Detection of Poly (A) RNA in Mesophyll Cells of *Nicotiana benthamiana* Using *in situ* Hybridization

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[Abstract] Export of transcribed mRNAs from nucleoplasm to cytosol is an essential process for the translation of genes into proteins. This process is tightly regulated by nuclear pores, composed of about 30 nucleoporin proteins (Nups). Whether or not the mRNAs are able to be appropriately exported to cytoplasm is of an importance for understanding the role of Nups. Here, we describe a practical protocol to detect the intracellular localization of mRNAs in mesophyll cells of *Nicotiana benthamiana* (*N. benthamiana*). This protocol is based on poly (A) *in situ* hybridization method using an oligo d(T) probe conjugated with Alexa Fluor-488.

Materials and Reagents

1. Leaves of 3-4 weeks old wild type or *Nup* gene silenced *N. benthamiana*
   Notes:
   a. *This method can be applied for other dicot plants with relatively soft leaves.*
   b. *For Virus-induced gene silencing (VIGS) of N. benthamiana. See Zhang and Liu (2014).*
2. 10 µM 48-mer oligo d(T) 5'-labeled with Alexa Fluor-488, HPLC-purified [purchased from custom oligo services (*e.g.* Eurofins Genomics)]
   Note: Dissolved in TE (Tris-EDTA) buffer and store at -80 °C, shaded.
3. 99.8% Methanol (4 ml per sample) (Wako pure chemical, special glade, catalog number: 131-01826)
4. 99.5% Ethanol (5 ml per sample) (Wako pure chemical, special glade, catalog number: 057-00456)
5. 99.5% Ethanol/Xylene (1:1 v/v, 1 ml per sample) (Wako pure chemical, special glade, catalog number: 244-00086)
6. 99.8%Methanol/Fixation solutionB (1:1 v/v, 1 ml per sample)
7. PerfectHyb Plus Hybridization Buffer (2 ml per sample) (Sigma-Aldrich, catalog number: H7033)
8. Fixation cocktail (see Recipes)
9. Fixation solution A (see Recipes)
10. Fixation solution B (see Recipes)

Equipment

1. 3 ml vial containers
2. Rotary shaker (*e.g.* BioCraft, model: BC-730)
3. Hybridization oven or incubator (50 °C) with rotary shaker (*e.g.* TAITEC, model: BR-23FP MR)
4. Confocal laser-scanning microscope (preferred) or ordinary fluorescent microscope with the filter set for GFP or Alexa Fluor-488 (*e.g.* Olympus, model: FV1000-D)

Procedure

A. Poly (A) RNA *in situ* hybridization

1. Cut leaves of *N. benthamiana* into small pieces (*e.g.* 3 mm x 3 mm) by knife and put ~10 leaf pieces/sample in 3 ml vial container.
   Note that the center of larger pieces won't be stained with AF488 probe.
2. Add 1 ml Fixation solution A in the vial container and shake (Approx. 80 rpm) for 30 min at room temperature (RT). Leaf pieces will float on the surface of the solution at the start, but will sink later (Figure 1).

Figure 1. Leaf discs of *N. benthamiana* in Fixation solution A before (left) and after (right) shaking for 30 min (step A2)

3. Remove Fixation solution A by pipeting.
4. Add 1 ml methanol. Shake (80 rpm) for 5 min at RT and remove methanol (twice). Note that the inner side of the vial should be washed well with methanol.
5. Add 1 ml ethanol, shake (80 rpm) for 5 min at RT and remove ethanol (three times).
6. Add 1 ml ethanol/xylene (1:1). Shake (80 rpm) for 30 min at RT and remove the solution. Note that leaf tissues will become white during steps A4-6.
7. Add 1 ml 100% ethanol. Shake (80 rpm) for 5 min at RT and remove ethanol (twice).
8. Add 1 ml 100% methanol. Shake (80 rpm) for 5 min at RT and remove ethanol (twice).
9. Add methanol/Fixation solution B (1:1). Shake (80 rpm) for 5 min at RT and remove the solution.
10. Add 1 ml Fixation solution A and shake (80 rpm) for 30 min at RT.
11. Remove Fixation solution A.

12. Add 1 ml Fixation solution B. Shake (80 rpm) for 5 min at RT and remove the solution (twice).
13. Add 1 ml PerfectHyb Plus, and shake (80 rpm) for 5 min at RT.
14. Remove the solution and add 1 ml of fresh PerfectHyb Plus. Shake (80 rpm) at 50 °C in hybridization oven (incubator with rotary shaker can be used) for more than 1 h.
15. Add 1 µl of 10 µM Alexa Fluor-488-labeled oligo d(T). Shake (80 rpm) at 50 °C overnight. It is preferred to perform fluorescent microscopy immediately after the preparation of samples, but the samples can be kept at least for a couple of days at RT, shaded.

B. Microscopic observation
1. Mount the sample in PerfectHyb Plus on slide glass with cover slip.
2. Observe the localization of mRNA with confocal laser-scanning microscopy. Use appropriate setting for the detection of Alexa Fluor-488 (excitation peak 490 nm, emission peak 525 nm). We generally use 488-nm excitation source, and Alexa Fluor-488 fluorescence is recorded between 500 and 600 nm.

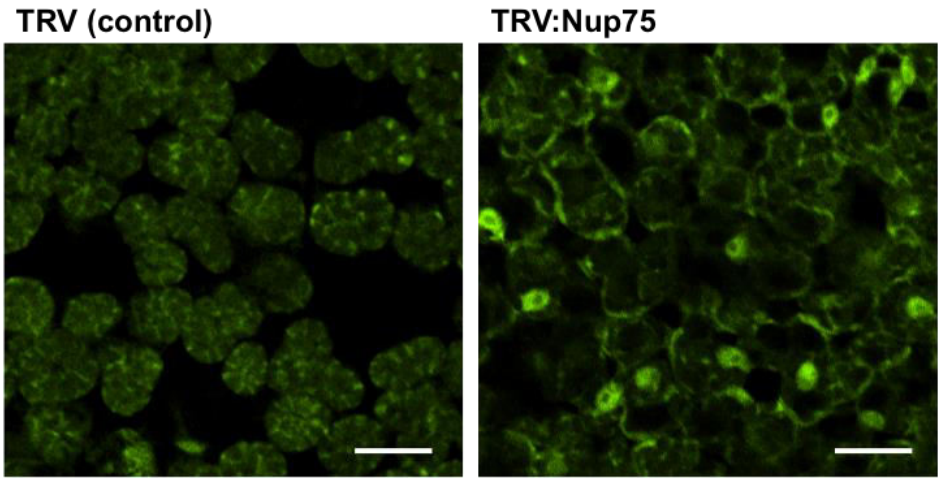

Figure 2. Distribution of Poly (A) RNA in mesophyll cells of control (TRV-infected) or *NbNup75*-silenced *Nicotiana benthamiana*. In *NbNup75*-silenced plant, abnormal accumulation of mRNA in nuclei is observed as a result of impaired export of mRNA. Bars=30 µm. (Ohtsu *et al.* 2014)

Recipes

1. Fixation cocktail (x 4)
   240 mM NaCl
   14 mM Na_2HPO_4
   6 mM NaH_2PO4
   5.4 mM KCl

160 mM EGTA

Autoclaved and stored at RT

2. Fixation solution A (for 10 ml)

   Fixation cocktail (x 4) 2.5 ml

   Formaldehyde 250 µl

   DMSO 500 µl

   Tween20 10 µl

   Heptan 5 ml

   H_2O 1.74 ml

   Prepare before use

3. Fixation solution B (for 10 ml)

   Fixation cocktail (x 4) 2.5 ml

   DMSO 500 µl

   Tween 20 10 µl

   Heptan 5 ml

   H_2O 1.99 ml

   Prepare before use

Acknowledgments

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