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一种从苏铁叶片中有效提取RNA的方法*

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摘要: 由于苏铁 (*Cycas revoluta*) 叶片中含有大量的多糖多酚等次生代谢物, 常规RNA提取方法很难获得优质的RNA。在常规的CTAB法中加入了硼砂和β-巯基乙醇来消除多酚和多糖的干扰, 得到了一个从苏铁叶片中有效提取RNA的方法, 每克鲜叶片可获得约930 μg RNA。$A_{260/280}$和$A_{260/230}$的纳米波长的吸收比值都约为2, 表明RNA的质量较好。获得的RNA可用于Northern blot和反转录PCR等分析, 也说明RNA的质量比较好。此外, 改进的提取方法也适合于含有次生代谢产物的其它植物, 同样可以获得优质RNA。

关键词: 苏铁; 硼砂; RNA提取
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An Effective Protocol for the Isolation of RNA from Cycad Leaves*

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Abstract: Conventional RNA extraction methods have been shown to produce poor-quality RNA when applied to *Cycas revoluta* because of abundant secondary metabolites included. With modification of the standard cetyltrimethylammonium bromide (CTAB) method by adding borax (disodium tetraborate decahydrate) and β-mercaptoethanol to eliminate the interference of polyphenol and polysaccharides, an effective protocol was developed. This modified protocol could extract high quantities of RNA from cycad leaves at about 930 μg per gram of fresh weight. Both $A_{260/230}$ and $A_{260/280}$ ratios were around 2, indicating that RNA is of high quality. The RNA quality was confirmed by Northern blotting analysis and reverse transcription polymerase chain reaction (RT-PCR). The modified protocol could be successfully extended to other plants containing secondary metabolites.

Key words: *Cycas revoluta*; Borax; RNA extraction

Extraction of high-quality RNA is necessary for making cDNA library, isolating genes by RT-PCR, or investigating gene expression profile. When performing the project "construction of the cDNA library for the valuable plants in Yunnan", we found it difficult to extract RNA from the leaves of *Cycas revoluta* (Cycadaceae) using available conventional protocols. The extract was highly viscous and brown because of the large amounts of polysaccharides, polyphenols and other unidentified compounds. Phenolic compounds are readily oxidized to form covalently linked quinines (Loomis, 1974), and avidly bind nucleic acids. The same situ-

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ation was also found in other two woody gymnosperms such as *Ginkgo biloba* (Ginkgoaceae), *Taxus wadlichiana* (Taxaceae) and three angiosperm species, including the ornamental orchid *Cypripedium flavum* (Orchidaceae) and two horticultural plants endemic to Yunnan: *Mussella lasiocarpa* (Musaceae), and *Lucalia gratissima* (Rubiaceae). In the present study, we successfully isolated high-quality and high-quantity RNA from these six plants with a modified CTAB method. Northern blotting and RT-PCR analysis were used to confirm the quality of the extracted RNA from *C. revoluta*.

1 Materials and methods

1.1 Materials

Young and mature leaves from six plants (*C. revoluta*, *G. biloba*, *T. wallichiana*, *Cy. flavum*, *L. gratissima*, *M. lasiocarpa*) were collected in Kumming Botanical Garden of Kumming Institute of Botany, the Chinese Academy of Sciences. Some cycad leaves harvested were treated with salicylic acid (SA) for 12 hr for Northern blotting analysis. All samples were frozen immediately with liquid nitrogen after collection and then stored at −80℃ until use.

1.2 RNA isolation

Modified extraction buffer: 2% (w/v) CTAB, 0.125 M disodium tetraborate decahydrate, 0.1 M Tris/HCl (pH 7.5), 2 M NaCl, 0.025 M EDTA, and 2% (v/v) β-mercaptoethanol (added just before use); 4 M LiCl; 5 M NaCl; 70% (v/v) ethanol. These solutions were treated with 0.1% (v/v) DEPC. Phenol/Chloroform/isoamyl alcohol (25∶24∶1 [v/v]), 0.1% DEPC-treated water.

Modified protocol: Three grams of frozen leaves were grinded to a fine powder and added to 10 mL pre-warmed extraction buffer, shaken vigorously and incubated at 65℃ for 6 min. When the homogenate was cooled for 10 min on ice, an equal volume of phenol/chloroform/isoamyl alcohol (25∶24∶1) was added and mixed gently. Following centrifugation at 10 000 g for 20 min at 4℃, the collected upper phase was precipitated with an equal volume of 4 M LiCl at 4℃ for 4 hours. After centrifugation at 10 000 g for 20 min at 4℃, the precipitate was suspended in 10 ml DEPC-treated water, and precipitated with 2.5 volume of ethanol and 1/10 volume of 5 M NaCl at −80℃ for 1 hour. After the same centrifugation as last time the pellet was washed with 70% (v/v) ethanol and air-dried on ice for 10 min. And then, the pellet was resuspended in an adequate volume of double-distilled DEPC-treated water. The RNA is ready for use or can be stored at −80℃ for up to 1 month.

1.3 RNA analysis

The purified total RNA was quantified with a spectrophotometer at wavelengths of 230 nm, 260 nm, and 280 nm. The integrity of total RNA was verified by running sample on a 1.5% formaldehyde/agarose gel.

1.4 Northern blotting analysis

The Northern blotting protocol was based on the method described by Sambrook *et al.* (1989). Partial *CrWRKY* sequence was obtained by PCR using the degenerate primers WRKY1-FP & WRKY 2-RP (Borrone *et al.*, 2004) from the genomic DNA of *C. revoluta* and cloned into pUCm-T Vector (Sangon, Shanghai). The positive clones were confirmed by sequencing and designated as *CrWRKY1*, *CrWRKY2* and *CrWRKY3*, respectively. The partial sequences of the three *CrWRKY* genes were used as probes in Northern blotting analysis respectively.

1.5 RT-PCR analysis

The single-stranded cDNA product was synthesized from the DNAase-treated RNA of *C. revoluta* using the AMV reverse transcriptase according to the manufacturer's instructions (Fermentas Corp.). The product was used to amplify the *CrrbcL* gene with the primers derived from the coding region of *CrrbcL* gene (*CrrbcL*-F: 5′-AGCAGCGTTCCGAGTAACTCCT-3′ and *CrrbcL*-R: 5′-TGAGCCAAGCTGGTATTTGCA-3′). The amplified products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

2 Results and discussion

Using the modified protocol, pure RNA was easily acquired within 8 hours. It efficiently eliminated most of the interference and produced white and water-soluble RNA precipitates with high-yield (approximately 0.42–0.956 mg/g fresh wt) (Table 1). Both $A_{260/230}$ and $A_{260/280}$ ratios were around 2 indicating that the RNA preparations were free of polysaccharide/polyphenol and protein contamination, respectively (Table 1). A formaldehyde-denatured agarose gel was used to check the integrity of ribosomal bands visulalized in

Table 1 Quality and yield of total RNA isolated from six plants*

Plant species	$A_{260/280}$	$A_{260/230}$	μg RNA/g FW
C. revoluta	1.90±0.023	1.88±0.026	956±35.6
G. biloba	1.99±0.019	2.27±0.021	921±26.9
T. wallichiana	1.85±0.018	2.30±0.014	570±19.8
M. lasiocarpa	2.08±0.021	2.11±0.068	420±23.5
L. gratissima	2.03±0.032	2.26±0.042	640±31.2
C. flavum	1.97±0.041	2.04±0.031	675±21.9

* Values are means±SD (n=6).

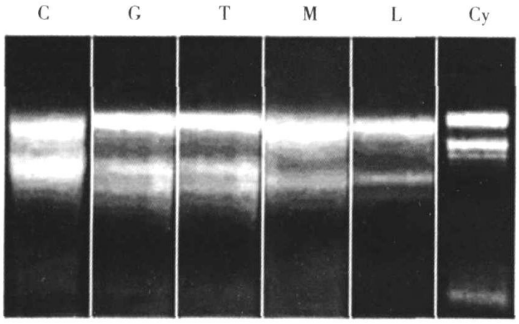

Fig. 1 Electrophoretic analysis of RNA isolated from six plants
C: *C. revoluta*; G: *G. bioloba*; T: *T. wallichiana*;
M: *M. lasiocarpa*; L: *L. gratissima*;
Cy: *Cypripedium flavum*.

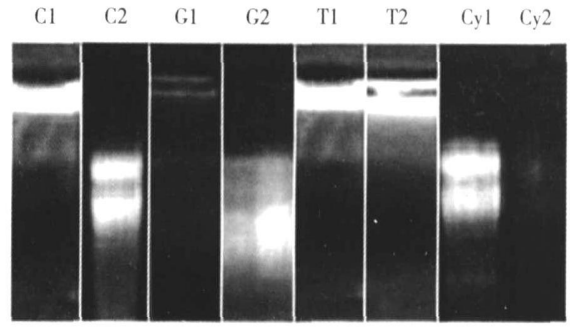

Fig. 2 Electrophoretic analsis of RNA from 4 plants by two different methods
C1, G1, T1, and Cy1: by standard CTAB method (Chang *et al.*, 1993); C2, T2, G2, and Cy2: by TRizol Kit (Invitrogen)

Fig. 1. The two main distinct bands (28S rRNA & 18S rRNA) could be visualized on the gel without obvious degradation.

In this study, we used the TriZol kit (Invitrogen) and conventional CTAB method (Chang *et al.*, 1993) to extract RNA from these six plants respectively. However, these two methods gave poor results and failed to yield useable RNA for further investigation (Fig. 2). The pellets were highly viscous and brown in gymnosperm plants and orchid. When the improved CTAB protocol applied, high-quality and high-quantity RNA was extracted from these plants. The most useful modification is the adding of borax and β-mercaptoethanol into the standard CTAB extraction buffer. In order to eliminate the oxidation of polyphenol, we tried different reagents, eg: PVP (polyvinylpyrrolidone), DTT (dithiothreitol), β-mercaptoethanol, and borax. Our results showed that the borax, together with β-mercaptoethanol, was more efficient than the other reagents to eliminate the interference of brown complexity. Previous studies in cotton have confirmed the significance of borax in preventing sample from oxidation, together with other reagents, such as Nonident-40 (NP-40), PVP, proteinase K, and DTT (Wan and Wilkins, 1994; Wu *et al.*, 2002). Therefore, using borax and β-mercaptoethanol in the CTAB extraction buffer is an effective modification to gain clean RNA from the six plants.

In addition, it is an efficient protocol to obtain pure RNA. The RNA can be directly isolated from DNA, proteins, and other secondary productions after LiCl precipitation. This protocol will be finished within one day. And then the RNA could be washed by 70% ethanol and resuspended in DEPC-treated water for Northern blotting. If RNA is used for cDNA library construction or RT-PCR, it should be precipitated by ethanol/NaCl and then washed. Long-term storage of RNA can be achieved by addition of 2.5 vol of absolute ethanol and 1/10 vol of 5 M NaCl.

The RNA isolated from cycad leaves was used in gene expression analysis by Northern blotting. *WRKY* genes encode a superfamily of plant-specific transcriptional factors, regulating physiological responses to biotic and abiotic stresses (Hara *et al.*, 2000; Rushton and Somssich, 1998) and senescence as well as development (Camerom, 2002; Lagac and Matton, 2004). Some WRKY genes expressions were induced by molecular signal SA to be associated with plant defense responses (Dong *et al.*, 2002). Studies on *WRKY* genes reported are restricted to only a few plant species such as *Arabidopsis*, rice, and tabacco (Yu *et al.*, 2001; Hara *et al.*, 2000; Zhang *et al.*, 2004). For this reason, we took an approach of analyzing the expression profiles of the three *WRKY* gene fragments (*CrWRKY1*, *CrWRKY2*, *CrWRKY3*) in both untreated and SA-treated leaves of cycad. The good-quality RNA of SA-treated or untreated leaves from cycad was used in *WRKY* genes expression analysis by Northern blotting, respectively (Fig. 2). The analysis revealed that the activities of the three *WRKY* gene fragments were not associated with SA, since the expression levels of

the three genes didn't show any significant difference between in SA-treated and untreated leaves. The results implied that the three genes might not be involved in SA-mediated disease resistant pathway.

The good quality of the RNA was also proved by confirmation from RT-PCR in cycad (Fig. 3). By use of the first strand cDNA, a part of the *CrrbcL* gene (around 400 bp) was successfully cloned.

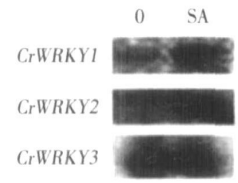

Fig. 3 Northern blotting analysis of RNA extracted from cycad leaves
0: RNA from untreated leaves; SA: RNA from SA-treated leaves

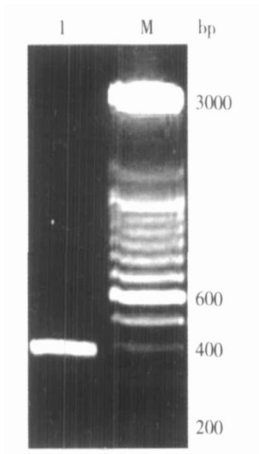

Fig. 4 Agarose electrophoresis of the RT-PCR products
1. rubulose 1, 5-bisphosphate carboxylase large subunit gene *rbc*L (400 bp), M. 100-bp DNA ladder.

In conclusion, the modified protocol could yield pure RNA from both mature and young leaves of these six plants.

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