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In Situ Reverse Transcription PCR on Plant Tissues

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Summary

In situ detection techniques allow specific nucleic acid sequences to be exposed in morphologically preserved tissue sections. In combination with immunocytochemistry, *in situ* detection can relate microscopic topological information to gene activity at the transcript or protein levels in specific tissues. The advantage of *in situ* methods over the conventional techniques (e.g., Northern blot, reverse transcription polymerase chain reaction [RT-PCR], or real-time PCR) is that they allow the investigatation of the putative spatial distribution of nucleic acid products activity in a heterogeneous cell population. In this chapter, we describe a protocol for *in situ* RT-PCR detection of specific messenger RNA in cucumber (*Cucumis sativus*), although this protocol can be used for any plant species, floral buds, and somatic embryo tissue sections on glass microscope slides. A successful *in situ* RT-PCR procedure requires the optimization of many conditions related to the tissue types used, for example, a cell's age, size, and composition, which may influence the detection of RT-PCR products, as well as specific transcript availability. Moreover, parameters, such as the fixation time, thermal cycling set-up, and the time of detection of RT-PCR products, also should be optimized. The importance of the other factors also is estimated in the protocol. In addition several types of controls that are necessary for a trustworthy *in situ* RT-PCR method are being discussed.

Key Words: Direct *in situ* RT-PCR; *Cucumis sativus*; floral buds; somatic embryos; paraffin tissue sections; digoxigenin-11-dUTP; alkaline phosphatase substrate.

1. Introduction

There are several common names for *in situ* polymerase chain reaction (PCR), which is performed based on RNA. Nuovo ***(1)***, who was one of the first developers of the method, called it reverse transcription (RT) *in situ* PCR, but since then, other names have been created for it, such as *in situ* complementary (c)DNA***(2)*** or *in situ* reverse transcriptase PCR ***(3)***, which are present in literature to date ***(4)***.

From: *Methods in Molecular Biology, vol. 334: PRINS and* In Situ *PCR Protocols, Second Ed.*
Edited by: F. Pellestor © Humana Press Inc., Totowa, NJ

In situ RT-PCR combines the sensitiveness of PCR amplification with spatial localization of products to monitor the appearance of specific transcripts in the tissue sections. Therefore, *in situ* RT-PCR defines a powerful tool for the low-abundance transcript detection ***(5)*** because the revealing threshold can be as low as one or two copies per cell. In comparison, *in situ* hybridization detects 10 to 20 copies per cell ***(6)***. This method has not been used to a large extent in plants, but it provides several advantages over the classic *in situ* RNA hybridization, which was widely discussed recently ***(7)***.

The first application of *in situ* RT-PCR for the plant tissue was reported by Woo et al. ***(8)*** and described the expression of the *HIS 3;2* gene (encoding the H1 histone) in the single detached border cells of pea seedlings. The subsequent papers on applying *in situ* RT-PCR technique to the plant material regarded several different tissues and genes ***(9–16)***.

In situ RT-PCR techniques can be classified into two groups based on labeling and detection systems used (**Fig. 1**). During the direct *in situ* RT-PCR, digoxigenin (biotin or fluorescein)-labeled nucleotides ***(13,14)*** or primers ***(15)*** are incorporated into the PCR product, leading to a direct signal detection. To the contrary, the indirect signal detection for *in situ* RT-PCR occurs when the PCR product is subsequently visualized by hybridization with specifically labeled probe ***(16)***. The direct *in situ* RT-PCR can be a quicker alternative to the indirect technique because it avoids the subsequent *in situ* hybridization step.

Although RT-PCR technique generally is adjusted by optimizing RT-PCR mixture and timing, for the successful procedure it also is crucial to set up optimal conditions for each new tissue in respect of its cells size and composition (e.g., lignified walls), as discussed previously ***(7)***. Engler et al. ***(17)*** described an *in situ* protocol suitable for obtaining the optimal results for different *Arabidopsis* tissues. Lee and Tegeder ***(7)*** observed that the thickness of the tissue sections and proteinase K pretreatment strongly increased the probability of successful application of *in situ* RT-PCR. Furthermore, the fixation and embedding processes also have a noticeable influence on the *in situ* RT-PCR results ***(14)***.

This chapter is aimed at providing a laboratory protocol for the *in situ* RT-PCR optimized for localization of transcripts in the cucumber floral buds and cucumber somatic embryo tissue sections, which also can be used for other plant species. Additionally, we provide a comprehensive procedure of *in situ* RT-PCR while discussing the main steps of this technique and providing a detailed list of materials effectively used in our laboratory for it.

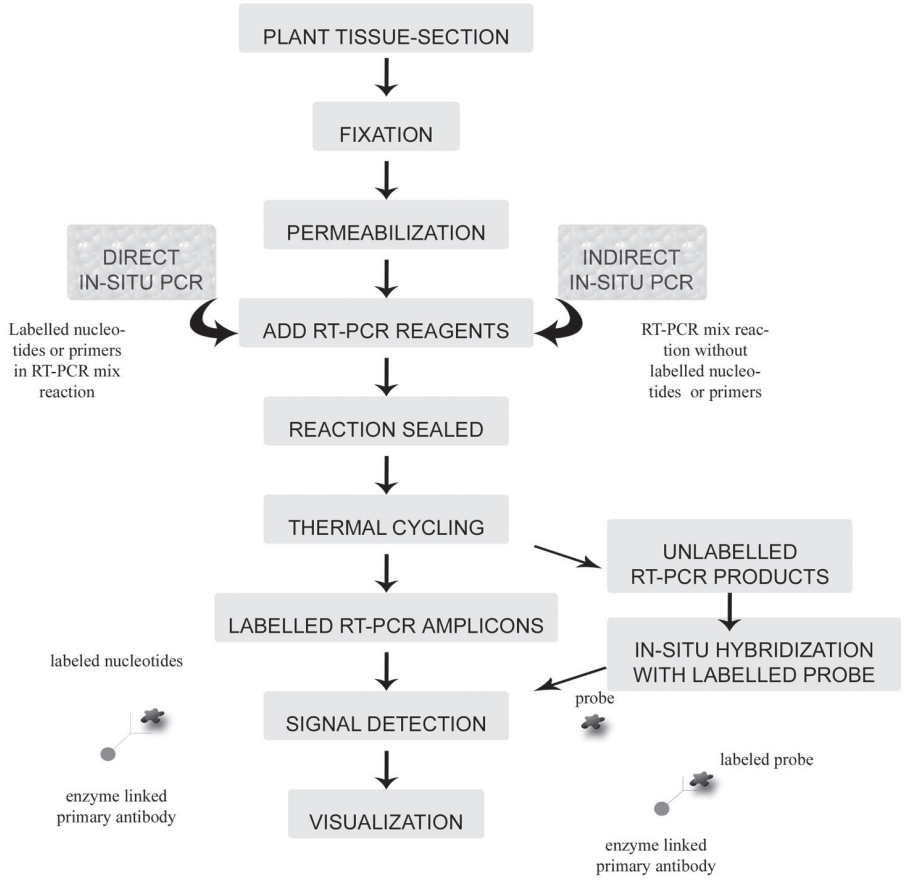

Fig. 1. Schema of *in situ* RT-PCR. Direct *in situ* RT-PCR-labeled nucleotide or primer is incorporated in RT-PCR mix reaction. Indirect *in situ* RT-PCR-labeled probe is incorporated during a hybridization step.

2. Materials

2.1. Tissue Preparation

As with all RNA work, precautions against RNase contamination should be taken. All solutions were prepared using 0/1% diethylpyrocarbonate-(DEPC) treated MilliQ H_2O. Always use gloves. Metallic tools, the glass dish, and cover are individually wrapped in aluminum foil and sterilized overnight at 180°C.

The majority of the chemicals are from Sigma-Aldrich Chemie in Steinheim, Germany. Chemicals from other company are noted.

1. To produce comparable material, all plants should be grown in tightly controlled environmental and developmental conditions. For cucumber (*Cucumus sativus*) plants are cultivated in a greenhouse under the same light regime with a minimum of 300 to 400 μ*M* photons m^{-2} s^{-1} at 20°C (night) and at 25°C (day) with a 8- to 16-h photoperiod. Healthy-looking, similar-size buds of the same shape and color are selected for experimental purposes. For each repetition, a pool of cucumber tissue should be taken. Embryos are collected with embryogenic suspension culture in phases: 0, 6, and 24 h and 3, 7, and 14 d after induction (after removing 2,4-dichlorophenoxyacetic acid).
2. Absolute ethanol (99.8% (Polmos, Kutno, Poland).
3. Formaldehyde 37% solution (12.3 *M*). Hazardous; store in chemical box at room temperature.
4. Acetic acid; store in chemical box at room temperature.
5. FAA fixative: 2% formaldehyde, 5% acetic acid, 60% ethanol. Prepare immediately before use and store on ice.

2.2. Tissue Embedding

1. DEPC. Very toxic. Stock store at 4°C.
2. RNase-free water (DEPC water): to MilliQ H_2O add DEPC to 0.1%, shake well, leave at 37°C overnight, then autoclave twice for 40 min to destroy excess the DEPC, and store at room temperature.
3. Ethanol solutions: 70%, 85%, 95%, and 99.8%.
4. HistoClear (Histochoice Clearing Agent): irritant; store in chemical box at room temperature.
5. HistoClear: ethanol ratio of 2:1; prepare immediately before use and store under exhaust at room temperature.
6. HistoClear: ethanol ratio of 1:2; prepare immediately before use and store under exhaust at room temperature.
7. Embedding medium: Paraplast Plus; store at room temperature, but melt before use to 58–60°C.

2.3. Microscope Slide Sectioning

There is no need for a special slides preparation in case of SuperFrost Plus glass slides being used. *Cucumis* embryo and flower bud paraffin sections perfectly attach to these slides, and Superfrost Plus slides optimize *in situ* methods. If you do not have SuperFrost glasses, you should prepare clean, RNase-free glass slides. To coat the slide for tissues adhesion, dip them in 2% 5-aminopropyltrithoxysilane (TESPA) in dry acetone for 5 to 10 s. Quickly rinse them (twice in acetone and once in distilled H_2O), then air-dry. Slides coated with TESPA can be kept for several months.

1. "Ready-to-use" glass slides (SuperFrost Plus, Menzel-Glaser, Germany), 25 × 75 × 1 mm.

2. MiliQ-water.
3. Embedding tissue.

2.4. Pretreatment of Slides

1. HistoClear.
2. Ethanol solutions: 99.8%, 95%, 85%, 70%, 50%, and 30%.
3. 0.02 *M* HCl: add 1.2 mL of concentrated HCl to 60 mL of water.
4. 20X standard saline citrate (SSC): sodium citrate solution: 3 *M* sodium chloride, 0.3 *M* Tri-sodium citrate. To prepare 20X SSC, add 175.3 g of NaCl and 88.2 g of sodium citrate adjust to pH 7.0 and add up to 1 L of DEPC-treated water, autoclave, stored 2 to 3 wk at 4°C.
5. 0.5 *M* Ethylene diamine tetraacetic acid (EDTA) pH 8.0: add 18.61 g of EDTA to 80 mL of DEPC-treated water, to prepare 100 mL solution; stir with a magnetic stirrer, dissolve by adjusting to pH 8.0 with NaOH (10 *M*), and complete volume to 100 mL, then autoclave 17 min at 121°C.
6. Pectinase from mold: (Fluka Chemika, Buchs, Switzerland), store at 4°C.
7. Pectinase buffer: 0.1 *M* sodium acetate, 5 m*M* EDTA. Per 50 mL, add 0.41 g of sodium acetate, 0.09 g of EDTA, and up to 50 mL of water. Adjust to pH 4.5, store at 4°C.
8. 10% Pectinase solution: put 1.5 mL of 10% pectinase buffer and add 15 mg of pectinase into Eppendorf tube. Mix these compounds together gently for a minimum of 4 h and store at 4°C.
9. Proteinase K. Store at 4°C.
10. Proteinase K buffer: 100 m*M* Tris-HCl, 50 m*M* EDTA, pH 8.0. Add 3.025 g of Tris-HCl, 4.652 g of EDTA, and up to 250 mL of DEPC water. Adjust to pH 8.0 and autoclave; store at –20°C.
11. 10% Glycine solution: add 25 g of glycine and up to 250 mL of DEPC water, autoclave, and store at 4°C.
12. 10X phosphate-buffered saline (PBS): adjust to pH 7.4 and autoclave. Keep at 4°C for 2 to 3 wk.
13. DNase buffer: 0.1 *M* sodium acetate, 5 m*M* $MgSO_4$, pH 5.5. Add 0.41 g of sodium acetate and 0.0615 g $MgSO_4 \cdot 7H_2O$ and up to 50 mL DEPC water. Autoclave and store at –20°C.
14. RNase-free Dnase I (Roche Molecular Biochemicals, Mannheim, Germany). Store at –20°C.

2.5. In-the-Tube RT-PCR

1. Pair of primers: designed by the user (*see* **Note 1**), of known concentration (can be stable at –20°C).
2. Gene Amp r*Tth* DNA Polymerase and EZ Buffer Pack: (EZ rTth kit, PE Biosystems, Nortwalk, CT) all reagents should be stored at –20°C. The kit includes r*Tth* DNA polymerase, 5X EZ Buffer, and 25 m*M* $Mn(OAc)_2$. r*Tth* polymerase is a dual-activity enzyme, that is, a reverse and high-temperature *Taq* polymerase. It makes the one-step RT-PCR reaction possible.

3. 10 m*M* each of dNTPs (Roche Molecular Biochemicals). Store at –20°C. Mix four dNTPs into Eppendorf tubes. Store in aliquots to avoid constant freezing and unfreezing.
4. Bovine serum albumin (20 mg/mL; Fermentas, Vilnius, Lithuania). Store at –20°C.

2.6. RT-PCR Step of In Situ RT-PCR

To the amplifying solution described in **Subheading 2.5.**, add digoxigenin-11-dUTP (25 n*M*; Roche Molecular Biochemicals) and store at –20°C.

2.7. Detection System

1. Prepare 2X, 1X, and 0.5X SSC solutions.
2. Maleic buffer: 100 m*M* maleic acid, 150 m*M* sodium chloride. Add 11.61 g of maleic acid and 8.76 g of NaCl, adjust to pH 7.5, autoclave, and store at 4°C.
3. Triton X-100. Store at room temperature.
4. Goat serum: (Vector Laboratories, Burlingame, CA). Store at 4°C.
5. Maleic-serum-Triton solution: 1 mL of maleic buffer, 40 µL of goat serum, and 3 µL of TritonX-100.
6. For digoxigenin system: use the antidigoxigenin–alkaline phosphatase (AP) *Fab* fragments (Roche Molecular Biochemicals) 150 U/200 µL. Store at 4°C.
7. Maleic-serum-Triton-AP solution: 500 µL of maleic buffer–serum–Triton solution and 0.5 µL of antidigoxigenin–AP *Fab* fragments.
8. 4-Nitroblue tetrazolium chloride (NBT; Roche Molecular Biochemicals). NBT is very toxic. Store at –20°C in the dark.
9. 5-Bromo-4-chloro-3-indolylphosphate-4-toluidine (BCIP; Roche Molecular Biochemicals). BCIP is very toxic. Store at –20°C in the dark.
10. Magnesium buffer: 100 m*M* Tris-HCl, 100 m*M* NaCl, 50 m*M* $MgCl_2$. Add 5 mL of 1 *M* Tris-HCl, pH 8.0; 0.29 g of NaCl, 2.5 mL of $MgCl_2$, and up to 50 mL of sterile water. Adjust to pH 9.5.
11. Prepare magnesium–NBT-BCIP solution: 500 µL of magnesium buffer, 1.75 µL of BCIP, and 2.25 µL of NBT.

2.8. Other Equipment

1. The frame and the cover: (65 µL of EasySeal Hybaid Limited, Middlesex, UK); the size of the frame will depend on the size of the tissue.
2. Microscope (Olympus BX 60) with image analysis system (AnalySIS Soft Image System GmbH, Münster, Köln, Germany) and video camera (CCD-IRIS/RGB Sony Image System GmbH, Munster).
3. Slide storage boxes: sealable (Kartell, Italy). Boxes that hold 25 and 100 slides are useful.
4. Pipets used for RNA only.
5. Air vacuum (Cole-Parmer, Chicago, IL).
6. Thermalcycler with *in situ* block: (Biometra, Annalytik GmbH, Göttingen, Germany).

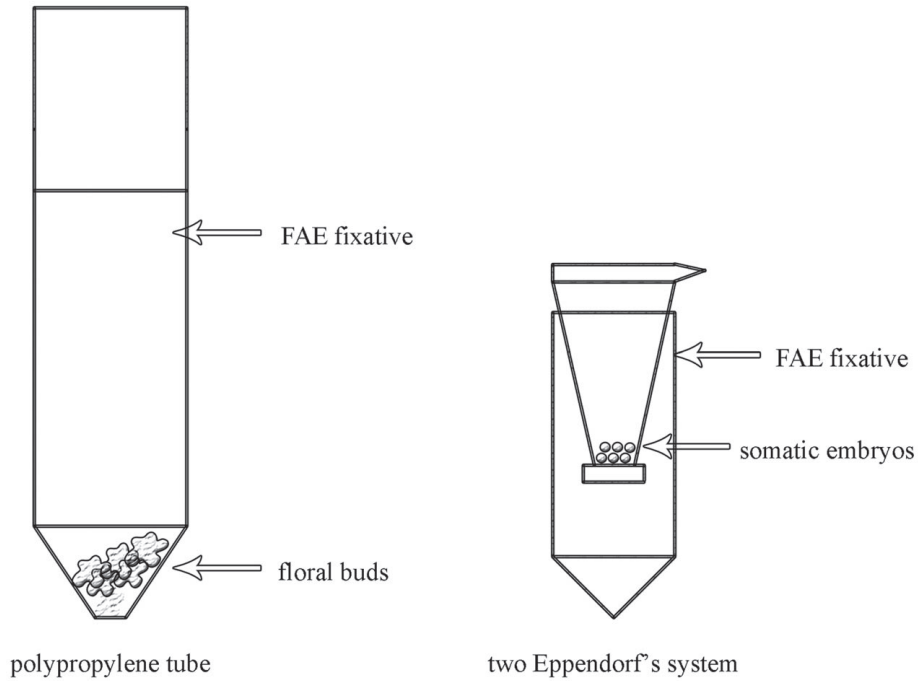

Fig. 2. Variations of possible placement manner for the fixation of cucumber floral buds and cucumber somatic embryos.

7. Microtome (Microm Laborgerate GmbH, Walldorf, Germany).
8. Humidified box: a small plastic box with a sterile, damp gauze at the bottom; this box can be used for holding slides during incubation with different reagents using in the reaction.

3. Methods

3.1. Tissue Preparation

1. Immediately after dissecting the plant material, throw the tissue into a fresh, cold FAA fixative and store on ice (*see* **Note 2**). Do not fix more than 1 cm^3 of tissue per 40 mL of fixing solution. Cucumber floral buds are put in polyester tubes and somatic embryos into a "two-Eppendorf system" (**Fig. 2**). A 1.5-mL Eppendorf tube is filled with the fixative solution and contains another 0.5-mL Eppendorf without a bottom, which is mounted with sticky nylon net (Ø stitch: 20 μm). The embryos are then laid on the net with small stitches, which helps collect tissues after fixation and transfer to the next solution.
2. Tubers with tissues in fixation solution should be transferred to the vacuum oven. Use a vacuum to degas the plant tissues for cucumber buds for approx 3 min and

for embryos for approx 1 to 2 min, which can improve penetration of tissues by fixative. After degassing, the fixed tissue should be sunk on the button of tubes and then stored at 4°C overnight (*see* **Note 3**).

3.2. Tissue Embedding

It is necessary to use at least 10 times greater volume of the solvent of that of the prepared tissue.

1. Remove the fixative solution and replace it with 70% ethanol. Use a vacuum for a better infiltration and store for approx 6 h at 4°C. Remove 70% ethanol by replacing it with a fresh 70% ethanol and then degas tissue samples in vacuum oven and store at 4°C overnight.
2. Dehydrate the fixed samples by incubating them in gradient series of ethanol solutions in H_2O (85%, 95%, followed by two cycles in 99.8% ethanol), each stage for 1 h at room temperature under the degas system.
3. Incubate tissues with 1 volume of HistoClear in 2 volumes of ethanol, employ a vacuum, and leave at room temperature for 1 h, then repeat this step with 2 volumes of fresh HistoClear in 1 volume of ethanol mixture.
4. Transfer the plant samples to 100% HistoClear, employ a vacuum, and leave at room temperature for 1 h. Repeat the 100% HistoClear incubation for 1 h for a total ethanol replacement.
5, The Paraplast Plus should be melted at 58 to 60°C before embedding tissues in a vacuum oven (*see* **Note 4**). After removing the plant samples from HistoClear, place them into glass bakers with melted Paraplast in the vacuum oven at 60°C overnight. Replace with freshly melted Paraplast twice a day for 3 to 4 d to obtain a perfect infiltration, then for the last step, the samples should be transferred into an aluminum mold or small Petri dishes. Finally degas in the vacuum oven overnight. On the next day, arrange the tissues position using a bacteria inoculation needle and then immediately put the embedded materials on ice (partially thawed with ethanol and water) for quick cooling. The paraffin blocks can be stored at room temperature or at 4°C indefinitely.

3.3. Microscope Slide Sectioning

For our system, we used glass slides (SuperFrost Plus), which can be used directly without the need for any special adhesives or protein coating.

1. Using a sharp single-edged razor blade trim the excess wax from the edges of the embedded tissue block, get a tissue sample of the pyramid shaped structure, and place it onto microtome block.
2. Cut a 7-µm thin ribbon section from the block. We have applied this thickness to both floral buds and cucumber embryos to obtain optimal results.
3. Sections submerged into warm water in microtome are ready for careful adherence onto the surface of the slides (*see* **Note 5**). Slides should be dipped into the water and sections caught onto the surface of the slides. Remove the excess water

from the tissue, by rattling gently on sterile scraps of paper or using the corner of a paper sheet.
4. Place the slides on a heat tray at 40–42°C overnight (*see* **Note 6**). Then, transfer them to the slide storage boxes located in a dry place for a long storage.

3.4. Pretreatment of Slides

We recommend that the following pretreatments should take place in laminar flow cabinets (to protect against dust and especially RNase contamination) at room temperature. Incubate paraffin sections in HistoClear to remove paraffin for 10 min, and then repeat this step in new HistoClear.

1. Immerse slides in 100% ethanol for 1 min, then repeat this step with a fresh 100% ethanol.
2. Rehydrate the mounted tissue sections by washing the slides in each of a graded series of solutions of ethanol in DEPC water by ethanol series (95%, 85%, 70%, 50%, and 30%) and finally, transfer to the DEPC-water. At each step, wash the slide by repeatedly dipping it for approx 1 min.
3. Incubate the slides in 0.02 *M* HCl for 20 min (*see* **Note 7**).
4. Transfer the slides into 2X SSC for 30 min. Remove excess 2X SSC from the slide surface by gently shaking down on sterile sheets of paper. Place each slide into the plastic humidified box, on the blotting paper moisten with DEPC water.
5. Firmly closing the humidified box lid is crucial (*see* **Note 8** and **Fig. 3**).
6. For each slide, add approx 70 to 100 µL of pectinase buffer to cover completely tissue section and incubate the slides for 10 min.
7. Remove the pectinase buffer from slides by shaking down on a sterile blotting paper.
8. Apply approx 50 µL of 1% pectinase in pectinase buffer and incubate for 10 min (*see* **Note 9**).
9. Remove the remains of pectinase (1%) from slides by shaking down on sterile blocking papers.
10. Apply approx 100 µL of proteinase K (2 µg/mL final concentration) in proteinase buffer for each slide (to cover the tissue section completely) and quickly transfer to 37°C, then incubate for exactly 5 min in a humidified box (*see* **Note 10**).
11. Wash slides in glycine (2%) in 1X PBS for 1 min. Wash another two times in 1X PBS for 5 min. Remove the excess of 1X PBS from the slide surface by gently shaking down on sterile blotting paper.
12. Put each slide for a second time into the humidified box and apply 150 µL of DNase buffer (tissue sections should be completely covered). After short incubation replace with a new DNase buffer and subsequently incubate for 45 min.
13. Remove DNase buffer by shaking down on a sterile paper towels.
14. Apply 8 U RNase-free DNase I in 50 µL of DNase buffer onto each section.
15. Tissues should be treated in a humidified box at 37°C overnight (*see* **Note 11**).
16. The digestion of tissues with DNase is recommended to avoid amplification of nuclear DNA.

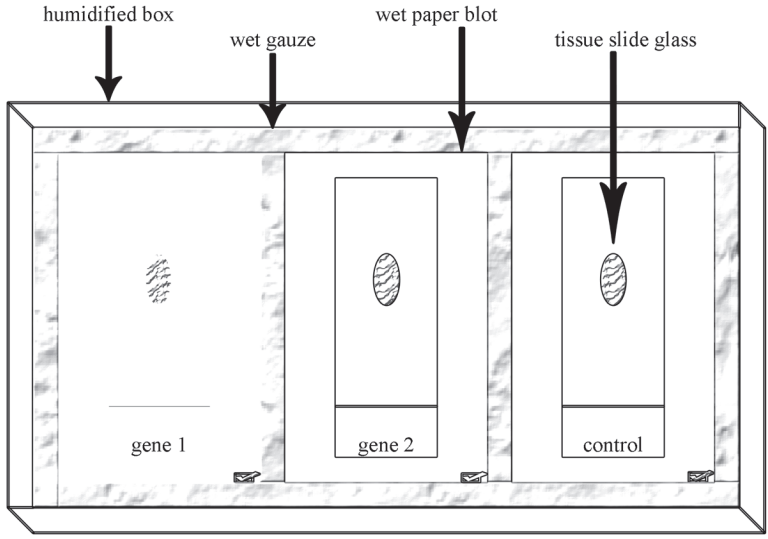

Fig. 3. Humidified box used for digestion steps during *in situ* RT-PCR.

17. The next day, wash the slides in the following solutions: 0.5 *M* EDTA, 2X SSC, 1X SSC, 0.5X SSC, and DEPC water for 10 min each.

3.5. In-the-Tube RT-PCR

Control specimens without r*Tth* polymerase and with or without primers always should be included. Use also an amplification mixture from under cover slip as control. (*See* **Note 12**).

One of many controls deployed is the "in-tube RT-PCR reaction," which can be assumed as performed, if the detected transcripts are present in the tissue.

1. RNA from selected tissues should be extracted using convenient protocol.
2. Reaction mix contains: 5U r*Tth* Polymerase, 5X EZ buffer, 2.5 m*M* $Mn(OAc)_2$ 0.3 m*M* dNTPs, 0.064% bovine serum albumin, 1.2 μ*M* of each forward and reverse primers, and 1 μg of RNA.
3. Cycling reaction set-up: 61°C for 30 min; 94°C for 3 min; 30 cycles of two-temperature PCR (94°C for 75 s, 61°C for 75 s); and the final extension in 61°C for 7 min. PCR is completed in an UNO II Thermalcycler.
4. The RT-PCR products were visualized by gel electrophoresis on 2% agarose gel with EtBr.

3.6. RT-PCR Step of In Situ *RT-PCR*

Parallel to washing (*see* **Subheading 3.4.**, **step 15**), the RT-PCR reaction mixture needs to be prepared. Necessary reagents are stored at –20°C and first have to be gently thawed on ice.

1. The reaction mixture should be prepared in Eppendorf tube. The mixture contains: 5X EZ buffer, 0.3 m*M* dNTPs, 2.5 m*M* $Mn(OAc)_2$, 5u r*Tth* Polymerase, 0.064 bovine serum albumin, 25 m*M* digoxigenin 11-duTP, and 1.2 m*M* each of primers in a total reaction volume of 50 μL.
2. Transfer the slides from water incubation onto a sterile blotting paper.
3. When the part of the glass slide around tissue sections is dry, mount EasiSeal frame and stick it tight to the glass (do not touch the tissue sections, which should be inside the frame). Remove the paper protecting the frame.
4. Transfer the slides into the plate heated up to 61°C. This step needs to be processed promptly and without pause.
5. Apply RT-PCR reaction mixture onto the frame, starting from one edge of the frame.
6. Gently and slowly stick a flexible plastic cover slip (EasiSeal) to the frame from the edge where the reaction mix was applied (*see* **Note 13** and **Fig. 4**).
7. Transfer slides to the *in situ* thermocycler block preheated to 61°C. Cycling reaction set-up: 61°C for 30 min; 94°C for 3 min; 32 cycles of two-temperature PCR (94°C for 90s, 61°C for 90s); and the final extension in 61°C for 7 min. The optimal lid temperature for cucumber floral bud sections is 105°C and 70°C for cucumber somatic embryos. PCR is completed in an UNO II Thermalcycler with *in situ* block. Several controls can be applied to this step (*see* **Note 14**). The reaction without primers is the most informative (for that, H_2O is added instead of primers).

Optimization of *in situ* RT-PCR requires many different experimental controls to ensure that no false-negative or false-positive results are obtained. After optimization, we routinely use only one control (reaction with water instead of primers), which in our opinion is capable of identifying any unspecific amplification. These controls served to confirm that the signal detected was derived from the amplified mRNA in the sections (**Fig. 5**).

3.7. Detection System

All detection steps are being performed at room temperature with freshly prepared buffers.

1. After the amplification RT-PCR, frames with covers are gently removed from each microscope slide.
2. Wash the slides twice in 2X SSC for 5 min. Rinse the slides in the following solution: 1X SSC and 0.5X SSC.
3. Remove remaining drops of 0.5X SSC buffer by shaking down on sterile blotting paper (by gently hitting the paper towel with vertically positioned glass slide) and put each slide in humidity chamber.
4. Quickly apply 300 μL of maleic buffer into the slide (sections should be covered with liquid), then remove its excess by shaking down on a sterile blotting paper.
5. Repeat the maleic buffer treatment with 100 to 200 μL (sections should be covered with liquid) for 10 min.
6. Remove maleic buffer by shaking down on a sterile blotting paper.

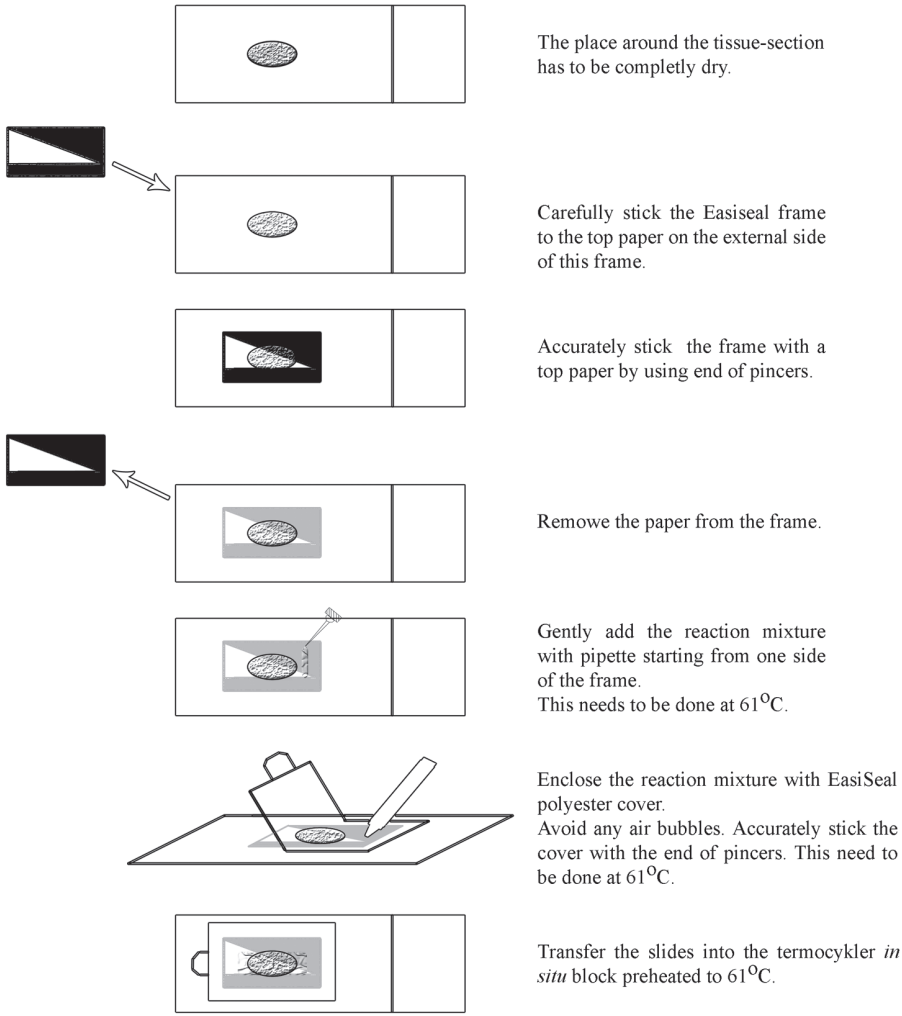

Fig. 4. The placement of RT-PCR reaction mixture on the tissue section.

7. Add 150 μL of maleic buffer–serum–Triton solution onto tissue, and incubate for 30 min.
8. Remove maleic buffer–serum–Triton solution by shaking down on sterile blotting paper.
9. Into fresh maleic buffer–serum–Triton solution, add antidigoxigenin antibody, conjugated with AP diluted to 1:1000 (maleic buffer–serum–Triton–AP solution), and incubate tissue for 2 h.
10. Remove a maleic buffer–serum–Triton–AP solution by shaking down on a sterile blotting paper.

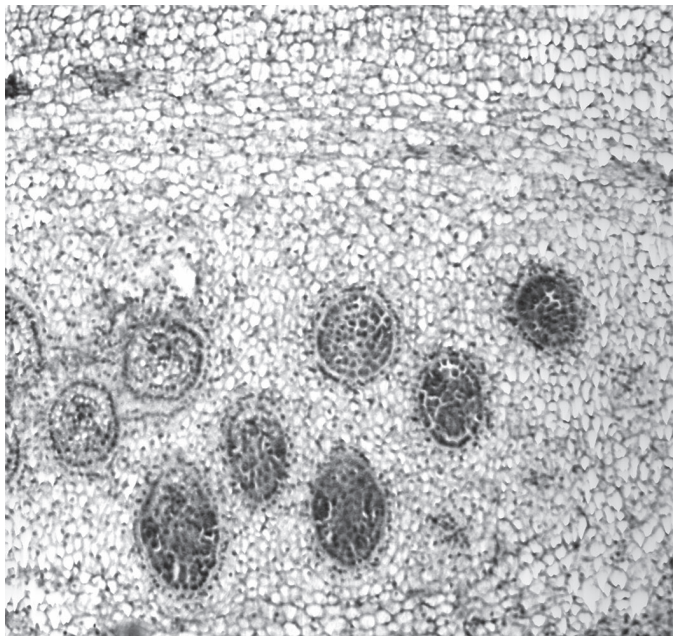

Fig. 5. Example of *in situ* RT-PCR control reaction without DNase pretreatment in cross-sectioned female bud of cucumber (*Cucumis sativus* L.). Signals were detected in every cell nucleus. Magnification ×100.

11. Wash the slides in magnesium buffer.
12. Remove the excess of the magnesium buffer by shaking down on a sterile blotting paper.
13. Repeat washing in magnesium buffer for 10 min. The unbound antibody conjugates will be removed this way.
14. Remove the magnesium buffer by shaking down on a sterile blotting paper and transfer slides into the humidified box.
15. Develop signal depicted by the color-substrate 0.3 mg/mL NBT and 0.2 mg/mL BCIP substrates. In the darkroom, gently apply (according to the supplier's recommendations) freshly prepared color-substrate on the tissue sections (slides need to be still in a humidified box, on the paper towels soaked in sterile water).
16. Monitor the color reaction development (blue-purple staining) on the tissue sections under microscope every few minutes. The color was developed for approx 15 min for cucumber embryos and 8 to 10 min for cucumber floral buds (*see* **Note 15**).
17. The color reaction can be stopped by incubation in water. Leave the slides in sterile water at 4°C for 12 h (color intensifies overnight in a fridge).
18. After washing in H_2O, the slides are air-dried and mounted in balsam oil (*see* **Note 16**), then covered with a cover slip. The drying time is approx 12 h.

19. For documentation-obtained results it is recommended that one use a microscope with the phase-contrast lenses and dark field. Try the lenses in the range of ×20 to ×100. Sections were observed and documented with a CCC-IRIS/RGB. Additionally, we have used the analySIS system connected to the Olympus light microscope to visualize the results after *in situ* RT-PCR procedure on the cucumber tissue-sections (**Fig. 6**).

4. Notes

1. The primers have to be designed to amplify a short fragment of complementary DNA not exceeding 250 base pairs. Larger fragments may be not completely reverse transcribed because of their secondary structure, and the nucleic acid can be partially degraded. To check how correctly the primers were designed, we performed the control reaction, with cucumber RNA as a target, in a tube.
2. Several fixatives can be used successfully for the preservation of plant tissues, for example, formaldehyde, glutaraldehyde, and a mixture of ethanol and acetic acid, as we described previously ***(14)***. Time of fixation should be optimized for each individual object, as it is one of the crucial factors. Most often the proper fixation time is 24 to 48 h. The fixation time conditions are very strictly dependent on protease digestion, and any change of the fixation protocol determines necessary changes in protease digestion circumstances. Insufficient digestion makes it difficult for the reagents to access the nucleic acids. However, overdigestion may cause outflow of the amplification products from the cells, when the reaction takes place and often leads to a loss of tissue architecture. Thus, any changes in fixation method should be correlated with optimization of protease digestion.
3. As mentioned previously, the volume of fixative depends on the amount of tissue being fixed. After applying vacuum gently, release it slowly as to facilitate the access of fixative into the tissue. Pieces of tissue that are well infiltrated with fixative should sink to the bottom of the tube as liquid replaces air in the tissue.
4. The manufacturer of Paraplast Plus warns against overheating. The temperatures higher than 62°C may adversely affect sectioning of the embedded tissues.
5. Heat the DEPC-water in microtome to release the paraffin sections. Sectioning process should be smooth and slides easy to observe under microscope. Transfer the ribbon into the water with a small paintbrush, and then allow it to float for 2 to 3 min and pick up the section with a microscope slide.
6. We found that treatment of slides at 42°C was important for optimal tissue adhesion to the slides (overnight). Slides were in a closed thermal box to prevent contamination.
7. The efficacy of the HCl is unknown.
8. For the incubation steps, we used closed plastic boxes containing gaze and moistened paper towels, which provided the necessary humidified conditions for several incubation steps (**Fig. 3**).
9. The pectinase treatment of the tissue prevents from an unspecific cell walls staining during the detection step.

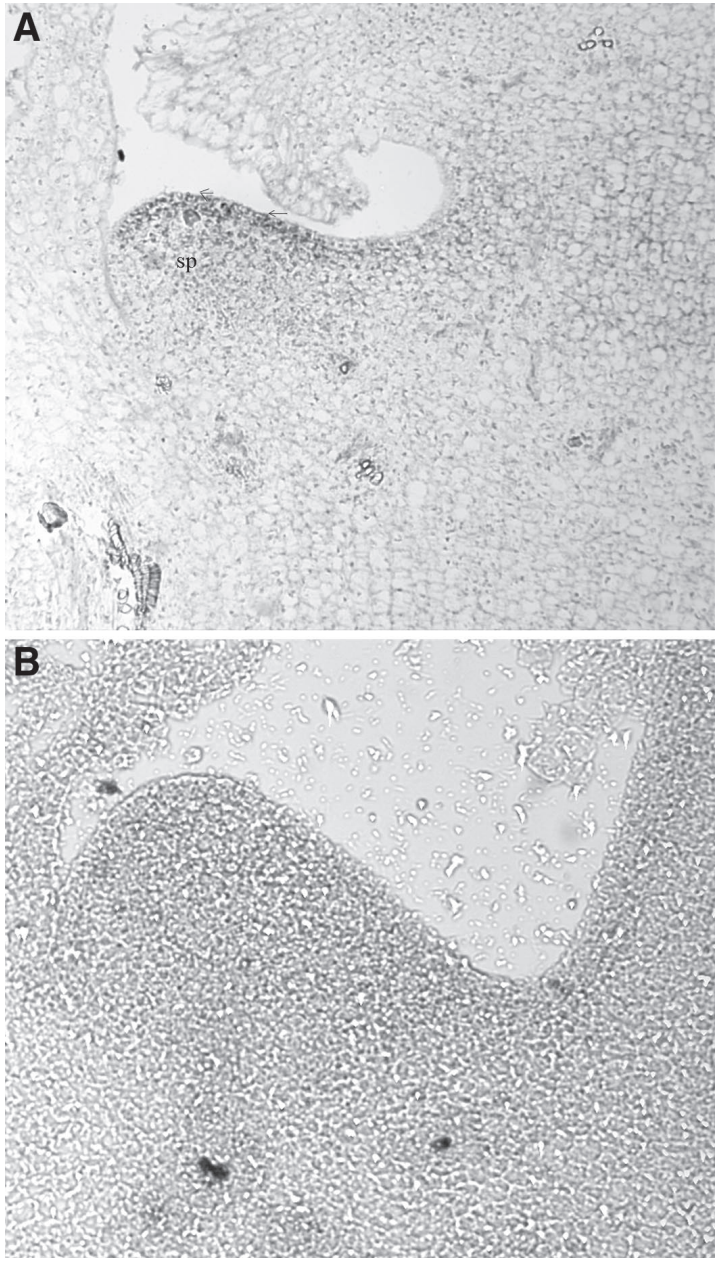

Fig. 6. *In situ* RT-PCR in cross-sectioned female bud of cucumber (*Cucumis sativus* L.). (**A**) reaction with specific 157B10 primers. Localization signals of transcripts in stamen primordium (sp). (**B**) Negative control reaction without specific primers in the RT-PCR mix. Magnification ×100.

10. The optimal time of proteinase K digestion was tested twice for the fixation of floral buds (for 48 and 24 h, respectively). The buds fixed for 48 h were treated with proteinase K for 15, 30, 45, and 60 min. The best results were achieved after 45 min of the proteinase K digestion. The morphology of the tissue was well retained, and interpretation of results was unambiguous. The signal was recognized as a purple-black dye at the site of the label. The 30- and 15-min durations turned out to be too short and led to lack of signal. The extension of the reaction time up to 60 min produced morphological distortions to the point that interpretation of results became impossible, so that unspecific signals appeared in every cell. We applied the same four time intervals of proteinase K digestions to the buds that were fixed for 24 h. It turned out that all tested times were too long. After signal development, only the unspecific signals were observed and the tissue morphology was poorly preserved. Three new times of digestion were tested (5, 15, and 20 min). Negative control without the proteinase K digestion also was prepared. The shortest time (5 min) proved to be optimal. The tissue was well preserved, and the signal was clear and specific. The reaction without proteinase K treatment was carried out, but no signals were noticed.
11. When the DNase digestion was skipped after the development, the signals were observed in all nuclei, although no primer was added to the reaction. As was discussed previously, this effect is very likely attributable to the repairing activity of r*Tth* polymerase used in this protocol, which may heal the nicks and gaps in the genomic DNA formed during the fixation and embedding processes ***(14)***.
12. Optimization of *in situ* RT-PCR needs to include many different control experiments to eliminate any false-positive or false-negative signals. Control experiments should be conducted in parallel on the same tissue section that is prepared for the experiments. For detecting false-positive signal reaction without labeled nucleotide, r*Tth* enzyme or primers could be performed. The *in situ* RT-PCR reaction with the antidigoxigenin–AP detection phase omitted can serve as an additional control for false-positives signals. As a control may use reaction mixture after RT-PCR step from under cover slip. Solution phase should be collected, next spotted on the nylon membrane, and hybridized with specific probe.
13. Cover the slide with a cover slip, avoiding formation of any bubbles. Make sure not to touch the section.
14. The parameters of the *in situ* RT-PCR reaction, especially the annealing temperature, should be optimized by applying the standard RT-PCR protocol in a solution phase (RNA isolated from the cucumber buds can be added). The reaction times applied were longer than usually used in a solution phase. It was a consequence of different conditions of *in situ* reaction. On the glass slide, the temperature fluctuation was much slower. A proper seal is very important to keep reaction concentrations consistent throughout the thermal cycling process. Concentrations of reagents are critical for a proper amplification.
15. The development time depends on the abundance of the mRNA and the structure of the cells. If a signal is not visible after 12 h, a fresh chromogenic substrate needs to be added. The slides can be stored in water at 4°C for up to 2 d. If slides are left longer than 2 d, the sections start to ruin and the stain diffuses.

16. Before covering the slides, remove all air bubbles, which can be detected as small, round bumps on their surface. If bubbles are present, try to cap the balsam oil.

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