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A generic plant RNA isolation method suitable for RNA-Seq and suppression subtractive hybridization

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ABSTRACT. A recently developed revolutionary approach to transcriptomics, RNA-Seq, and suppression subtractive hybridization are powerful tools for gene expression research. However, currently, the difficulty of isolating high-quality RNAs from plant tissues bearing abundant complex polysaccharides, polyphenolics, and secondary metabolites is a serious problem that not only limits the application of these technologies but also hinders studies dealing with RNA in general. We have developed a consistent protocol to prepare highly intact and pure RNAs from tissues of a variety of field-grown plant species, with high yields, in 2 to 3 h. Additionally, this method can be readily applied to mammalian, yeast, and bacterial cells.

Key words: Plant RNA; Isolation; Polyphenols; Polysaccharides; RNA-Seq

INTRODUCTION

Identifying differentially expressed genes and comparing their expression patterns are important to deciphering their functions relevant to evolution, differentiation, and development. Sequence- or hybridization-based approaches including RNA-Seq, cDNA array, and suppression subtractive hybridization (SSH) have been employed to analyze transcriptomes. RNA-Seq, utilizing recently developed deep-sequencing technologies, provides researchers with a genome-scale transcription map that consists of the transcriptional structure and precise levels of transcripts. On the other hand, hybridization-based SSH is also a powerful technique for identifying differentially expressed genes. For these technologies, obtaining abundant, intact, pure, and consistent RNAs is a basic prerequisite. Unfortunately, extracting plant RNA is a very tedious and challenging task, primarily because of the polysaccharides and polyphenolics that irreversibly interact and co-precipitate with nucleic acids (Katterman and Shattuck, 1983; Levi et al., 1992; Gehrig et al., 2000). Moreover, plants have complex metabolic pathways through which a great number of various secondary metabolites are generated. The enormous compositional differences among plants also profoundly influence the ribonucleic acids preparation procedures (Loomis, 1974; Sharma et al., 2003). Because of a plant's "chemotypic heterogeneity," it is believed that even closely related plant species might require different nucleic acids isolation protocols, and just one method that is suitable for all plant species would never be developed (Loomis, 1974; Weishing et al., 1995; Sharma et al., 2003).

The common methods for plant RNA isolation published so far are mainly based on the following approaches: guanidinium/guanidine salts (Puissant and Houdebine, 1990; Chomczynski and Sacchi, 1987, 2006) or urea (Almarza et al., 2006); acid phenol (Geuna et al., 1998); detergents like cetyl trimethyl ammonium bromide (CTAB) (Chang et al., 1993; Ildiko et al., 1999; Kiefer et al., 2000) and sodium dodecyl sulfate (Newbury and Possingham, 1977; Wan and Wilkins, 1994). These protocols, including the commonly used commercial kits (Qiagen RNeasy Plant Kit, Invitrogen TRIzol Reagent, and Ambion RNAqueous Kit), may be suitable for particular plant species and tissues. However, when applied to untested materials, these protocols usually need some adjustments, and, even after improvement, they may not work well, all because of the "chemotypic heterogeneity" problem (Khanuja et al., 1999; Li et al., 2006).

We describe here a procedure that we have developed for the isolation of plant RNA, which combines and incorporates the advantages of several reported methods. It was tested and validated by applying to tissues from 18 plant families (5 field-grown woody plant families, 13 herbaceous plant families), mammalian Hela cells, and *Escherichia coli* DH5α and yeast *Saccharomyces cerivisiae* Y190 cells. The quality of the isolated RNAs was consistently high, as judged by spectrophotometric readings, the formaldehyde denaturing agarose gel separation analysis, and the Agilent Bioanalyzer NanoDrop assay. Furthermore, the RNA samples yielded fine results when used for mRNA extraction, double-stranded (ds) cDNA synthesis, 3' cDNA ends amplification, reverse transcription polymerase chain reaction (RT-PCR), RNA-Seq, and SSH.

MATERIAL AND METHODS

Plant and cell materials

The field-grown plant tissues were harvested from University Town of Shenzhen Gar-

den, or purchased as indicated (Table 1). Mid-exponential phase *E. coli* DH5α and *S. cerivisiae* Y190 cells cultured in liquid medium were collected by centrifugation, respectively. All plant tissues, yeast, and bacterial cells were thoroughly ground into fine powders, under liquid nitrogen, using a mortar and pestle. The ground samples were stored at -80°C for future use. Confluent HeLa cells (80%) in a 5x5cm flask were digested with trypsin, collected by centrifugation, and directly lysed with a homogenization solution at room temperature.

Solutions and reagents

Homogenization solution: 2% CTAB (w/v); 1.5 M NaCl; 100mM Tris, 20 mM EDTA, pH 8.0; and 2% β-mercaptoethanol [(v/v); added before use]. Precipitation buffer: 10M lithium chloride (LiCl). Denaturing solution: 4.0 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5% sodium N-lauroyl sarcosine (w/v); and 2% β-mercaptoethanol [(v/v); added before use]. Organic reagents: water-saturated phenol/chloroform [1:1 (v/v), pH 4.5]; chloroform; absolute ethanol, and 75% ethanol.

RNA isolation protocol

Preheat 2.5 mL homogenization solution contained in a 10 mL tube at 65°C for 3 min. Transfer approximately 500 mg frozen tissue powder into the tube, vigorously shake the tube, and then incubate it at 65°C for 3 to 5 min. Spin the tube in a microcentrifuge at 14,000 rpm for 2 min at room temperature. Transfer the supernatant to two fresh microcentrifuge tubes.

Add 0.1 volume of 10 M LiCl to the supernatant. Invert the tube several times and incubate it at -20°C for 15 to 20 min, or go directly to the next step without incubation. Centrifuge the liquid in a microcentrifuge at 12,000 to 14,000 rpm for 30 min at 4°C to pellet the RNA. Discard the supernatant completely and suspend the pellet in 100 to 200 µL denaturing solution.

Add an equal volume of water-saturated phenol/chloroform [1:1 (v/v), pH 4.5]. Vortex the tube and then spin it at 12,000 rpm for 5 min at 4°C. While avoiding disturbing the interphase, transfer the aqueous phase into a fresh tube. Add an equal volume of chloroform, and then vortex and spin the tube at 12,000 rpm for 1 min at 4°C. For most of the samples listed in Table 1, the organic extraction could be omitted. The RNAs qualitiy was almost as good as these organic extraction-treated RNAs (data not shown).

Transfer the supernatant to a fresh microcentrifuge tube, add 2.5 volumes of 100% ethanol, and then vortex and centrifuge at maximum speed for 15 min at 4°C. The RNA pellet at this stage could be stored at -80°C for several months (data not shown).

Discard the supernatant, wash the pellet with 0.5 mL 75% ethanol, and then spin the tube at 8000 rpm for 3 min at room temperature. Repeat this step twice. Decant the supernatant, spin briefly, and collect and aspirate the residual supernatant. Air-dry the pellet at room temperature and dissolve it in diethylpyrocarbonate-treated water.

Estimation of RNA quality

RNA purity, integrity, and yield analyses

The RNA purity and quantity were assessed spectrophotometrically at wavelengths

of 230, 260, and 280 nm. The RNA integrity was verified by analyzing 4 to 8 µg RNA via 1% formaldehyde (w/v) denaturing 1.2% agarose gel electrophoresis (Sambrook et al., 1989) and 1 to 5ng RNA via the NanoDrop test with the Agilent 2100.

RT-PCR

Total RNA (2µg) extracted from the young shoot tip and young leaf of mango (*Mangifera indica* L.) was reverse transcribed using the Moloney murine leukemia virus reverse transcriptase (M-MLV) RNase H^- (TaKaRa) with the oligo$(dT)_{18}$ primer. Using the *psb*A gene-specific 5' primer (5'-ATGACTGCAATTTTAGAGAGA-3') and 3' primer (5'-TTATCCATTTATAGATGGAGC-3'), and the *Actin*1 gene-specific 5' primer (5'-GTGACAATGGAACTGGAATG-3') and 3' primer (5'-AGACGGAGGATAGCGTGAGG -3'), subsequent PCRs were carried out with *Taq* DNA polymerase (TaKaRa). The PCR conditions were as follows: 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and a final cycle of 72°C for 7 min.

3'-cDNA ends amplification

In order to amplify the 3'-cDNA ends of gene *Actin*1, a specific 5' primer and an anchor 3' primer (5'-TTTTTTTTTTTA-3') were used for the PCR. The thermocycling conditions were as follows: 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and a final cycle of 72°C for 7 min.

mRNA purification and ds cDNA synthesis analysis

According to manufacturer protocols, 2 mg total RNA from the fruits of seeded or seedless lichi (*Litchi chinensis* Sonn cv. *Seedless*) was used for mRNA extraction, respectively (PolyA Ttract mRNA Isolation Systems III, Promega), followed by reverse transcription, second-strand cDNA synthesis, and *Rsa*I digestion (PCR-Select™ cDNA Subtraction Kit, Clontech).

All the above products were separated on a 1.2% agarose gel, stained with ethidium bromide, and photographed under UV light.

RNA-Seq

Using the Oligotex mRNA Mini Kit (Qiagen), mRNA samples were purified from 20µg total RNAs extracted from *Cymbidium sinense*, *Neuwiedia veratrifolia*, and *Holcoglossum amesianum*. The ds cDNAs were synthesized and then sequenced via Solexa according to the manufacturer protocols.

RESULTS AND DISUSSION

RNA purity, integrity, and yield

The A_{260}/A_{280} ratio of the RNA samples ranged from 1.9 to 2.3, and the A_{260}/A_{230} ratio

was above 4.0 for each of the samples, which suggested minimum contaminants of salts, proteins, polyphenolics, or polysaccharides. The RNA yields were in the range of 20 to 300 µg/g (Table 1). The formaldehyde denaturing gel-electrophoresis results showed distinct 28S, 26S, 25S, 23S, 18S, and 16S rRNA bands. The respective intensities of the 28S, 26S, 25S, and 23S rRNA bands were 1.5 to 2 times that of the corresponding 18S or 16S rRNA bands, which implies high RNA integrity (Figures 1, 2, and 3). Additionally, there were no visible bands around the loading wells, which indicates no genomic DNA contamination (Figures 1 and 2). The NanoDrop test showed the 25S/18S rRNA ratio for *H. amesianum* RC, *N. veratrifolia*, and *C. sinense* SW to be 1.9, 1.4 and 2.1, respectively. The respective RNA integrity number (RIN) was 7.6, 7.1, and 8.4 (Table 2). The NanoDrop test results indicated that the RNAs were qualified for RNA-Seq.

Table 1. Spectrophotometer readings used to evaluate plant RNA quantity and quality.

Families	Genera	Plant species	Tissues	Purity ($A_{260/280}$)	Yield (µg/g)
Sapindaceae	*Litchi*	*Litchi chinensis* Sonn cv. *Seedless*	Young seedless fruit	2.15	330
			Young seeded fruit	2.18	320
			Seedless fruit embryo	2.08	340
			Seeded fruit embryo	2.23	306
			Young leaf	2.14	221
Musaceae	*Musa*	*Musa nana* Lour.	Young leaf	2.03	165
			Ripening pulp	2.14	232
			Mature pulp	2.13	20
Pinaceae	*Pinus*	*Pinus massoniana* Lamb.	Shoot tip, needle	2.14	213
Malvaceae	*Gossypium*	*Gossypium hirsutum* L.	Young leaf	2.11	220
Solanaceae	*Nicotiana*	*Nicotiana tabacum* L.	Young leaf	2.01	254
Gramineae	*phyllostachys*	*Phyllostachys pubescens*	Young leaf	2.12	231
Anacardiaceae	*Mangifera*	*Mangifera indica* L.	Young shoot tip	2.19	276
			Young leaf	2.18	227
Leguminosae	*Cercis*	*Cercis chinensis* Bge.	Expanded flower	1.97	40
Amaryllidaceae	*Agave*	*Agave sisalana* Perrine.	Fleshy stem	2.09	221
Liliaceae	*Allium*	*Allium sativum* L.	Fleshy bulb	2.03	214
Zingiberaceae	*Zingiber*	*Zingiber officinale* Rosc.	Fleshy rhizome	2.21	236
Orchidaceae	*Cymbidium*	*Cymbidium goeringii* Rchb.f.	Unexpanded flower	2.17	234
Leguminosae	*Glycine*	*Glycine max* L.	Ripe fruit	1.93	273
Commelinaceae	*Zebrina*	*Zebrina pendula* Schnizl cv. *Quadricolor*	Young leaf	2.20	221
Taxodiaceae	*Metasequoia*	*Metasequoia glyptostroboides* Hu et Cheng	Young leaf	2.10	243

cDNA synthesis

We also demonstrated that the total RNAs prepared in large or small scale were qualified for mRNA extraction, 3' cDNA ends amplification, and RT-PCR. Figure 4 shows that the litchi ds cDNA smeared from 0.3 kb to the gel wells and the digested ds cDNA mainly ranged from 0.1 to 2 kb, which indicates excellent RNA quality and suitability for subtractive cDNA library construction. On the one hand, using specific paired primers for the *psb*A and *Actin*1 genes, amplicons of the expected size were amplified (Figure 5). On the other hand, using the *Actin*1 gene-specific 5' primer and anchor 3' primer, the 3' cDNA ends were also successfully amplified, which showed that the obtained RNAs were efficiently reverse-transcribed into cDNA (Figure 5).

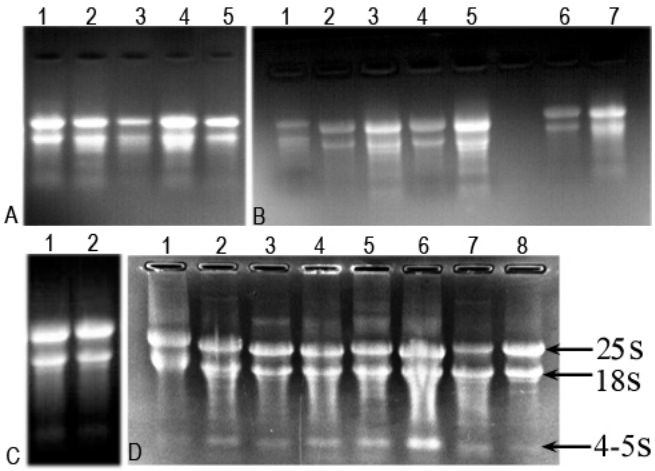

Figure 1. RNA isolated from different plant species tissues. **A.** *Lanes 1* to *5* = RNA extracts of young seeded fruit, seedless fruit, young leaf, seeded fruit embryo, seedless fruit embryo from *Litchi chinensis* Sonn cv. *Seedless*. Materials were collected 3 weeks after the female flower's expanding; **B.** *Lane 1* = ripe and soft *Musa nana* Lour. pulp; *lane 2* = young *M. nana* Lour. pulp; *lane 3* = young *M. nana* Lour. leaf; *lane 4* = young *Pinus massoniana* Lamb. needle and young green shoot tip; *lane 5* = young *Nicotiana tabacum* L. leaf; *lane 6* = *Cercis chinensis* Bge flower (the first fully expanded flower from apical meristem); *lane 7* = young leaf of *Gossypium hirsutum* L. **C.** *Lane 1* = young *Phyllostachys pubescens* leaf; *lane 2* = young *Zebrina pendula* Schnizl cv. *Quadricolor* leaf. **D.** *Lane 1* = ripe *Glycine max* L. seed; *lane 2* = *Allium sativum* L. bulb; *lane 3* = first fully developed but unexpanded flower from *Cymbidium goeringii* Rchb.f; *lane 4* = young *Mangifera indica* L. shoot tip; *lane 5* = young *M. indica* L. leaf; *lane 6* = young *Zingiber officinale* Rosc. rhizome; *lane 7* = young *Agave sisalana* Perrine flesh stem; *lane 8* = green young *Metasequoia glyptostroboides* Hu et Cheng leaf. Arrows showed ribosomal RNA different size.

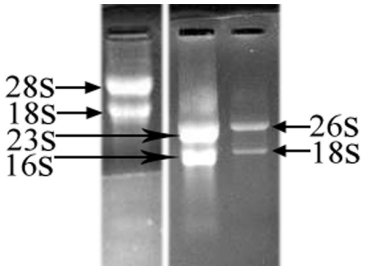

Figure 2. RNA from HELA, *Escherichia coli* Dh5α; *Saccharomyces cerivisiae*. *Y190* cells. From left to right, RNA obtained from HELA a, *E. coli* Dh5α; *S. cerivisiae Y190* cells. Arrows showed ribosomal RNA different size.

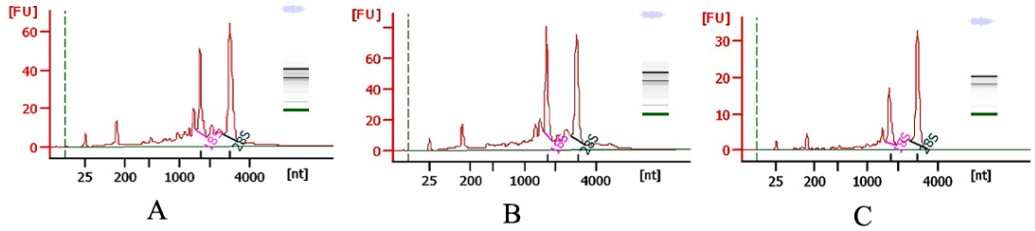

Figure 3. RNA integrity judged by Nano drop test. **A.** RNA extracts of *Holcoglossum amesianum* RC young leaf. **B.** RNA extracts of *Neuwiedia veratrifolia* young leaf. **C.** RNA extracts of *Cymbidium* SW young leaf.

Table 2. NanoDrop test used to evaluate plant RNA integrity.

Genera	Plant species	rRNA Ratio25s/18s	RNA integrity number
Holcoglossum	*Holcoglossum amesianum* RC	1.9	7.6
Neuwiedia	*Neuwiedia veratrifolia*	1.4	7.1
Cymbidium	*Cymbidium* SW	2.1	8.4

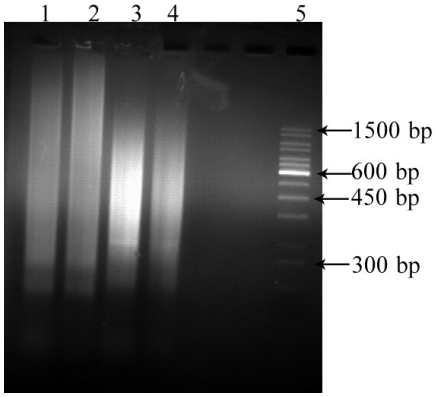

Figure 4. mRNA purification and ds cDNA synthesis and *RsaI* digestion analysis. *Lane 1* = ds cDNA generated with mRNA purified from young *Litchi* seeded fruit RNA. *Lane 2* = ds cDNA generated with mRNA purified from young *Litchi* seedless fruit RNA. *Lane 3* = ds cDNA of young *Litchi* seeded fruit digested by *RsaI*. *Lane 4* = ds cDNA of young *Litchi* seedless fruit digested by *RsaI*. *Lane 5* = 50 bp DNA marker (TaKaRa, China).

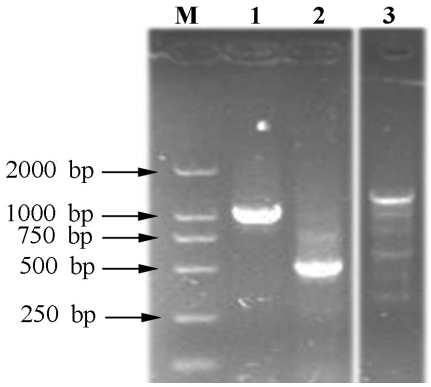

Figure 5. RT-PCR product analysis through agarose gel electrophoresis. Agarose gels electrophoresis analyze RT-PCR product from total RNA. *Lane M* = DL 2000 DNA marker (TaKaRa, China). *Lane 1* = 1020-bp RT-PCR amplicon of gene psbA (RNA purified from *Mangifera indica* L. young leaf). *Lane 2* = 500-bp amplicon of Actin1 gene amplified with specific primers (RNA isolated from *M. indica* L. young shoot tip). *Lane 3* = approximately 1000-bp amplicons of Actin1 gene amplified with 5' specific primer and 3'anchor primer (RNA obtained from *M. indica* L. young shoot tip).

RNA-Seq

The total reads were 16,000,000 and the total nucleotides were 1,200,000,000 nt for

each of the RNA samples. The respective Q20 scores for *H. amesianum* RC, *N. veratrifoli*, and *C. sinense* SW were 88.82, 84.31, and 90.23%, which demonstrates excellent sequence quality (Table 3).

Table 3. RNA-Seq via solexa.

Samples	Total reads	Total nucleotides (nt)	Q20 (%)	N (%)	GC (%)
Holcoglossum amesianum RC	16,000,000	1,200,000,000	88.82	0.09	48.29
Neuwiedia veratrifoli	16,000,000	1,200,000,000	84.31	0.06	44.45
Cymbidium SW	16,000,000	1,200,000,000	90.23	0.06	47.37

Comparison of RNA with guanidinium thiocyanate method

Generally, highly intact RNAs can be isolated from mammalian cells and animal tissues through the acid-guanidinium thiocyanate-phenol-chloroform method (Figure 6A). However, when we applied it to the lichi leaf and mango shoot tip, the homogenates and the subsequently precipitated RNA pellets became brown and extremely viscous, which suggests severe contamination by polysaccharides and oxidized polyphenols. On the contrary, our purification method rendered RNA of good quality from plant materials, but it failed in animal tissues (Figure 6B).

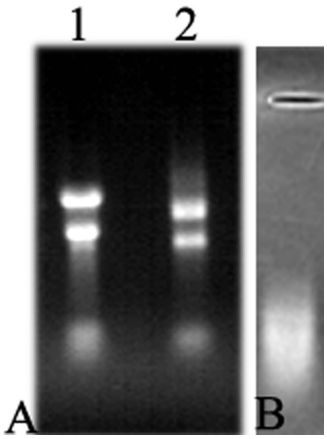

Figure 6. RNA purified from HELA cells and mouse liver using different methods. **A.** Guanidinium thiocyanate-phenol-chloroform extraction method. *Lane 1* = RNA of HeLa cells. *Lane 2* = RNA of mouse liver tissues. **B.** our method. RNA of mouse liver.

Through this procedure, we successfully purified high-quality RNAs from materials representing 18 plant families, including nutrition storage tissues such as seeds, fruits, tubers, fleshy stems, and flowers that contain a large amount of proteins, lipids, polysaccharides, and secondary metabolites. In particular, the mature flower petal and the succulent ripe banana pulp (the peel was completely yellow and scattered with speckles) have extremely abundant polysaccharides. Moreover, the cotton leaf, litchi leaf, purple dayflower leaf, pine needle and shoot tip, orchid flower, ginger rhizome (purchased from a market), and garlic bulb (stored

for several months at room temperature, purchased from a market) carry a great variety of secondary metabolites. The biochemical compositions differ greatly among these materials, and most of the tissues listed in Table 1 are typically considered as recalcitrant materials for RNA isolation (Loomis, 1974; Newbury and Possingham, 1977; Katterman and Shattuck, 1983; Chang et al., 1993; Gehrig et al., 2000; Kiefer et al., 2000). Apart from plant materials, we also accomplished RNA isolation from mammalian cells (HeLa), and yeast (*S. cerivisiae* Y190) and bacterial (*E. coli* Dh5α) cells.

Integrating several reported methods, this procedure demonstrates the following characteristics contributing to the successful RNA isolation. First, the homogenization solution, which contains CTAB, NaCl, and β-mercaptoethanol, can efficiently restrain RNase and polyphenols oxidase activities and inhibit polysaccharides and secondary metabolites from combining with nucleic acids (Fang et al., 1992; Ildiko et al., 1999). Then, simply through centrifugation and selective precipitation by LiCl, most of the cell debris, polyphenols, polysaccharides, proteins, DNA fragments, and other contaminants are left in the supernatant (Ainsworth, 1994; Mehar et al., 2000). Finally, the RNA pellet is suspended in denaturing solution and re-precipitated with ethanol. With these treatments, high-quality RNAs are eventually purified.

The main significance of this simple procedure is that it can be readily applied to a wide range of plant species and tissues, in contrast to other reported methods (as pointed out in the Introduction). Moreover, in most of cases, no organic extraction is required, and the RNAs are still excellent for downstream applications. In conclusion, this method is a versatile procedure suitable for RNA isolation from plant tissues, as well as from mammalian, bacterial, and yeast cells.

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