CTAB extraction buffer: 2% CTAB (cetyltrimethylammonium bromide); 2% polyvinylpyrrolidone (PVP); 100 mM Tris-HCl (pH 8.0); 25 mM EDTA; 2 M NaCl (mixed and uatoclaved); 2% 2-mercaptoethanol (added prior to using the mixture)
- chloroform:isoamyl alcohol (24:1)
- 10 M LiCl
- SSTE buffer: 1 M NaCl; 0.5% SDS; 10 mM Tris-HCl (pH 8.0); 1 mM EDTA

- – 96% ethanol

Protocol for the RNA isolation using acetone:

1. Grind plant tissue (50-70 mg) in a mortar with liquid nitrogen and transfer it to a 2 ml test tube.
2. Add 1 ml of acetone and mix. Centrifige at 13,000g for 10 minutes.
3. Remove the supernatant and dry the pellet at room temperature for 5minutes.
4. Add 800 µl of CTAB extraction buffer and mix by rotating the tube.
5. Incubate at 65°C for 5 minutes.
6. Add equal volume of chloroform:isoamyl alcohol mixture, mix well. Centrifuge the mixture at 13,000g for 10 minutes.
7. Transfer the upper phase to a clean test tube.
8. Repeat step 6.
9. Transfer the upper phase to a clean test tube and add 1/4 volume of 10 M LiCl. Precipitate overnight at 4°C.
10. Centrifuge at 13,000g for 10 minutes.
11. Remove the supernatant. Dissolve the pellet in 500 µl of the SSTE buffer.
12. Repeat step 6.
13. Transfer the upper phase to a clean test tube and add 2 volumes of 96% ethanol. Incubate at -20°C for 2 hours.
14. Centrifuge at 13,000g for 15 minutes.
15. Remove the supernatant, dry pellet at room temperature and dissolve in 20 µl of Milli-Q water.

Then, remaining genomic DNA was eliminated by DNase (AM 1907, “Ambion”, Life Technology, USA) treatment, according to the manufacturer protocol.

Estimating quality of RNA preparations

Quality of isolated total RNA was monitored by electrophoretic separation in 1% agarose gel containing ethidium bromide. Purity and RNA concentration was determined using NanoDrop 1000 (Thermo-Scientific, USA) spectrophotometer. Purity of the RNA preparations obtained was estimated by 260nm/280nm absorbance ratio. This ratio has to be close to 2 for pure preparations.

RNA integrity was monitored by an automated capillary electrophoresis using TapeStation 2200 (“Agilent Technologies”, USA) instrument.

RESULTS AND DISCUSSION

Two methods for obtaining total RNA preparations from birch leaf tissue were tested in the present work: a method based on application of TRIzol [17-19], and a method using CTAB buffer [20]. The former one is a widespread universal method for RNA isolation and commonly used in the case of woody plants [21-25]. It was successfully used in our laboratory for RNA isolation from aspen leaves. CTAB method was selected on the basis of analysis published data as it is frequently applied for obtaining RNA preparations from birch plant tissues [26-30]. However, the authors show neither the protocol used nor data regarding quality of the preparations obtained.

Table 1: Comparison of RNA isolation methods used for obtaining total RNA preparations from birch leaf tissue.

RNA isolation method	Total number of isolations	Number of successful isolations	% of successful isolations	Mean RNA concentration (ng/µl)	Mean RIN value
TRIzol	7	0	0	-	-
CTAB	73	35	48	248	4,5

Using the TRIzol-based method, we failed to obtain RNA preparations from birch leaves (Table 1). Using CTAB method, we were able to obtain RNA preparations from 48% of samples. The preparations feature

medium degradation extent confirmed by gel electrophoresis analysis (Figure 1) and by results of automated capillary electrophoresis (Figure 2). Mean RNA integrity number (RIN) was 4.5. Absorbance ratio at 260 nm/280 nm for these samples was never less than 1.9, which shows lack of impurities [31, 32]. The mean RNA concentration was 248 ng/µl.

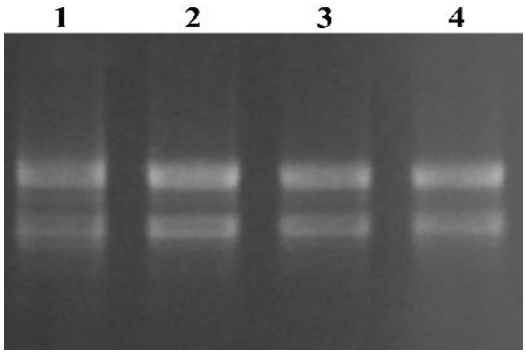

Figure 1: Typical electrophoregram of RNA samples (1-4) isolated from birch leaf tissue using CTAB method.

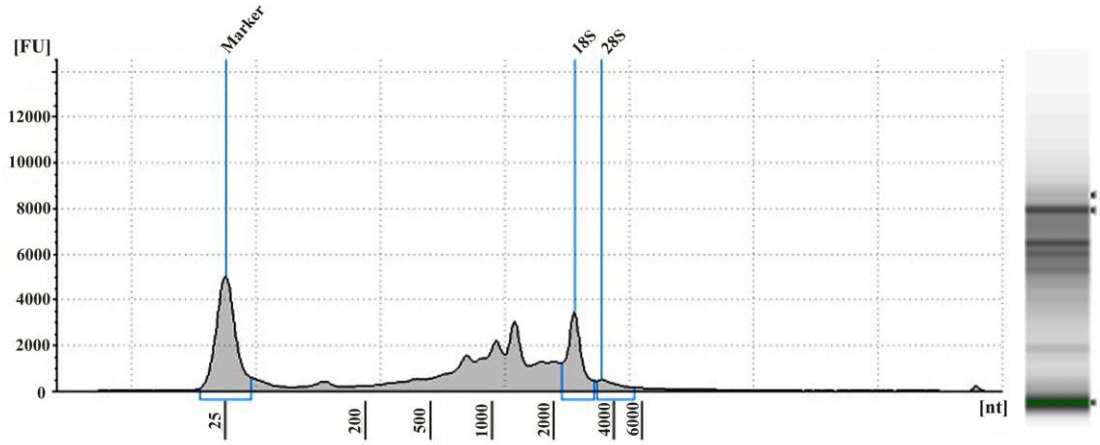

Figure 2: Example of TapeStation 2200 spectra of total RNA isolated using the CTAB method (RIN-4.7; 28S/18S-0.1). The graph shows the intensity of the peaks of the ribosomal RNA subunits: nuclear large-28S, small-18S, cytoplasmic, mitochondrial, and chloroplastic (smaller subunits). The electrophoretic gel is shown to the right indicating the subunit bands or degradation (i.e., smear). nt = number of estimated nucleotides based on ladder; FU = fluorescence unit (i.e., intensity of peak).

The RNA integrity number (RIN) is a reliable and broadly accepted criterion for estimating RNA quality [33, 34]. It varies from 10 (intact RNA) to 1 (completely degraded RNA). RNA with RIN≥5 corresponds to a good quality RNA and it is suitable for further work [35]. Samples obtained by the CTAB method possessed RIN<5. Thus, they were not suitable for downstream applications associated with studying the gene expression. Furthermore, chance of successful preparations from birch leaf tissue only 48%. Hence, one had to modify this RNA isolation method.

During the RNA preparations using CTAB method [20], it was noted that the plant tissue powder after homogenization in liquid nitrogen cannot be completely suspended in the extraction buffer. The powder forms a clump. For this reason, ground and frozen in liquid nitrogen tissue samples were treated with acetone, which is able to dissolve numerous organic compounds and also causes denaturation of proteins. These properties of acetone allowed avoiding clumping of the ground plant tissue particles. After such treatment, complete suspending in the extraction buffer was achieved; tissue could be easily mixed with the buffer. To increase lysis efficiency, the mixture was incubated at 65°C. This modified RNA isolation protocol allowed obtaining high-quality preparations from 80% of birch leaf tissue samples. Thus, isolation efficiency was increased by 32% (Table 2).

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Table 2: Results of RNA isolation from birch leaf tissue by the modified CTAB method using acetone.

Total number of isolations	Number of successful isolations	% of successful isolations	Mean RNA concentration (ng/µl)	Mean RIN value
56	45	80	346	6,4

Analysis of electrophoregrams of RNA samples (Figure 3) and automated electrophoresis data (Figure 4) demonstrated that preparations obtained by this method had low degradation extent as compared to preparations obtained by the original CTAB method: mean RIN value was 6.4. Absorbance ratio at 260 nm/280 nm was close to 2.0, which confirms that these preparations are of high quality. Mean RNA concentration was 234 ng/µl.

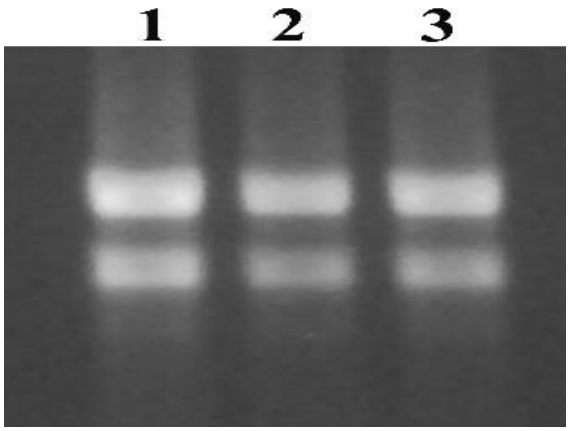

Figure 3: A typical electrophoregram of separation of RNA samples (1-3) isolated from birch leaf tissue by the modified CTAB method.

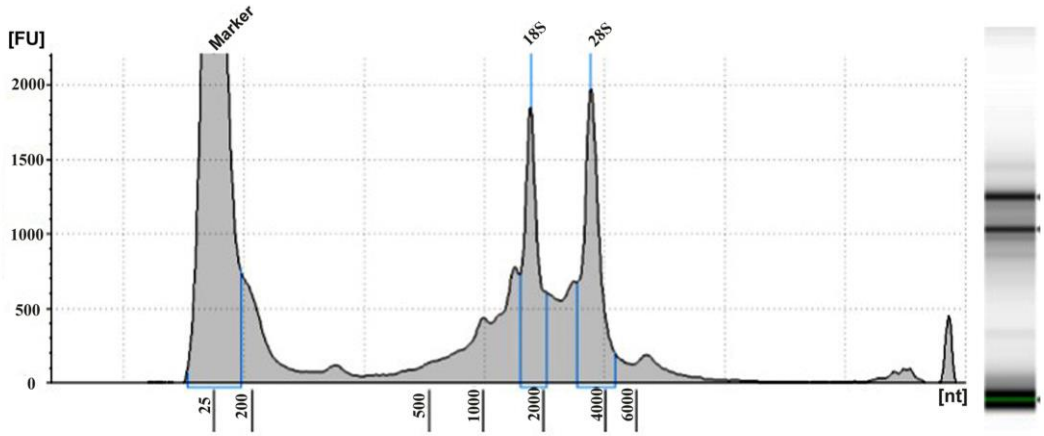

Figure 4: Example of TapeStation 2200 spectra of total RNA isolated using the modified CTAB metod (RIN-7.4; 28S/18S-1.2). The graph shows the intensity of the peaks of the ribosomal RNA subunits: nuclear large-28S, small-18S, cytoplasmic, mitochondrial, and chloroplastic (smaller subunits). The electrophoretic gel is shown to the right indicating the subunit bands or degradation (i.e., smear). nt = number of estimated nucleotides based on ladder; FU = fluorescence unit (i.e., intensity of peak).

CONCLUSIONS

Thus, our modified protocol for isolation of total RNA has allowed boosting productivity of obtaining preparations from birch leaf tissue along with increasing their quality by reducing degradation extent and rising RNA concentration. RNA samples obtained using the modified protocol are suitable for further experiments associated with studying the gene expression as their average RIN value was 6.4.

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