

Preparation of total RNA from a very small wheat embryo suitable for differential display

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Summary

A simple method was developed for extracting total RNA from a single mature wheat seed embryo, which is very small and hard. Guanidine thiocyanate and chloroform were employed and the grinding of the samples was performed in a microcentrifuge tube with a plastic pestle. A "jacket" of liquid nitrogen and simplified procedures were applied to ensure the thorough grinding of the embryo tissue and to minimise the loss of samples. These measures substantially increased the recovery of total RNA in the extraction process. Reliable differential display was successfully achieved with the total RNA after DNase treatment and reverse transcription. This method makes it viable to study gene expression and gene regulation in a single wheat seed embryo. It may also give researchers the ability to analyse mRNAs in tissues or organs which were previously too small for RNA isolation using conventional procedures.

Key words: Total RNA, single wheat embryo, differential display, reverse transcription

Introduction

The expression of many genes may be differentially regulated in response to the change of environmental elements, developmental stages, chemical treatment and genotypes. Analysis of gene expression is very important in biological studies (White, 2001). The difficulty of isolating RNA from a small amount of tissue is an obstacle to the analysis of gene expression. For example, the analysis of gene expression associated with dormancy and its breakage in a wheat embryo is of very important agronomic interest, because this breakage may relate to pre-harvest sprouting which damages the quality of grains (Derera, 1993). Dormancy and its breakage are very sensitive to many factors and the condition of the seeds, some of which are indistinguishable, such as the maturity of seeds and any injury to the seed coat. In order to monitor the possible bias caused by the variation in seed condition, it is necessary to analyse individual seed embryos besides the analysis of pooled embryos. There are many available protocols for extracting RNA from different plant materials, but large amounts of tissues are required (Wadsworth *et al.*, 1988; Vicient & Delseny, 1999). There is no method available for extracting RNA from a wheat embryo. A wheat embryo is very small (600 µg) and hard. For such small material, a high yield RNA isolation method

is essential for obtaining sufficient RNA. Effective lysis of the cells and minimum sample loss are crucial for such a method. It has been reported that total RNA can be isolated from a small piece of human eye tissue (several milligrams). The eye tissue was homogenised in extraction buffer, and a silica gel binding technique was used to isolate total RNA (Gonzalez *et al.*, 1999). However, it is impossible to homogenise a hard wheat embryo well in extraction buffer. It is also difficult to grind a wheat embryo in liquid nitrogen. The frozen embryo tissues may quickly melt during grinding, or boiling liquid nitrogen may cause sample loss. In order to solve this problem, we designed a liquid nitrogen "jacket" to ensure the thorough grinding of an embryo into a fine powder. In the subsequent extraction, we utilised guanidine thiocyanate, which has been widely applied for RNA isolation (Maniatis *et al.*, 1989; Puissant & Houdebine, 1990), and adopted an ethanol precipitation procedure to collect the RNA. We simplified the procedures of the available methods (Maniatis *et al.*, 1989) to minimise the RNA loss and obtain purified DNA-free total RNA from a single wheat embryo. The RNA was suitable for differential display reverse transcription PCR (DDRT PCR), which is a useful method in gene expression analysis (Liang & Pardee, 1992; Liang *et al.*, 1994).

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Materials and Methods

General

Pipette tips, tubes, plastic pestle and MilliQ water were treated with diethyl pyrocarbonate (DEPC) (Sigma, St. Louis, USA) and autoclaved. Guanidine thiocyanate (4 M) (Sigma, St. Louis, USA) solution and 800 ml litre⁻¹ ethanol were made with DEPC treated and autoclaved MilliQ water.

Plant materials

Mature seeds of two spring wheat varieties, which differ in their susceptibility to pre-harvest sprouting, AUS1408 (resistant) and Cascades (susceptible), were used to provide a source of embryo material.

Sample preparation

A single wheat seed was separated, by using a sterile scalpel blade. The embryo was isolated under a dissecting microscope and placed in a polypropylene 1.5 ml centrifuge tube filled with liquid nitrogen. To provide a cooling jacket during the grinding process, a 5 ml polypropylene tube (Sarstedt, Ingle Farm, SA, Australia) was fixed in a foam plastic block, and filled with liquid nitrogen. The 1.5 ml centrifuge tube containing an embryo was then placed into this tube and the liquid nitrogen in both tubes was replenished, until it no longer boiled. The embryo was ground into a fine powder using a pre-chilled disposable pellet pestle (Kontes, Vineland, NJ, USA).

Total RNA extraction

After the embryo sample was fully ground, the tube was allowed to warm up to room temperature. A total RNA isolation buffer of 600 µl, containing 488 µl 4 M guanidine thiocyanate, 12 µl 2-mercaptoethanol (ICN Biomedical, Aurora, Ohio, USA) and 100 µl chloroform was freshly prepared. Initially, 100 µl RNA isolation buffer was added to the ground embryo material and the plastic pestle was used to ensure that a homogenised suspension was formed. Subsequently, the remaining total RNA isolation buffer (500 µl) was added and used to rinse the material remaining on the pestle into the tube. The solution in the tube was mixed and left at room temperature for about 30 min. The tube was then centrifuged at 16 100 g for 10 min and the supernatant was transferred into a new tube. 1 ml absolute ethanol was added to the supernatant and, following mixing, placed at -20°C for 2 h. The tube was then centrifuged at 16 100 g for 10 min and the supernatant discarded. The pellet was washed twice with 200 µl 800 ml litre⁻¹ ethanol and allowed to dry briefly, following which 42 µl of DEPC treated MilliQ water was added and the solution was stirred with a pipette tip to assist in re-dissolving the pellet. Finally, the tube was centrifuged at 16 100 g for 5

min and the supernatant transferred to a new tube for DNase treatment.

DNase treatment and analysis

RQ1 RNase Free DNase (Promega, Madison, WI, USA) was used to treat the RNA sample. To a 42 µl sample of the total RNA solution, 5 µl 10 × RQ1 DNase Reaction Buffer, 2 µl RQ1 DNase (one unit µl⁻¹) and 1 µl RNasin (Promega, Madison, WI, USA) (35 unit µl⁻¹) were added and mixed. The tube was incubated at 37°C for 15 min. Then 100 µl DEPC treated MilliQ water and 150 µl chloroform were added to the mixed solution and the tube centrifuged at 16 100 g for 10 min. After centrifugation, the aqueous phase was carefully transferred to a new tube and 150 µl DEPC treated MilliQ water was added to the remaining chloroform. The centrifugation was repeated and the two aqueous supernatants were pooled. Two volumes of absolute ethanol were then added to the supernatant and mixed. The tube was placed at -20°C for 2 h, followed by centrifuging at 16 100 g for 10 min. The pellet was washed with 800 ml litre⁻¹ ethanol twice. After a brief drying, the pellet was dissolved in 10 µl DEPC treated MilliQ water. Finally, the integrity of extracted RNA was assessed by agarose (15 mg ml⁻¹), MOPS, formaldehyde, gel electrophoresis (Maniatis *et al.*, 1989). The total RNA sample (2 µl) was mixed with 1 µl 6 × bromophenol blue dye prior to loading. The RNA bands were displayed by using an ethidium bromide stain. The yield of total RNA was measured with a spectrophotometer Ultrospec 4050 (LKB Biochrom, Cambridge, England) at a wavelength of 260 nm.

Reverse transcription

M-MLV Reverse Transcriptase (RTase) (Promega, Madison, WI, USA) was used to synthesise the cDNA. The reaction included: 5 µl total RNA treated with DNase, 2 µl 100 µM anchor primer T14A (custom primer, Invitrogen Australia, Mulgrave, Vic, Australia), 200 units M-MLV RTase, 1 × M-MLV RT Buffer, 2 µl dNTPs (10 mM each) (Invitrogen, Carlsbad, CA, USA) and 35 units RNasin. Initially anchor primers, total RNA and water were mixed and placed at 70°C for 5 min and then transferred immediately to ice for 2 min. A pre-mixed solution of the other components of the reaction was then added to make a total volume of 25 µl. The reaction was incubated at 37°C for 1 h followed by heating at 65°C for 10 min.

DDRT PCR

Red Hot® DNA polymerase (ABgene, Epsom, Surrey, UK) was used for the PCR. The 20 µl PCR reaction contained 1 × PCR Buffer IV, 1.5 µM MgCl₂, 200 µM dNTPs, 250 nM primer T12AA (custom primer, Invitrogen Australia, Mulgrave, Vic.,

Australia). 250 nM 10-mer random primer OPD-08 (Operon, Alameda, CA, USA) and 1 unit Red Hot® DNA polymerase. The cDNA was diluted 1:2, with autoclaved MilliQ water and 2.5 µl was used in each PCR reaction. PCR was performed using a PTC-160™ Programmable Thermal Controller (MJ Research, Watertown, MA, USA). The solution was denatured at 94°C for 2 min, and then subjected to the following temperature profile: 94°C for 30 s, 40°C for 1 min, 72°C for 35 s in 40 cycles and finished with a 72°C extension for 5 min. DDRT-PCR products were separated by non-denaturing polyacrylamide gel electrophoresis and visualised by silver staining (Bassam *et al.*, 1991). The 60 ml gel system included 12 ml acrylamide-bis (Ultra pure grade, Amresco, Solon, Ohio, USA), 6 ml 10 × TBE, 600 µl ammonium persulfate solution (100 mg ml⁻¹) and 60 µl N,N,N',N'-Tetramethylethylenediamine (TEMED) (ICN Biotechnologies, Aurora, Ohio, USA)

Results

Agarose gel electrophoresis of DNase treated total RNA displayed the presence of both 28S rRNA and 18S rRNA, with the 28S rRNA band being almost twice as intense as the 18S rRNA band in all the samples (Fig.1A). This indicates that the method is reliable and the total RNA was not significantly degraded during the extraction and DNase treatment. Approximately 5 µg of total RNA was extracted from a single wheat embryo. The A_{260}/A_{280} ratio was 1.9. Half of the DNase treated total RNA was used for reverse transcription. The synthesised cDNA could be used for about 30 DDRT PCR reactions. The DDRT PCR products of individual wheat embryos of different varieties are shown in Fig.1B. When the cDNA was replaced with a DNase treated total RNA in DDRT PCR, no amplification product was detected (data not shown).

Discussion

The success of the RNA extraction from a small embryo to a great extent depends on the thorough grinding of the embryo tissue. It is essential that the embryo should be frozen completely throughout the whole grinding process. Any extra addition of liquid nitrogen during grinding could cause splashing and result in sample loss. The use of a liquid nitrogen “jacket” substantially reduced the rate of evaporation and sample loss caused by splashing during the grinding process. Furthermore, the 5 ml tube is a suitable size for holding the 1.5 ml tube for grinding. This device is easy, effective and reliable for the grinding of all kinds of small tissues using liquid nitrogen. It is also important to minimise the loss of the small quantity of samples during the extraction

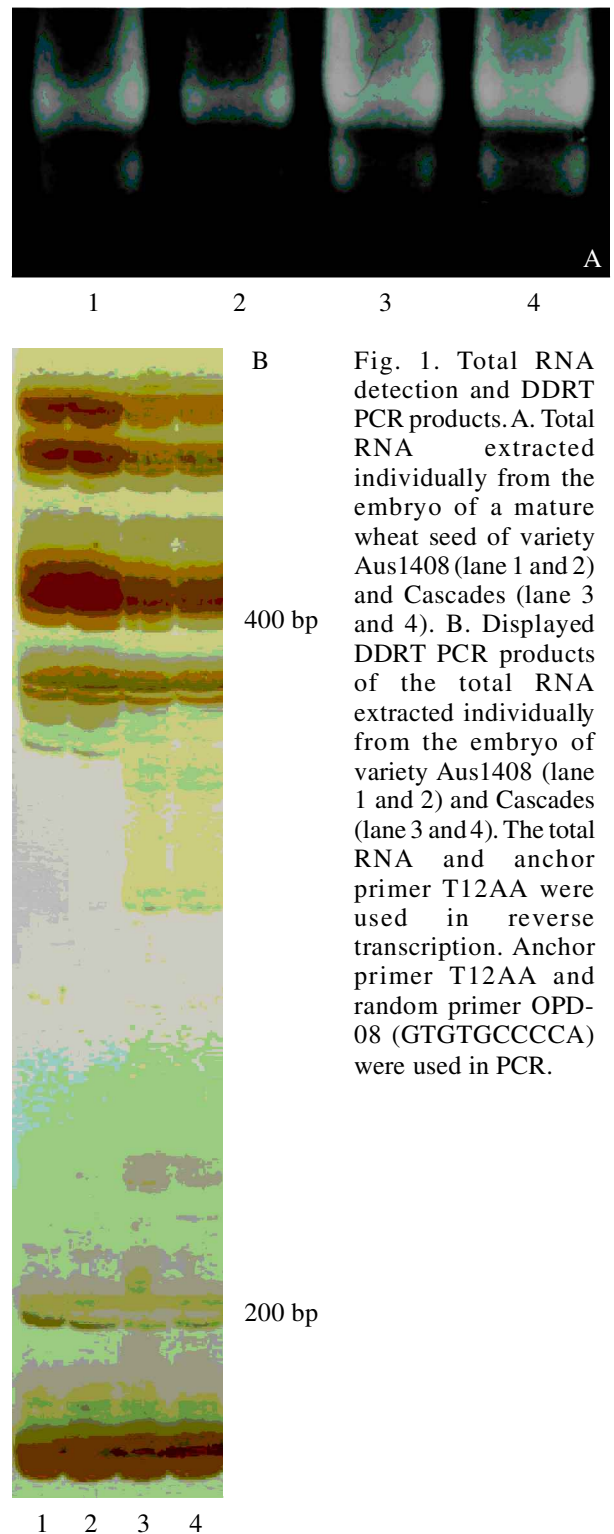


Fig. 1. Total RNA detection and DDRT PCR products. A. Total RNA extracted individually from the embryo of a mature wheat seed of variety Aus1408 (lane 1 and 2) and Cascades (lane 3 and 4). B. Displayed DDRT PCR products of the total RNA extracted individually from the embryo of variety Aus1408 (lane 1 and 2) and Cascades (lane 3 and 4). The total RNA and anchor primer T12AA were used in reverse transcription. Anchor primer T12AA and random primer OPD-08 (GTGTGCCCCA) were used in PCR.

and purification process. There are many commercial kits for isolating RNA using different techniques, but DNase treatment and subsequent purification is still needed for RNA samples isolated by using most of the kits. Therefore, we adopted an inexpensive method using ethanol precipitation to collect total RNA from the extraction buffer followed by DNase treatment. In order to reduce the sample loss, purification was performed only once. The total RNA

precipitated from the extraction buffer was washed with 800 ml litre⁻¹ ethanol prior to DNase treatment, which was followed by the purification procedure. The isolated total RNA was shown to be pure enough and free of DNA for subsequent differential display. This method is simple, inexpensive and no special equipment is required. It can be used for gene expression analyses in wheat embryos, such as in our study on gene expression associated with dormancy. It may also facilitate many other gene expression studies using small amounts of tissue.

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