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RESEARCH ARTICLE

Comparison of the methods of RNA isolation from *Juglans regia* L. buds

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Abstract **In this paper, four RNA extraction methods (CTAB method, TRIzol method, D326A reagent and RP3301 Kit) for walnut buds were compared by agarose gel electrophoresis and spectrophotometry, and the optimal method was verified further by Reverse transcription and PCR amplification (RT-PCR). There was a certain degree of degradation for the extracted RNA by CTAB method, TRIzol method and D326A reagent. Moreover, protein was found in the CTAB method. For the three unsatisfactory methods, longer operation time could be the main reason that RNA could not be separated in time with polyphenols, polysaccharides and other secondary metabolites. RP3301 kit was suitable for the RNA extraction from walnut buds with high purity, simplicity, innocuity and no degradation. This research will provide a fundamental basis for the cloning and expression analysis of genes and other follow-up tests.**

Keywords *Juglans regia* L., buds, total RNA, RP3301 kit

Introduction

Walnut (*Juglans regia* L., $2n = 32$), with a long cultivation history in China, is one of the main oil and woody species on the international market (Xi et al., 1996). It is difficult to isolate the walnut RNA due to its complexity of tissue components. Only when the secondary metabolites like polyphenols and polysaccharides are removed can the high quality RNA be extracted (Li et al., 2004), which can provide a fundamental basis for the cloning and expression analysis of genes and other follow-up tests. Molecular studies are mainly focused on using molecular markers to evaluate the resources of walnut varieties (Fjellstrom et al., 1994; Nicese et al., 1998; Potter et al., 2002; Yang et al., 2007; Wang et al., 2009; Wang et al., 2010; Zhang et al., 2010). It was by DNA that these researchers explored the genetic relationship among many walnut cultivars and evaluated these resources. Only Xu et al. (2010) constructed a cDNA library to clone genes and study the gene expression by RNA, and only Xu et al. (2008) and Liu et al. (2008a) reported the RNA extraction from walnut tissues. The molecular mechanism of the early fruit has not been reported although the precocious walnut possesses the characteristic of short juvenility (Xu et al., 2001; Li et al., 2008). To study the molecular mechanism and clone the related gene, high-quality RNA should be extracted from walnut tissues. In this paper, we isolated the RNA from walnut varieties of Zhonglin 5 and Qingxiang buds by four RNA extraction methods (CTAB method, TRIzol method, D326A reagent and RP3301 Kit), and compared the four methods using agarose gel electrophoresis and spectrophotometry and finally selected the optimal method by Reverse Transcription and PCR amplification.

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Materials and methods

Experimental materials

The apical buds of walnut cultivars Zhonglin 5 and Qingxiang were cut and collected at the campus of Agricultural University of Hebei, China on May 11th, 2009, with the lowest content of phenolic compounds and the weakest photosynthetic capacity (Wang et al., 2003). The cut apexes were frozen in liquid nitrogen immediately and stored at −70ºC until use. All reagents, EP tube and tips were treated with 0.1% (v/v) diethylpyrocarbonate (DEPC), and the glassware were thoroughly rinsed and heated overnight at 180ºC.

Methods

Total RNA extraction

The RNA of walnut buds was extracted by using CTAB method modified according to Xu (2008), TRIzol (Invitrogen cat.No.15596-026) method, D326A reagent (TaKaRa D326A) and RP3301 Kit (BioTeKe RP3301) referring to their instructions, respectively. 1% and 2% gradients were set for the CTAB concentration, and there were no gradients for the other three methods. Ten samples (5 copied buds of Zhonglin 5 and Qingxiang cultivar, respectively) were used to isolate RNA, and each sample was 0.1 g of bud. The RNA was resuspended ultimately in 50 μL RNase (free water). The RNA by RP3301 Kit was resuspended in 100 μL RNase (free water) according to the kit's directions. The integrity was verified by running samples on 1.5% agarose gel and stained with ethidiumbromide.

The purity and yield of RNA

Total RNA was quantified with a spectrophotometer at wavelengths of 230, 260 and 280 nm. Then the purity was detected according to the value of $OD_{260/280}$. When the ratio was between 1.9 and 2.1, the purity was better. There was protein impurity at the ratio of lower than 1.9, and the RNA was degraded seriously at the ratio higher than 2.1. The value of $OD_{260/230}$ was used as reference value. The ratio less than 2.0 indicated that there were other impurities such as peptide and phenol. The concentration (μg/mL) and yield (μg/g) of RNA were calculated by the following formula (Wang et al., 1998):

$$\text{Concentration of total RNA} = OD_{260} \times \text{dilution ratio} \times 40\ \mu g/mL$$

Reverse transcription and PCR amplification

Single-stranded cDNA was synthesized with 0.25 μg total RNA using the M-MLV reverse transcriptase and oligo (dT) according to the manufacturer's instructions (TaKaRa, D314). M-MLV (-) control was set at the same time. The cDNA was used for the PCR amplification of *JrLFY* gene (GenBank GU194836). The specific primers derived from the coding region of the gene were: F (5′ - ATGGATCCCGACCCCTT-TAC- 3′) and R (5′-TCCACCACTTTCCTCAGGC-3′). PCR program parameters were: pre-denaturation at 94ºC for 3 min, followed by 35 cycles of DNA denaturing at 94ºC for 30 s , primer annealing at 52ºC for 30 s, and extension at 72ºC for 30 s with final extension at 72ºC for 10 min. The amplified products were separated on a 1% agarose gel and visualized by ethidium bromide staining. The amplified *JrLFY* gene fragment was cloned into pGM-T vector (Tiangen) as described by the manufacturer and the ligation product was used to transform DH5α *E. coli*. Clones carrying recombinant plasmids were collected and sent to Shanghai Sangon for sequencing.

Results

RNA electrophoresis analysis

The integrity of RNA was checked on a 1% agarose gel (Fig. 1). There was a serious degradation for No.1-No.8 samples. However, two distinct bands (28S rRNA and 18S rRNA) were visualized on the gel for No.9 and No.10 samples. The significant difference in the results was mainly due to the impact of RNase and phenols and other substances on RNA. The RP3301 Kit reduced the impact because of shorter operating time and fewer frequency of changing EP tube.

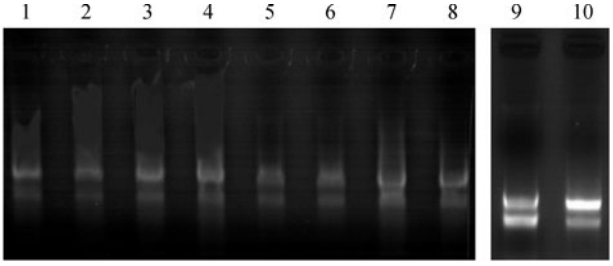

Figure 1 The agarose gel electrophoresis for RNA extracted by different methods. Note: 1–4 are in CTAB method (1–2 in 1% CTAB, 3–4 in 2% CTAB). 5–6 are in TRIzol method. 7–8 are in D326A reagent. 9–10 are in RP3301 kit. 1, 3, 5, 7 and 9 stand for Zhonglin. 5, 2, 4, 6, 8 and 10 stand for Qingxiang.

The purity and yield of RNA

The RNA extracted by the four methods did not contain impurity. The quality of 2% CTAB was better than that of 1% CTAB; unfortunately, protein occurred in the RNA by CTAB method. There was a significant degradation for the RNA by CTAB method, TRIzol method and D326A reagent, which was directly related to the complexity of operating procedures. There was almost no degradation for the RNA by RP3301 Kit, with better purity and yield of the RNA than those of other methods (Table 1).

Reverse transcription and PCR amplification

Clean RNA is the most important for successful construction of full-length cDNA and other follow-up tests. Reverse transcription is highly sensitive to impurities. Therefore, we further verified the RNA quality by reverse transcription and PCR amplification. No PCR products from the No.1–No.8 were observed. While the PCR products of No.9 and No.10 were detected by the 1% agarose gel (Fig. 2) and sequenced after being recovered. The amplified product was of 557 bp and was verified as one part of *JrLFY* gene (55–611 fragment of GU194836 from GeneBank). Target band did not appear in the Lane 1 with Lane 3 as negative control. The above result suggested that the 557 bp bands were not obtained from the

Table 1 $OD_{260/280}$, $OD_{260/230}$ and yield of RNA extracted by different methods

Value	1% CTAB		2% CTAB		TRIzol		D326A reagent		RP3301 Kit	
	1	2	3	4	5	6	7	8	9	10
$OD_{260/280}$	1.59	1.66	1.84	1.81	2.38	2.15	2.18	2.15	1.91	1.96
$OD_{260/230}$	2.01	1.97	1.94	1.91	2.02	2.09	2.07	2.08	1.95	2.08
Yield(ug/g)	74.7	67.4	102.7	113.5	47.5	42.5	87.5	69.6	300	356.8

Note: 1, 3, 5, 7 and 9 stand for Zhonglin and 5, 2, 4, 6, 8 and 10 stand for Qingxiang.

genomic DNA but from the cDNA, and that the RP3301 Kit was very suitable to isolate RNA for the following test.

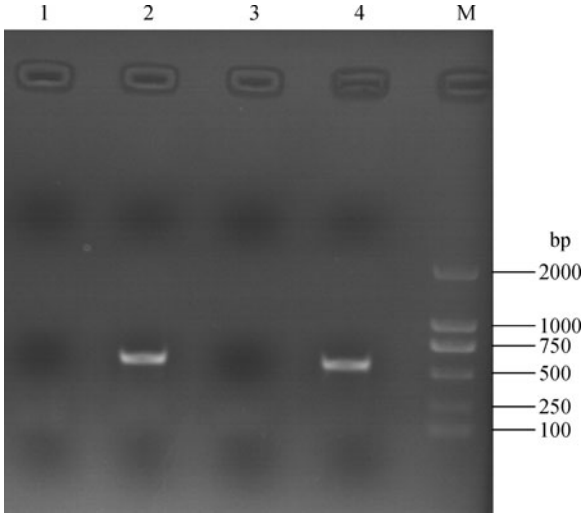

Figure 2 The RT-PCR bands of No.9 and No.10 RNA. Note: M stands for marker DL2000, Lanes 1 and 3 mean RT-PCR without M-MLV, and Lanes 2 and 4 mean RT-PCR with M-MLV. 1–2 stand for Zhonglin 5 and 3–4 stand for Xiangling 5.

Discussion

The report about RNA extraction from walnut tissues was rare, which was directly related to the impact of the polyphenols and polysaccharides. Xu et al. (2008) and Liu X et al. (2008) isolated the RNA by modified CTAB from different species and different tissues, but there was a certain degradation. In our study, no high-purity RNA from walnut buds was found by using different methods. Even sometimes a little RNA was observed, which lies in that walnut tissues contained more polyphenols and polysaccharides and other secondary metabolites than other species in the same growth period (Liu et al., 2008a). RNA was easy to form jelly precipitation with polysaccharide because of many physical and chemical properties (Logemann et al., 1987; Fang et al., 1992). Phenolic compounds were easily oxidized into quinone, bitten with the RNA irreversibly (Chen et al., 2005). Therefore, it is a key step to isolate RNA from proteins, polysaccharides, polyphenols, pigments, DNA and other impurities as soon as possible. RNA not only was contaminated by various impurities, but also was vulnerable to the effects of RNase (Maliyakal, 1992; Woodhead, 1997). To inhibit the activity of RNase, great efforts should be made to avoid the contamination of human-induced exogenous RNase in addition to the endogenous RNase. At the same time, each step should be fulfilled in ice to further suppress the RNase.

However, we did not obtain the high-quality RNA by CTAB, TRIzol and D326A reagent even under the extremely strict conditions and operation. Among them CTAB was poor in the removal of impurity in addition to the serious degradation. In comparison, 2% CTAB was better than 1% CTAB in RNA quality. We also set different concentrations of gradient for PVP and β-mercaptoethanol, but the effect was not obvious. TRIzol performed well in the removal of impurities; however, the problem of degradation could not be solved. D326A reagent was specially designed to extract the RNA from the plant tissues rich in polyphenols and polysaccharides but the RNA was still degraded. These showed that polyphenols and polysaccharides must be rich in walnut buds.

High levels of phenols were also constraints for RNA extraction from cotton and pears. Gossypol content in cotton was about 0.60%–1.04% (Yao et al., 2010). 0.173%–0.229% phenol contents in cotton leaves and 0.96%–1.41% phenol contents in cotton seeds were measured, respectively (Liu et al., 2008b). The phenol content in *Pingguoli* pear peel was about 2.5% (25 mg/g) and phenolic content in sarcocarp was about flesh 0.6% (6 mg/g) (Zhao et al., 2009). However, the average phenol content in walnut tissues was at the range of 2.95%–11.86% (29.5 mg/g–118.6 mg/g) (Wang et al., 2003), with the phenolic content in walnut much higher than that in cottons and pears. The strong interference of polyphenols and polysaccharides suggested that we need to find a way to shorten the process of RNA extraction. RP3301 Kit made RNA isolated from other substances quickly by adsorption column, which greatly weakened the interference of other substances. By electrophoresis and spectrophotometry, it was found that the RNA extraction was complete with very high quality. Results showed that the RNA extraction by RP3301 Kit can be used in the follow-up test by RT-PCR, with the advantages of simplicity, innocuity, etc. Therefore, RP3301 Kit as the most ideal method of RNA extraction from walnut buds, will provide a fundamental basis for the cloning and expression analysis of genes and other follow-up tests.

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