Detection and Visualization of Specific Gene Transcripts by *in situ* RT-PCR in Nematode-Infected *Arabidopsis* Root Tissue

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[Abstract] This protocol describes an effective method of *in situ* RT-PCR that was developed to localize specific gene expression directly in thin cross-sections of nematode feeding sites induced by the cyst nematode *Heterodera schachtii* (*H. schachtii*) or the root-knot nematode *Meloidogyne incognita* (*M. incognita*) in *Arabidopsis* roots using DIG (Digoxigenin-11dUTP) labeling coupled with AP (alkaline phosphatase) and nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate-based detection. This method is applicable to any other *Arabidopsis* root tissue.

Materials and Reagents

1. *Arabidopsis* roots
   Note: Non-infected root fragments or root fragments containing feeding sites of H. schachtii (syncytia) or M. incognita (giant-cells embedded within galls).
2. Ethanol
3. Formaldehyde
4. Diethylpyrocarbonate-treated water (DEPC-H_2O)
5. Low melting agarose
6. Petri dishes (∅ 50 mm)
7. 12/24-well plates
8. Parafilm
9. Superglue
10. RNase Away (Thermo Fisher Scientific-Molecular BioProducts, catalog number: 7005)
11. DNase I, RNase-free, HC (50 U/µl) (50 U/ul, 1,000 U) ((Thermo Fisher Scientic, Fermentas, catalog number: EN0523)
12. RiboLock RNase Inhibitor (40 U/µl) (RiboLock: 40 U/µl) (Thermo Fisher Scientic, Fermentas, catalog number: EO0381)
13. Ethylenediaminetetraacetic acid (EDTA)
14. SuperScript™ III Reverse Transcriptase Kit (Life Technologies, Invitrogen™, catalog number: 18080-093)
15. Deoxynucleotides (dNTPs)

16. Bovine serum albumin (BSA)
17. DIG (Digoxigenin-11dUTP-alkali stable; 25 nmol/25 µl) (Roche Diagnostics, catalog number: 11093088910)
18. BioTherm™ Taq DNA Polymerase (GeneCraft, catalog number: GC-002-0100)
19. Anti-Digoxigenin-AP, Fab fragments (150 U/200 µl) (Roche Diagnostics, catalog number: 11093274910)
20. BCIP®/NBT Liquid Substrate System (Sigma-Aldrich, catalog number: B1911-100 ML)
21. Fixation solution (see Recipes)
22. 10x phosphate buffered saline (PBS) (see Recipes)
23. 20x SSC (see Recipes)
24. 10x PCR buffer (see Recipes)
25. 10x washing buffer (see Recipes)

Equipment

1. Vibratome (Leica, model: VT 100)
2. Heating plate
3. Forceps
4. Laminar flow
5. PCR cycler
6. Stereo microscope

Procedure

Precautions: RNA is very sensitive and easily degraded by RNases! Therefore always wear gloves, use RNase-free water as well as glass- and plasticware. Recommended is the use of commercially available reagents for eliminating RNase and DNA contamination from the surface of glassware or plasticware e.g. RNase away!

A. Fixation
   1. Cut segments of *Arabidopsis* roots containing feeding sites of *H. schachtii* (syncytia) or *M. incognita* (giant-cells embedded within galls) and corresponding non-infected root fragments.
   2. Immediately put the root pieces into a microcentrifuge tube containing freshly prepared and pre-chilled fixation solution and vacuum infiltrate it on ice for 15-20 min to ensure the proper penetration of the fixative. Incubate samples 42 h or longer at 4 °C on a shaker with gentle shaking.

B. Embedding

1. Subsequently wash fixed samples 3 x 10 min in 63% ethanol in PBS and 1 x 10 min in PBS.
2. Embed the tissue in low melting agarose (5% w/v in PBS) in small petri dishes (∅ 50 mm) placed on a heating plate (35-40 °C) in the laminar flow. Grab the root fragments with the forceps, swab them carefully with a paper towel to remove the excess of PBS and dip them into warm agarose parallel to the bottom of the Petri dish or 12/24-well plate and approx. at the half depth of the agarose. Approx. 10 root segments/dish or 1-2 root segments/12/24-well plate (Figure 1A).
3. Cool down the agarose in the laminar flow.
4. Petri dishes sealed with Parafilm can be stored in the fridge (4 °C) for several weeks.

C. Preparation of cross sections with the vibratome and DNase treatment

1. Cut out small agarose blocks (approx. 8 mm high x 5 mm wide) containing the syncytia or non-infected root segments with a scalpel. Trim the agarose block under the microscope (Figure 1B) and glue it on the round vibratome plate with a drop of common superglue. To produce cross sections the root segments within the agarose block have to be placed perpendicular to the bottom of the vibratome plate (Figure 1B-C).

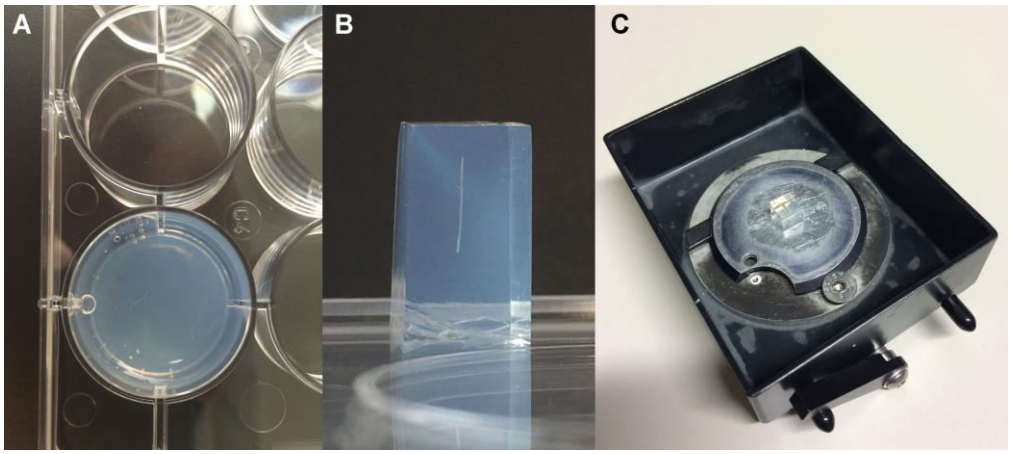

Figure 1. Placement of the root fragments within a well of the 12-well plate (A; view from above), trimmed agarose block with a root fragment in the perpendicular position (B; view from the side) and agarose block glued onto the vibratome plate (C)

2. Cut 20-25 µm thick tissue sections in the vibratome tray filled with DEPC-H_2O.
   Using forceps (in case the tissue section remains in the agarose section) or a pipette (in case the tissue section disconnects from the agarose section) collect the sections

in a watchglass or similar container filled with DEPC-H_2O supplemented with RNase inhibitor.

3. With a pipette remove the excess of DEPC-H_2O (as much as possible). To prevent non-specific DNA signals digest the DNA within the tissue by adding 2 µl RNase-free DNAse and 1 µl RNase Inhibitor to the remaining DEPC-H_2O covering the cross-sections. Subsequently incubate the samples overnight at 37 °C in a watchglass in a Petri dish sealed with parafilm.

D. Washing

1. Add several drops of 0.5 M EDTA and incubate the cross-sections at 65 °C for 10 min to inactivate the DNase.
   Subsequently wash sections as follows:
   2 times with 2x SSC buffer
   1 time with 1x SSC buffer
   1 time with 0.5x SSC buffer
   2 times with ddH_2O
   Each step should be performed with 1 ml of buffer or ddH_2O in a sealed Petri dish at 37 °C for 10 min.

E. Reverse transcription

1. Transfer several sections into a PCR tube using a pipette (in ca. 11.5 µl ddH_2O).
2. Add 0.5 µl 3' primer and 1.0 µl dNTPs and start the first step of the RT reaction: 65 °C for 5 min.
3. Cool down the samples, incubate them at least 1 min on ice and add 4 µl of the 5x First-Strand Buffer, 1 µl 0.1 M DTT, 1 µl RNase Inhibitor and 1 µl SuperScript™ Reverse Transcriptase. Total volume 20 µl.
4. Run the following program in a PCR cycler:
   25 °C - 5 min
   55 °C - 1 h
   75 °C - 5 min (inactivation of DNase)

F. PCR

1. Prepare a dNTPs mix containing 10 mM dGTP, dATP and dCTP. Add to the RT-mix as follows:
   5.0 µl - 10x PCR buffer
   1.0 µl - 3' primer (final concentration 0.5 µM)
   1.0 µl - 5' primer (final concentration 0.5 µM)
   1.0 µl - dNTPs mix
   2.36 µl - 2 mM dTTP
   0.5 µl - DIG

0.25 µl - BioTherm™ Taq DNA Polymerase

18.89 µl - ddH_2O

Total volume 50 µl (30 µl of PCR-mix and 20 µl of RT-mix).

In case of the negative control the polymerase will be omitted.

G. Washing and detection

The following steps are carried out under the stereo microscope at room temperature (from step G5 in dark room with a weak source of light *e.g.* small table lamp). Short centrifugation could be necessary between each washing step to collect the sections at the bottom of the PCR tube. This procedure might prevent the accidental removal of tissue sections during several washing rounds. You might also check the discarded liquids for unintended removed sections after each washing step.

1. Wash the samples twice in 1x PBS buffer for 5 min.
2. To block unspecific binding sites incubate the sections for 30 min in freshly prepared blocking solution containing 0.1% w/v BSA in 1x PBS.
3. Remove the blocking solution and add 50 µl *Anti-DIG-AP, Fab fragments* diluted 1:500 in blocking solution. Incubate 1 h at RT.
4. Remove the blocking solution containing *Anti-DIG-AP, Fab fragments* and wash the sections 2 x 15 min with 10x washing buffer.
5. Using a pipette place a drop of the washing buffer containing section(s) on a microscopic slide and replace the washing buffer with 50 µl of the NBT/BCIP solution (1:50 in washing buffer). Keep the slide in darkness (use *e.g.* lid from chemical bottle as a cover) since the NBT/BCIP is light sensitive!
6. From time to time observe the progress of the staining reaction under the microscope, it appears usually within first 2-3 min (keep the light exposure as short as possible).
7. If the violet staining is clearly visible stop the reaction with ddH_2O and photograph your sections. Figure 2 shows an example of an *in situ* RT-PCR analysis made for two β-1, 4- endoglucanases, *AtCel1* and *KOR3*, which are specifically up-regulated in syncytia induced by *H. schachtii* in *Arabidopsis* roots.

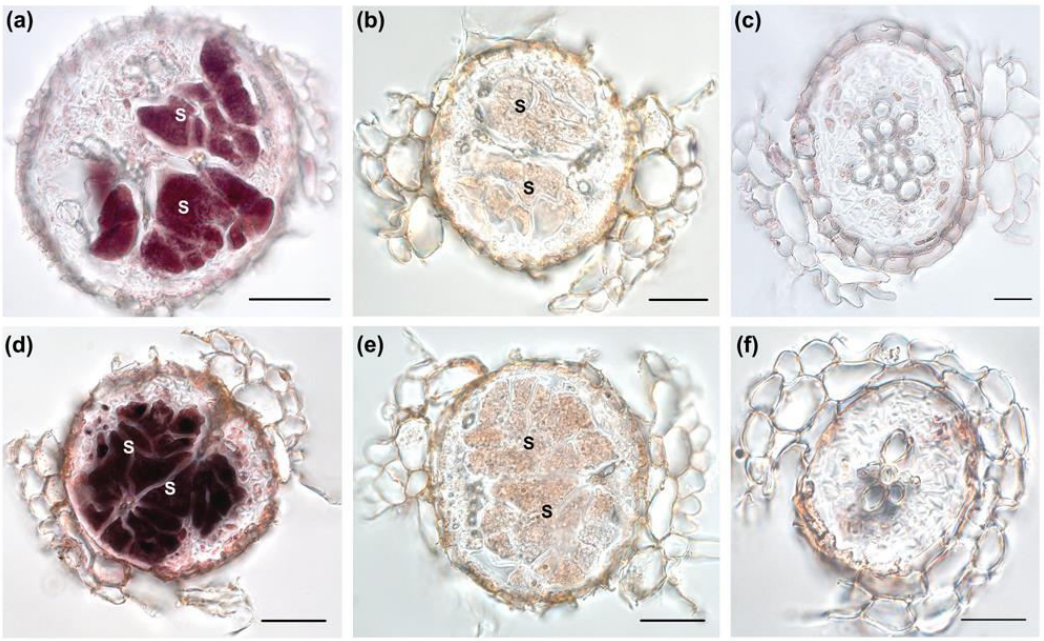

Figure 2. *In situ* RT-PCR analysis of *AtCel2* and *KOR3* on sections of syncytia induced by *Heterodera schachtii* at 10 days after inoculation (dai). (a) *AtCel2* transcripts accumulate within syncytium. (b) Control reaction for (a) performed without polymerase. Staining is not detected. (c) Control reaction for (a) on a root section above the syncytium. Transcripts of *AtCel2* were not detected. (d) Strong staining associated with transcripts of *KOR3* is visible in the syncytium. (e) Control reaction to (d) performed without polymerase; no staining is visible. (f) Control reaction for (d) on a root section above the syncytium. No transcripts of *KOR3* are visible. (a), (b), (d) and (e) scale bar = 50 µm; (c) and (f) scale bar = 20 µm. From Wieczorek *et al.* (2008), with permission.

Notes

1. RNA is easily degraded by RNAses and several different feeding sites/roots should be used to enhance the success rate of this method. However, if the feeding site/root tissue that is used contains intact RNA the reproducibility of this protocol is high and the variability low.
2. Start the detection with the positive reaction and subsequently use exactly the same exposure time for the controls.
3. The coloration of gene transcript-specific staining may vary from light violet to dark violet (almost black). The unspecific staining in the control sections (without Taq Polymerase) may vary from light brown, reddish to very faint/almost transparent.

Recipes

1. Fixation solution
   63% ethanol
   5% formaldehyde in PBS (containing DEPC-ddH_2O, pH 7.2)
2. 10x phosphate buffered saline (PBS)
   10 mM Na_2HPO_4
   130 mM NaCl in DEPC-ddH_2O
   pH 7.5
3. 20x SSC
   3 M NaCl
   300 mM $C_6H_5O_7 \cdot 2H_2O \cdot 3Na$ (sodium citrate dihydrate) in DEPC-ddH_2O
4. 10x PCR buffer
   160 mM $(NH_4)_2SO_4$
   670 mM Tris-HCl (pH 8.8) (at 25 °C)
   15 mM $MgCl_2$
   0.1% Tween 20
5. 10x washing buffer
   0.1 M Tris-HCl
   0.15 M NaCl
   pH 9.5

Acknowledgments

This protocol is a combination of methods previously published by Koltai and Bird (2000) and Urbanczyk-Wlochniak *et al.* (2002) and has been additionally modified. This work was supported by grant QLK-CT-1999-01501 ('NONEMA') from the European Union within the 5th Framework and FWF grants P16296-B06, P16897-B06 and P21067-B12.

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