Extraction and purification of total RNA from banana tissues (small scale)

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Abstract — Introduction. This protocol aims at preparing total RNA for gene expression analysis by Northern blots, RT-PCR and real-time quantitative PCR; cDNA isolation by RT-PCR; and cDNA library construction. The principle, key advantages, starting plant material, time required for obtaining total RNA and expected results are presented. **Materials and methods**. This part describes the required materials and the 27 steps necessary for preparing RNA from peel and pulp fruit tissue: preparation of plant tissue powder, preparation of the complete RNA extraction buffer and isolation of RNA from ground banana fruit tissue. **Results**. Extraction of total RNA by the method described makes it possible to achieve electrophoresis under denatured conditions and *in vitro* reverse transcription. An example for Northern blot analysis is illustrated.

France / *Musa* sp. / methods / plant tissues / RNA / extraction

Extraction et purification de l'ARN total de tissus de banane (à petite échelle).

Résumé — Introduction. Le protocole vise à préparer de l'ARN total pour l'étude de l'expression de gènes par les techniques de *Northern Blots*, de *Reverse Transcription PCR*, et de PCR quantitative en temps réel ; pour l'isolement de cDNA par *Reverse Transcription PCR* ; pour la construction de bibliothèque de cDNA. Le principe, les principaux avantages, le matériel végétal requis, le temps nécessaire à l'obtention de l'ARN total et les résultats escomptés sont présentés**. Matériel et méthodes**. Cette partie décrit le matériel et les 27 étapes nécessaires pour préparer l'ARN à partir de tissus de peau et de pulpe de banane : préparation de la poudre de tissus végétaux, préparation du tampon d'extraction de l'ARN total, isolement de l'ARN à partir du tissu de banane broyé. **Résultats**. L'extraction de l'ARN total par la méthode décrite permet de réaliser son électrophorèse avec un gel à base de formaldéhyde comme dénaturant et sa transcription réverse *in vitro*. Un exemple d'application de *Northern Blots* est donné.

France / *Musa* sp. / méthode / tissu végétal / ARN / extraction

1. Introduction

Application

This protocol aims at preparing total RNA for:

– gene expression analysis by Northern blots, RT-PCR and real-time quantitative PCR,

- cDNA isolation by RT-PCR,

- cDNA library construction.

Principle

This method is adapted from that described by Wan and Wilkins [1]. Frozen tissue previously pulverized to a fine powder can either be stored at –80 °C or processed immediately. During RNA isolation, the powder is first suspended in the basic hot borate/proteinase K extraction buffer, whose alkaline pH renders phenolic compounds ineffective and inhibits nuclease activity. Most cell debris, detergent and

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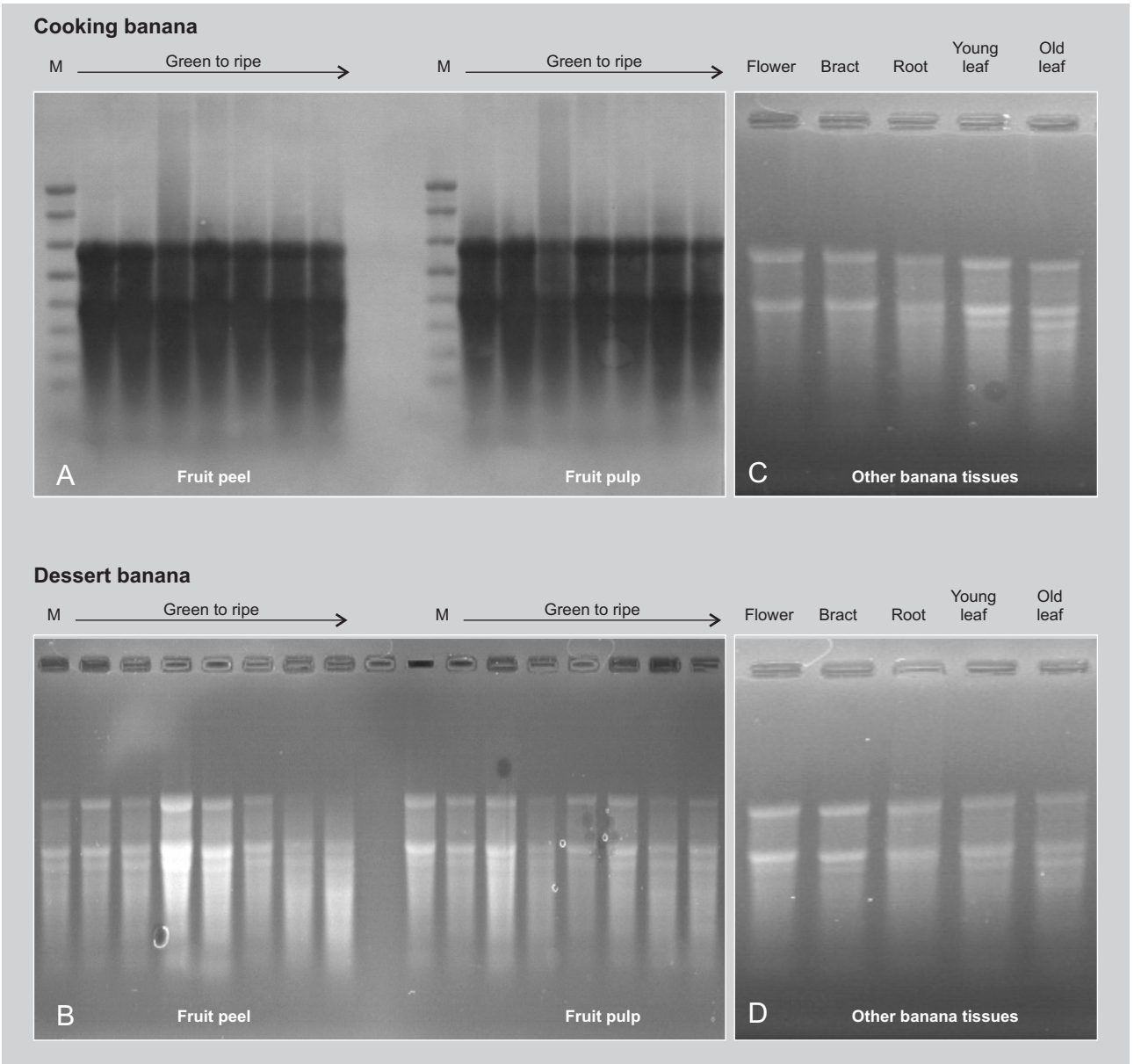

Figure 1.
After extraction according to the method described, total RNA was separated by electrophoresis under denatured conditions on 1.2% formaldehyde-agarose gel and colored with acridine orange (panels B, C and D) or with methyl blue after transfer onto a nylon membrane (panel A).

protein are discarded from the supernatant by the addition of potassium chloride followed by a centrifugation of the homogenate. An enriched RNA pellet is precipitated from the supernatant with lithium chloride and dissolved in tris-HCl buffer. The residual polysaccharides and proteins are discarded by two phenol/chloroform/isoamilic alcohol extractions. Total RNA is precipitated with ethanol and dissolved in DEPC-treated water.

Key advantages

– This method was successfully used for extracting, from different banana varieties, total RNA from different dessert and banana tissues including the fruit pulp, fruit peel,

bract, flower, young and old leaves, callus (compact and friable) and, with less efficiency, root and pseudostem tissue (*figure 1*).

– Because of the two overnight precipitations, the extraction of total RNA protocol spreads out over three half-days. However, the long incubation times envisaged in this protocol make it possible to carry out other experiments simultaneously.

– By adjusting the different reagent volumes, this protocol can be easily adapted to a large-scale total RNA extraction from up to 25 mg of tissue.

Starting material for a small-scale RNA extraction

This protocol uses 5 mg of banana tissues.

Note: the method can be used to isolate total RNA in a large-scale procedure from 25 mg of tissues. In this case, adjust tube size and reagent volumes in the remaining steps proportionally.

Time required

Total duration of the protocol spread out over 3 days is distributed as follows: for day 1, approximately 5 h; for day 2, approximately 4 h; for day 3, approximately 3 h.

Expected results

Yields are variable, according to the cultivar, the tissue and its development stage. For green to over-ripe fruit tissue, the average yields determined by spectrophotometry comprise between (55 and 120) µg RNA·g^{-1} for fruit peel and between (100 and 200) µg RNA·g^{-1} for fruit pulp. The highest yields were obtained with the bract, callus, flower and leaf (young or old), while the method appeared less efficient in the root, pseudostem and senescent tissues, including leaf and fruit.

2. Materials and methods

2.1. Materials

Laboratory materials

The protocol requires the products dithiothreitol, sodium deoxycholate acid, Igepal CA-630, ethanol to precipitate RNA, proteinase K to denature RNases, 20% sodium dodecyl sulfate, 10 M lithium chloride to precipitate RNA, 0.1% diethyl pyrocarbamate (DEPC)-treated water to inhibit RNases, 5 M potassium acetate (pH 5.5) for the salt precipitation step, 1 M tris-HCl buffer pH 7.5 to resuspend the enriched RNA pellet, distilled and autoclaved ultra-pure water (Milli Q-Water system from Millipore), and liquid nitrogen.

In addition, it is necessary to have sterile and autoclaved 50-mL centrifuge tubes (resistant to liquid nitrogen), a sterile and autoclaved 30-mL centrifuge tube, sterile and autoclaved 1.5- and 2-mL microcentrifuge tubes, a coffee blender, an Ultra-Turrax blender, a heating water bath, a refrigerated centrifuge (–10 °C) that can accommodate (30- and 50-) mL tubes, a rotary shaker, and a vortex.

Preparation of the incomplete RNA extraction buffer

The incomplete RNA extraction buffer used to suspend the tissue powder is composed of 200 mM of sodium borate decahydrate (BORAX) 2% (w/v), polyvinylpyrrolidone (MW 40,000) and 30 mM EGTA, adjusted to pH 9.0.
Note: the incomplete RNA extraction buffer can be prepared before, autoclaved and stored at room temperature for up to 3 months.

2.2. Protocol for preparing RNA from peel and pulp fruit tissue

Preparation of plant tissue powder

• Step 1
To prepare fruit material:
– harvest fruit and carefully separate peel and pulp tissues,
– immediately cut peel and pulp tissues into small pieces, and freeze them in liquid

nitrogen. Frozen material must be stored at –80 °C until used.

• Step 2
To grind pulp material and extract sugars:
– weigh approximately 6 g of frozen material,
– grind this material in the coffee blender,
– weigh 5 g in a frozen, sterile and autoclaved 50-mL centrifuge tube (resistant to phenol).

• Step 3
Keep the tube containing the frozen ground tissue in liquid nitrogen until you are ready to isolate RNA (see protocol below).
Note: frozen ground samples can be stored at –80 °C for up to 24 h without a negative effect on the yield or the integrity of total RNA extraction.

Preparation of the complete RNA extraction buffer

• Step 4
Complete the RNA extraction buffer by supplementing the incomplete buffer with 1% sodium dodecyl sulfate + 10 mM dithiothreitol + 1% sodium deoxycholate acid + 0.5% Igepal CA-630.

• Step 5
Heat the complete RNA extraction buffer at 80 °C for 10 min.
Note: it is essential to use a freshly prepared complete RNA extraction buffer. During this time, weigh 12.5 g of proteinase K.

Isolating RNA from ground banana fruit tissue

• Step 6
To lyse the cells and remove proteins, resuspend the frozen powder in complete RNA extraction buffer as follows:
– at room temperature, add 25 mL of the complete preheated RNA extraction buffer in a 50-mL centrifuge tube containing the frozen ground sample;
– vortex the tube and pass the sample through an Ultra-Turrax blender at maximal speed for 2 min to homogenize the sample. *Note:* It is essential to ensure that the powder is completely dispersed in RNA extraction buffer;
– add 12.5 mg of proteinase K to the homogenate;
– incubate the sample with mild agitation at 42 °C on a rotary shaker for 1.5 h;
– add potassium chloride at a final concentration of 160 mM;
– homogenize the mixture with the vortex and incubate the sample on ice for 1 h;
– centrifuge the tube for 20 min at 12 000 *g* and 4 °C.
Caution: keep the tube on ice before and after centrifugation;
Note: it is possible to add phenol/chloform/isoamilic alcohol just after the 1-h ice incubation to improve the purity of the RNA extract with respect to proteins. In this case, ensure that the 50-mL centrifuge tube is resistant to phenol, chloroform and isoamilic alcohol.

• Step 7
During the centrifugation, prepare a fresh, sterile and autoclaved 50-mL centrifuge tube to receive the supernatant from step 6 and put it to be cooled on ice.
Caution: keep the tube on ice before and after centrifugation.

• Step 8
When the centrifugation (step 6) is finished, place the spun tubes immediately on ice, and carefully transfer the supernatant with a pipette to a fresh 50-mL centrifuge tube (from step 7).

• Step 9
To precipitate the total RNA-enriched pellet:
– add to the supernatant a cool 10 M lithium chloride (LiCl) to a final concentration of 2 M. *Note:* the addition of LiCl must be done in small fractions with an agitation between fractions;
– incubate the tube overnight at 4 °C;
– centrifuge the tube for 20 min at 12 000 *g* and 4 °C to precipitate the total RNA-enriched pellet;
– discard the supernatant. *Note*: in general, the pellet RNA obtained is firm and relatively colored according to the tissue and its development stage.

• Step 10
To fade the total RNA-enriched pellet, wash it as follows:

– add 4 mL of cool 2 M LiCl to the sample tube,
– resuspend the RNA-enriched pellet by repeatedly passing it through a pipette,
– centrifuge the tube for 5 min at 12 000 *g* and 4 °C,
– discard the supernatant.

• Step 11
Repeat the above step until the supernatant becomes relatively colorless. *Note:* In general, this occurs after two or three washings.

• Step 12
To dry the total RNA-enriched pellet at room temperature: under a hood, invert the tube on a paper towel for 10 min.

• Step 13
To dissolve the total RNA of the pellet, perform the following steps:
– add 1 mL of cool 10 mM tris-HCl pH 7.5 to the tube,
– tap the tube gently to dislodge the total RNA-enriched pellet into the buffer,
– resuspend the RNA-enriched pellet by repeatedly passing it through a pipette,
– incubate the sample for 15 min on ice with regular and gentle agitation to dissolve the total RNA-containing pellet,
– transfer the total RNA extract into a phenol/chloroform/isoamilic alcohol-resistant 2-mL microcentrifuge tube.

• Step 14
To remove protein contaminant from the RNA-enriched extract, perform the following steps:
– add 500 µL of a mixture of phenol/chloroform/isoamilic alcohol (25/24/1) to the tube,
– vortex the tube briefly to mix the sample,
– centrifuge the tube for 5 min at 12 000 *g* at room temperature,
– discard the supernatant.

• Step 15
Repeat the above step once.

• Step 16
To remove the trace of phenol, perform the following steps:
– add 500 µL of a mixture of phenol/chloroform/isoamilic alcohol (25/24/1) to the tube,
– vortex the tube briefly to mix the sample,
– centrifuge the tube for 5 min at 12 000 *g* at room temperature,
– discard the supernatant.

• Step 17
To remove a salt-insoluble material including polysaccharide and detergents:
– add 5 M potassium acetate (pH 5.5) to the tube to a final concentration of 0.2 M,
– vortex the tube briefly to mix the sample,
– incubate the tube on ice for 15 min,
– centrifuge the tube for 10 min at 12 000 *g* and 4 °C.

• Step 18
During the centrifugation, prepare a new 30-mL sterile and cool centrifuge tube to receive the supernatant from step 17:
– pipette 2.5 mL fresh and cold ethanol into a sterile 30-mL centrifuge tube,
– set the tube containing ethanol on ice.

• Step 19
When the centrifugation (step 17) is finished, place the spun tubes immediately on ice, and transfer the supernatant into the 30-mL sterile and cool centrifuge tube containing ethanol (from step 18).
Note: it is possible that no polysaccharide pellet is obtained after the centrifugation of step 17.

• Step 20
To precipitate the total RNA, perform the following steps:
– vortex the tube to mix the supernatant and ethanol,
– incubate the tube overnight at –20 °C,
– centrifuge the tube for 1 h at 20 000 *g* and –10 °C.

• Step 21
At the end of the centrifugation (step 20), carefully discard the supernatant and place the spun tubes immediately on ice.

• Step 22
To remove a residual salt material, wash the total RNA pellet three times as follows:
– add 2–3 mL of 70% cool ethanol to the tube,
– tap and swirl the tube gently to dislodge the total RNA pellet,

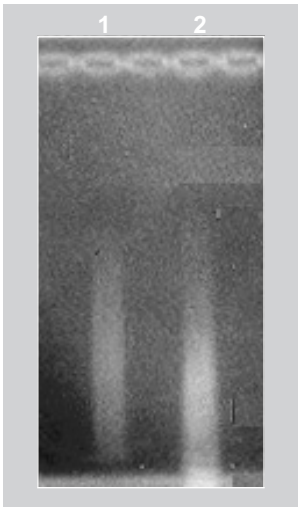

Figure 2.
In vitro reverse transcription from total RNA extracted from over-ripe fruit according to the method described. Lane 1, first strand of cDNA obtained by reverse transcription of mRNA. Lane 2, second strand of cDNA synthesized from the first strand. *Note:* the pattern of intact ribosomal RNA confirms the absence of discernable degradation and thus the integrity of the RNA used for *in vitro* reverse transcription and Northern blot analysis.

– centrifuge the tube for 5 min at 12 000 *g* and 4 °C. *Caution:* keep the tube on ice before and after centrifugation.

• Step 23
At the end of the centrifugation (step 22), carefully discard the supernatant and place the spun tubes immediately on ice.

• Step 24
To dry the total RNA pellet:

– decant and discard the supernatant,

– invert the tube on a paper towel,

– let the total RNA pellet dry for 5 min at room temperature.

• Step 25
At the end of drying, immediately put the tube containing the total dried RNA pellet on ice.

• Step 26
To dissolve the total RNA:

– add 300 µL of cool and sterile DEPC-treated water to the tube,

– tap the tube gently to dislodge the total RNA pellet into the water,

– swirl the tube gently to resuspend the total RNA pellet,

– leave the tube on ice with regular and gentle swirling, until the total RNA pellet is completely dissolved.

Note: in general, a DNase treatment of total RNA occurs at this step to eliminate the putative contaminant DNA.

• Step 27
Recover the supernatant containing total RNA solution into a 1.5-mL sterile and cool microcentrifuge tube, and store it at –20 °C.

2.3. Troubleshooting

Three problems can occur with this protocol:

(a) Few or no total RNA pellets. This can be due to:

– Failure of the lithium chloride in the precipitation step (step 9). *Solution:* check that the volume of LiCl added to the homogenate corresponds to a final concentration of 2 M.

– Tissue insufficiently ground before addition of complete RNA extraction buffer. *Solution*: grind plant tissue in liquid nitrogen.

– Incomplete suspension of tissue in complete RNA extraction buffer. *Solution:* ensure that the plant powder is completely suspended in complete RNA buffer.

– Failure of the ethanol precipitation step (step 20). *Solution:* check that the ethanol used at step 18 is pure and fresh.

Note: if the problem persists in spite of the above verifications, the protocol is not adapted to the tissue.

(b) Sheared RNA caused by:

– The contamination of the material and/or solution used with RNase. *Solution*: autoclave the whole of the material and solutions before their use.

– RNase in the resuspension buffer (steps 13 or 26). *Solution:* prepare the cool 10 mM tris-HCl buffer used at step 14 with sterile and cool DEPC-treated water, and use sterile and cool DEPC-treated water to dissolve the total RNA pellet at step 14.

(c) No RT-PCR product when total RNA is used as a template. Three explanations are possible:

– No RNA was isolated in the above protocol. *Solution:* check the presence and concentration of RNA by spectrophotometry and electrophoresis, respectively.

– RT-PCR reagents or conditions are not optimal. *Solution:* follow your RT-PCR troubleshooting procedures.

– Residual reagent (salt or alcohol) is present in the RNA. *Solution:* repeat RNA precipitation. For this, add to the 300 µL RNA extract 30 µL (0.1 RNA extract volume) of 3 M sodium acetate (pH 5.2) and 750 µL (2.5 RNA extract volume) of pure and fresh ethanol to the RNA extract, and mix, incubate and centrifuge the tube as described in step 17. Then perform step 19 more than three times and step 21 with a small increase in drying time.

3. Typical results obtained

Extraction of total RNA by the method described makes it possible to achieve electrophoresis under denatured conditions ((*figure 1)* and *in vitro* reverse transcription (*figure 2*). The RNA extracted could be used for Northern blot analysis (*figure 3*), for example.

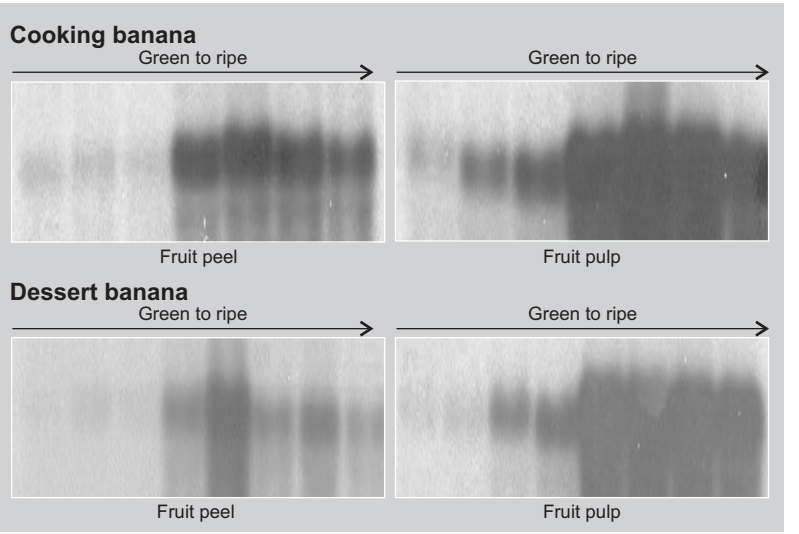

Figure 3.
Use of total RNA extracted from peel and pulp of banana fruit for Northern blot analysis. After extraction according to the method described, total RNA was separated by electrophoresis under denatured conditions on 1.2% formaldehyde-agarose gel, and blotted onto a nylon membrane. The blot was hybridized with a α-[^{32}P] dCTP-labeled cDNA probe. *Note:* the hybridization demonstrates that the total RNA isolated with this method is fully functional for radioactive Northern blot analysis.

Reference

[1] Wan C.-Y., Wilkins T.A., A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Grosypium hirsutum* L.), Anal. Biochem. 223 (1994) 7–12.