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Full Length Research Paper

A method for high-quality RNA extraction from tall fescue

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The isolation of high-quality RNA was a precondition in molecular biology research of tall fescue. Two common approaches were adopted for the total RNA extraction by using leaves of tall fescue as the material in this experiment in order to seek the optimized total RNA extraction method of tall fescue, as well as the improvement of the extraction method used by the predecessors. The results showed that the Trizol method cost much and was not suitable for the large quantity of plant tissues extraction. The traditional isothiocyanate method resulted in protein contamination and RNA degradation. By employing the improved isothiocyanate method, we found that there were three bright bands in agarose gel electrophoresis (28S rRNA, 18S rRNA and 5S rRNA). The band of 28S rRNA was brighter than that of 18S rRNA, and the value of OD_{260}/OD_{280} was 1.8 to 2.0. Clear bands and high polymorphisms were obtained by cDNA-AFLP analysis. These results indicated that the RNA isolated by the improved isothiocyanate method had a good integrity and high purity, which could be used for the later molecular researches.

Key words: Tall fescue, RNA extraction, improved isothiocyanate method.

INTRODUCTION

Extraction of high-quality RNA is a necessary pre-condition and key technology of molecular biology research. For example, high quality RNA is necessary for target gene clone, blot analysis, cDNA library construction and RT-PCR analysis (Gambino et al., 2008). Although RNA extraction has become a very mature technology, in practice, to get high-quality RNA is particularly challenging (Sivakumar et al., 2007; Wang et al., 2010). For many plants, the study of molecular biology is limited due to lack of an effective method for RNA extraction (Bahloul and Burkard, 1993). There are a variety of secondary metabolites and macromolecules, which could influence efficiency of RNA extraction and interference reverse transcription and enzyme digestion (Sivakumar et al., 2007). Besides that, it is difficult to have a uniform method for all plants RNA extraction due to significant discrepancy of different plant's metabolites as well as specificity of different plant's tissue (Ainsworth, 1994; Zhang and Rouf, 2003). At present, there are many reports on total RNA extraction methods of plant issues, but most of those methods are time-consuming (Li et al., 2008). Since RNA can be degraded easily, the shorter the operating time, the smaller possibility for RNA degradation.

Tall fescue (*Festuca arundinacea* Schreb.) is a cool-season grass species of great economic importance in Europe, Asia, North Africa and North America (Dong et al., 2007). It is widely distributed in the southwest, northwest, and northeast of China for establishment of artificial grassland and reseeding of natural grassland. Tall fescue is an important grass with dual-purpose, turf and forage purpose, due to its brilliant adaptation to various soil conditions, tolerance to continuous grazing, resistance to disease and coldness, and high yields of forage and seed (Wang and Ge, 2004; Huang et al., 2008).

Tall fescue has been one of the major target grass species for genetic and agronomic studies for its agronomic importance (Wang and Ge, 2004). Extraction of total RNA of tall fescue has been used for the studies of molecular basis of the plant-endophyte interaction (Johnson et al., 2003; Dinkins et al., 2010) and resistance

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of abiotic and biotic stresses, such as heavy metal stress (Lee et al., 2007), fungal diseases (Dong et al., 2007; Ergen et al., 2007) and so on. Although the extraction of total RNA is a critical step in the studies of molecular biology of the tall fescue, few protocols have been developed (Wang et al., 2007). The time-saving Trizol method is the most commonly used method for the total RNA extraction of tall fescue (Wang and Ge, 2005; Dong et al., 2007; Neslihan et al., 2007; Wang et al., 2007; Dinkins et al., 2010), but it is not suitable for large quantity plant tissues extraction because of its relatively high cost (Li et al., 2008). The isothiocyanate method makes use of guanidine isothiocyanate, β-mercapto-ethanol and sodium lauryl sarcosine to make RNase denaturation, and to free RNA into the aqueous phase under acidic conditions, while denatured RNase and other proteins with genomic DNA go into the organic phase (Chomczynski and Sacchi, 1987). We can get high-quality RNA when the aqueous phase which was extracted repeatedly by chlorophenols was precipitated with isopropanol. The advantage of this method is simple and time-saving, and can conduct multi-samples simul-taneously (Gasic et al., 2004). In this study, we improved the conventional isothiocyanate method in order to create a simple, rapid method which is suitable for total RNA extraction of tall fescue, which could lay foundations for the later researches of molecular biology.

MATERIALS AND METHODS

Plant material

Young leaves were collected from field-grown tall fescue; all samples were frozen by liquid nitrogen treatment at the time of collection, and stored in 80°C ultra low freezers for usage.

Establishment of RNase-free environment

An environment without RNase must be created before RNA extraction; all plastic ware were treated with 0.1 to 0.2% (v/v) diethyl pyrocarbonate (DEPC) and kept at 37°C for over 12 h, then autoclaved to inactivate DEPC; mortar, pestles, spoon and all glasswares used in the isolation of total RNA from plant material were kept overnight at 160°C; solutions used in RNA extractions were prepared using the water treated by DEPC; electrophoresis tank, gel tray and comb were also treated with DEPC for overnight or soaked in 0.5% SDS (sodium dodecyl sulfate, inactivate RNases) for overnight after cleaned by detergent, then cleaned with ddH_2O and dried for usage (Yin et al., 2008; Malnoy et al., 2001).

RNA extraction protocols

We optimized PCR system for EST-SSR primer by using DNA of *Eucalyptus tereticornis* Smith (P_2), mainly for Mg^{2+} concentration and annealing temperature of PCR procedures. We selected 32 EST-SSR which had different length of PCR products for further sequencing experiments. The PCR products after PCR optimization, primer sequences and sizes are listed in Table 1. PCR system and procedure were done according to to Li et al. (2008). Annealing temperatures were 56°C uniformly, whereas Mg^{2+} concentration in 10×buffer was changed to 15 or 20 mmol/L.

The isothiocyanate method was according to Chomczynski and Sacchi (1987) and Chao et al. (2009) methods for total RNA extraction. For the Trizol method, Trizol reagent were purchased from Shanghai ShengGong bioengineering corporation and extrac-tion of total RNA were carried out following the manufacturer's instructions. In the traditional isothiocyanate method, the plant materials were added to denaturing solution and β-mercaptoethanol after lapping and packing separately, but in the improved method, the denaturing solution and β-mercaptoethanol were added firstly, and then even grinded and packed separately after the reagent completely melted; meanwhile, the amount of β-mercaptoethanol were increased from the original 3.0 to 5.0 µl. Agarose gel electrophoresis (1% mass fraction) was used to detect the integrity of total RNA and then photographed with the BioRad gel imaging system to for recording. After addition of 0.1% DEPC-water into 5 µl total RNA solution to get 500 µl, optical density (D) were assessed by determining the spectrophotometric absorbance of the samples at 230, 260 and 280 nm and ratios of D_{260}:D_{280} and D_{260}:D_{230} were used to calculate degree of purity.

RNA yield (µg/g) = [OD_{260} × N (sample dilution multiple) × 40 (µg/mL) × V (volume)] / sample weight (g).

cDNA-AFLP analysis

Double-stranded cDNA was synthesized from 2 µg total RNA extracted from tall fescue by TaKaRa company's M-MLV RTase cDNA synthesis kit, and then diluted 20 - 50 times after purification, and digested by EcoR I and Mse I. Finally, artificial linker ligation was conducted to get a pre-amplification template. Pre-amplification products were diluted 20 times for selective amplification, and the PCR products were separated by electrophoresis on 8% agarose gel, and then detected by silver staining method (Wang et al., 2009).

RESULTS

Integrity testing of tall fescue total RNA extracted by different methods

The results of the agarose gel electrophoresis testing showed that we can extract total RNA from the mature leaves of tall fescue by the Trizol method. The band intensity of 28S rRNA and 18S rRNA was close to 2:1, indicating that RNA was not degraded (Figure 1A). Although the Trizol method was effective, it costs too much and was not suitable for RNA extraction from large amounts of plant tissues.

The electrophoresis of tall fescue's total RNA extracted by the isothiocyanate method showed that (Figure 1B); the two bands, 28S rRNA and 18S rRNA, had conditions of streaking, and 5S rRNA had a brighter band, indicating that there were partly degradation of the total RNA and DNA contamination. Meanwhile a bright band existed in the loading well, indicating that there was severe protein contamination.

By employing the improved isothiocyanate method, none of the bright bands were found at the loading well through the agarose gel electrophoresis testing, which proved that there was no protein contamination.

Table 1. Purity and yield of RNA samples extracted by different methods.

Method	RNA yield (μg/g)	Absorbance	
		OD_{260}/OD_{280}	OD_{260}/OD_{230}
Isothiocyanate method	98.10	2.291	1.706
Trizol method	118.60	1.831	2.198
Improved isothiocyanate method	224.40	1.871	2.118

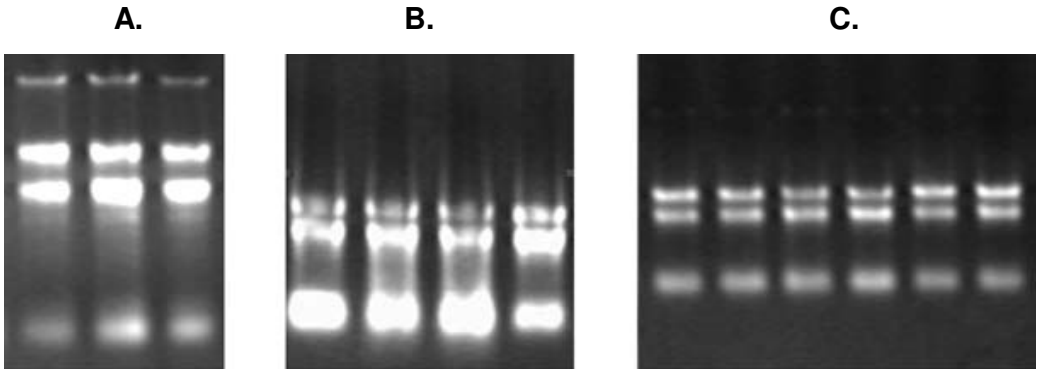

Figure 1. Agarose gel electrophoresis of total RNA extracted by (A) Trizol method, (B) isothiocyanate method and (C) improved isothiocyanate method.

Furthermore, two bright bands (Figure 1C); 28S rRNA and 18S rRNA, were very clear, and the brightness value of these two bands was close to 2:1, while the band of 5S rRNA was very weak, which proved that the total RNA had no DNA contamination, and had a good integrity and high purity without obvious degradation.

Assessment of tall fescue total RNA purity and concentration extracted by different methods

Absorbance ratio directly reflects contamination degrees of polysaccharides, polyphenols and proteins. The OD_{260}/OD_{280} values of RNA extracted by using the improved isothiocyanate method and the Trizol method were 1.8 and 2.0, respectively (Table 1), indicating that the RNA had a good integrity and could be used for further molecular biology research. The value of OD_{260}/OD_{230} was 2.0 to 2.5, which indicated that the RNA obtained by the two methods had high purity. RNA samples extracted by the isothiocyanate method had a smaller OD_{260}/OD_{280} value demonstrating the existence of proteins or phenols. The value of OD_{260}/OD_{230} was less than 2.0, which showed that there was a large number of micromolecule or salt, and we need a further purification of the RNA.

cDNA-AFLP analysis for total RNA of tall fescue

The most critical step of cDNA-AFLP experiment is to obtain high-quality cDNA template and then make a thorough enzyme cutting in the cDNA template, while extraction of high quality total RNA is the prerequisite of getting high-quality cDNA template. In order to validate the quality of the total RNA extracted by the improved isothiocyanate method, we synthesized double stranded cDNA and then conducted cDNA-AFLP analysis. The results showed that the total RNA extracted by the improved isothiocyanate method had features of high-quality and non-contamination. We obtained a clear AFLP map after reverse transcription and enzyme cutting (Figure 2), which was fully available for subsequent cDNA-AFLP analysis.

DISCUSSION

High-quality RNA is the precondition of gene cloning, gene expression and other studies. Conventional RNA extraction methods consist of isothiocyanate method, CTAB method, hot borate method, phenol method, anionic detergent method, LiCl-urea method, Trizol method, Gomez method and so on (Ainsworth, 1994; Gambino et al., 2008). However, different plant species and different parts of the same plant have their own characteristics of physical structure and contain various endogenous substances. Therefore, in order to get the best RNA extraction methods for different plant species, we should screen and improve methods according to their own characteristics (Yin et al., 2008). In addition, RNA can be easily degraded by trace amounts of RNase, meanwhile, plant tissues contain large amounts of

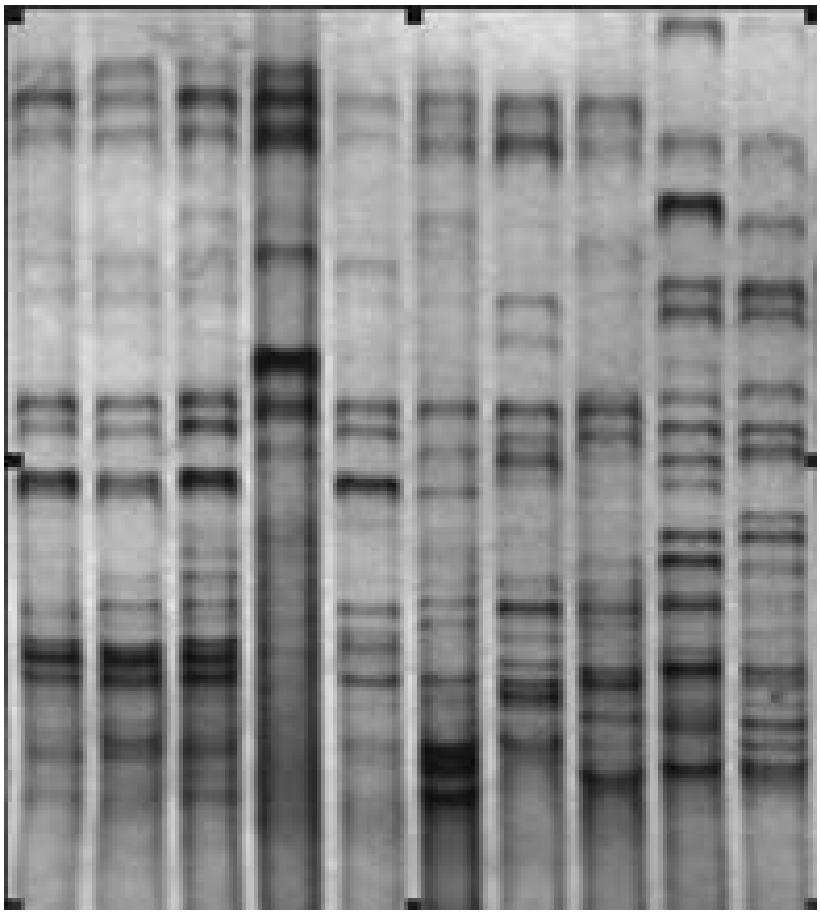

Figure 2. cDNA-AFLP analysis of total RNA extracted by modified AGPC protocol.

secondary metabolites; both interfere with the purification steps and hampered the extraction of total RNA, therefore the total RNA extraction of plant tissue is relatively more difficult (Li et al., 2010; Lewinsohn et al., 1994).

The Trizol method is one of the preferred conventional methods for RNA extraction at present because of its advantages of simplicity and rapidity (Ramchandra and Willem, 2010). Experiments shows that the Trizol method is the easiest and the most time saving method which only required about 2 h and total RNA of tall fescue was obtained with better integrity and purity but it is not suitable for RNA extraction from large amounts of plant tissues because of its relatively high cost (Dinkins et al., 2010; Wang et al., 2010). The isothiocyanate method is one of the conventional methods commonly used in laboratory for total RNA extraction of plant tissues. The main mechanism of this method is the interaction effect of guanidine isothio-cyanate, sodium lauryl sarcosine and mercaptoethanol leading to denaturation of proteins and inactivation of RNase; and the proteins was denatured and centrifuged down together with DNA under acidic conditions, where after RNA was isolated and dissolved in the supernatant fluid (Chomczynski and Sacchi, 1987). The isothiocyanate method was comparatively inexpensive and required only 3 to 4 h, but resulted in poor quality of total RNA of tall fescue, partial degradation and protein contamination. Longer grinding time may lead to RNA degradation. RNase activity was not inhibited and resulted in the release of endogenous RNase which degraded RNA. Furthermore, insufficient grinding of plant materials resulted in incomplete precipitation of polysaccharide and protein which affected purity of total RNA (Sivakumar et al., 2007).

This experiment made improvement on details of the isothiocyanate method, such as joining denaturing solution and mercaptoethanol after grinding, further grinding, separate packaging, and reduction of volatile mercaptoethanol loss by the force of frost action of liquid nitrogen. In the process of freezing and thawing by liquid nitrogen, denaturing solution and mercaptoethanol can play a better role in the inhibition of RNase activity; further grinding was more conducive to mix plant cells and increase efficiency of RNA extraction. In addition, increasing amounts of mercaptoethanol could inhibit activity of RNA enzymes in a shorter time, thereby reducing RNA degradation by RNase, and get high-

quality total RNA can be gotten. The modified isothiocyanate method could highlight its advantages when we need to extract large amounts of total RNA, reduce test time and opportunities of RNase contamination. We obtained total RNA of tall fescue with high purity and good integrity by using this system, and fully satisfied the requirements of molecular biology experiments, facilitating studies in tall fescue molecular marker-assisted breeding, gene mapping and cloning, high-density gene map drawing and studying of gene function, furthermore reference were provided for the extraction of total RNA of other turf grasses.

In conclusion, we reported an improvement of the standard isothiocyanate method for isolation of high-quality RNA from tall fescue, without expensive cost. This isothiocyanate method is an easy and time-saving extraction protocol and could facilitate studies in tall fescue genome research.

REFERENCES

Ainsworth C (1994). Isolation of RNA from floral tissue of *Rumex acetosa* (sorrel). Plant Mol. Biol. Rep. 12: 198-203.

Bahloul M, Burkard G (1993). An improved method for the isolation of total RNA from spruce tissues. Plant Mol. Biol. Rep. 11: 212-215.

Chao YH, Kang JM, Sun Y, Yang QC, Wang PQ, Wu MS, Li Y, Long RC, Qin ZH (2009). Molecular cloning and characterization of a novel gene encoding zinc finger protein from *Medicago sativa* L. Mol. Biol. Rep. 36: 2315-2321.

Chomczynski P, Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenolchloroform extraction. Anal. Biochem. 162: 156-159.

Dinkins RD, Barnes A, Waters W (2010). Microarray analysis of endophyte-infected and endophyte-free tall fescue. J. Plant Physiol. 167: 1197-1203.

Dong SJ, Tredway LP, Shewb HD, Wang GL, Sivamani E, Qu RD (2007). Resistance of transgenic tall fescue to two major fungal diseases. Plant Sci. 173: 501-509.

Gambino S, Perrone I, Gribauto I (2008). A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. Phytochem. Anal. 19: 520-525.

Gasic K, Hernandez A, Korban SS (2004). RNA extraction from different apple tissues rich in polyphenolsand polysaccharides for cDNA library construction. Plant Mol. Biol. Rep. 22: 437-438.

Huang X, Ye HX, Shu XL, Wu DX (2008). Application of biotechnology in genetics and breeding of tall fescue. (In Chinese) J. Nuclear Agric. Sci. 22: 806-810.

Johnson LJ, Johnson RD, Schardl CL, Panaccione DG (2003). Identification of differentially expressed genes in the mutualistic association of tall fescue with *Neotyphodium coenophialum*. Physiol. Mol. Plant Pathol. 63: 305-317.

Lee SH, Ahsan N, Lee KW, Kim DH, Lee DG, Kwak SS, Kwon SY, Kim TH, Lee BH (2007). Simultaneous overexpression of both Cu Zn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. J. Plant Physiol. 164: 1626-1638.

Lewinsohn E, Steele CL, Croteau R (1994). Simple isolation of functional RNA from woody stems of gymnosperms. Plant Mol. Biol. Rep. 12: 20-25.

Li C, Zeng HM, Han LB (2008). Comparative analyzing of total RNA extraction method in turfgrass. (In Chinese) Biotechnol. Bull. 1: 219-223.

Li XY, Cao X, Fang JG, Zhang Z (2010). Study on methods for RNA extraction from apricot leaf and fruit. (In Chinese) Chinese Agric.Sci. Bull. 26: 152-156.

Malnoy M, Reynoird JP, Mourgues F, Chevreau E, Simoneau P (2001). A method for isolating total RNA from pear leaves. Plant Mol. Biol. Rep. 19: 69.

Neslihan ZE, Gizem D, Robert CS, Hikmet B (2007). Identifying,cloning and structural analysis of differentially expressed genes upon *Puccinia* infection of *Festuca rubra* var. *rubra*. Gene, 393: 145-152.

Ramchandra P, Willem SA (2010). Validation of RNA integrity from low yield experiments with *Mycobacterium tuberculosis* for downstream application in real time PCR. Afr. J. Biotechnol. 4: 1594-1598.

Sivakumar S, Franco OL, Thayumanavan B (2007). Isolation of RNA from polysaccharide-rich seeds. Preparative Biochem. Biotechnol. 37: 323-332.

Wang FY, He Y, Xu BB, Xu F, Tian ZH (2007). Comparison of methods of RNA extraction from turfgrass plant. (In Chinese) J. Yangtze Univ. 4: 56-60.

Wang TK, Shang ZZ, Zou ZR, Wang XX, Du YC (2009). One modified method for isolating total RNA from tomato fruits for cDNA-AFLP analysis. (In Chinese) Acta Agric. Boreali-occidentalis Sinica. 18: 290-293.

Wang XX , Wang B, Liu LJ, Cui XP, Yang JY, Wang H, Jiang H, Luo BB, Long Z, Dou WX, Zhang N, Peng DX (2010). Isolation of high quality RNA and construction of a suppression subtractive hybridization library from ramie (*Boehmeria nivea* L. Gaud.). Mol. Biol. Rep. 37: 2099-2103.

Wang ZY, Ge YX (2005). Agrobacterium-mediated high efficiency transformation of tall fescue (*Festuca arundinacea*). J. Plant Physiol. 162: 103-113.

Yin H, Chen L, Li XY, Chen QM, Yi MF (2008). Analysis and improvement of high-quality RNA extraction in leaves of lily. (In Chinese) J. China Agric. Univ. 13: 41-45.

Zhang Y, RoufMian MA (2003). Functional genomics in forage and turf-present status and future prospects. Afr. J. Biotechnol. 12: 521-527.