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Fluorescent whole-mount RNA *in situ* hybridization (F-WISH) in plant germ cells and the fertilized ovule

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ABSTRACT

First evidence on gene function and regulation is provided by the cellular expression pattern in complex tissues. However, to understand the activity of a specific gene, it is essential to analyze the regulatory network, which controls the spatio-temporal translation pattern during the entire life span of the transcribed mRNA. To explore mechanisms which control mRNA abundance and localization in space and time, it is necessary to visualize mRNAs quantitatively with a subcellular resolution, without sectioning the tissues. We have adapted and optimized a protocol for colorimetric whole-mount RNA *in situ* hybridization (WISH) using egg cell-specific digoxigenin (DIG) labeled probes (Hejátko et al., 2006) [1] on ovules and early seeds of *Arabidopsis*. Furthermore, we established a fluorescent whole-mount RNA *in situ* hybridization (F-WISH) protocol, which allows mRNA visualization on a subcellular level. The polar localized mRNA of *SBT4.13*, encoding a subtilase, was identified using this protocol. Both methods are described and discussed in detail. Additionally a (F)-WISH flow-chart is provided along with a troubleshooting table.

1. Introduction

The transcribed gene product, the mRNA, is decorated with RNA-binding proteins (RBPs), forming large mRNA/protein (mRNP) complexes, at any given time. The composition of the mRNPs dictates the fate of the mRNA (translationally active, degraded, stored or transported to a specific site). The temporal and spatial control of mRNA translation thus regulate the presence of gene products precisely, especially during development. This mechanism is found in all kingdoms of life and appears to be essential, e.g. for asymmetric cell division, cell fate determination and many other developmental processes [reviewed in [2,3]]. In most model organisms it is well described that RNAs can move to specific sites within a cell or into neighboring cells, e.g. from the nurse cells into the oocyte of *Drosophila* [4]. Global mRNA expression studies with a subcellular resolution revealed that more than 71% of the expressed genes are distributed in a polar manner during early *Drosophila* embryogenesis [5]. In contrast to animals and fungi little is known so far about the localization and translational control of mRNAs in plants. After fertilization the association of polyribosomes to microtubules in the zygote suggests mRNP formation and transport during early ovule development [6]. During pollen maturation, synthesized mRNAs are stored in an non-translated form until pollen germination [7] or gamete fusion occurs [8]. Recently, it was shown that mRNA can move over long distances through different plant body parts [9]. The visualization of such mRNAs would give insights into the underlying molecular transport mechanism. To our knowledge, in plants the only visualization of localized mRNA in a non-tagged approach was published in 2000. Using *in situ* RT-PCR, the authors could show that mRNA of the prolamine seed storage protein localizes to subdomains of the endoplasmic reticulum (ER) of rice endosperm cells [10,11]. The restricted translation of specific mRNAs due to mRNA localization has been shown to be a key element of cell fate determination in all stages of development. To identify such localized mRNA during ovule development, we visualized particular mRNA molecules on a subcellular level in intact tissues. Therefore we used linearized plasmids, carrying gene-specific sequences for RNA probe synthesis. The *in vitro* transcribed probes carry DIG, biotin or fluorescein linked nucleotides for antibody detection [1]. The tissue is fixed by formaldehyde and dehydrated. To ensure probe infiltration and RNA/RNA hybrid formation, tissues have to be dewaxed by a strong solvent and mRNA decorating proteins have to be degraded. Every permeabilization step has to be associated with an additional fixation step. RNA hybrids are detected by immunohistochemistry using an alkaline phosphatase (AP)-conjugated primary antibody and a chromogenic substrate for colorimetric detection. For fluorescent labeled RNA hybrid

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detection on a subcellular level, we used a horseradish peroxidase (HRPO)-conjugated primary antibody combined with the Tyramide Signal Amplification (TSA)-system (PerkinElmer). The HRPO/TSA-system is based on the activation and covalent binding of TSA-reagent to tyrosine residues in close vicinity, most likely on the antibody/enzyme itself. Unbound, activated fluorophores form dimers and are washed away. By using this method, we are able to visualize mRNA distribution in *Arabidopsis* ovules and developing seeds on a subcellular level.

2. Material and methods

All liquids should be prepared in RNase-free bottles with RNase-free water. We commonly use baked bottles (180 °C for 8 h) and Milli-Q® Integral ultra-pure water with Biopak® final filters (Merck Millipore). If possible all buffers, which are listed in Table 1, were treated with DEPC (no –N, –S or –O in the molecule). All chemicals used have a purity level of "pro analysis".

2.1. Probe synthesis

We commonly use two methods to generate gene-specific labeled RNA probes. The selected sequence is cloned into the pCR™-Blunt II-TOPO® (ThermoFisher) vector. In the flanking region, the plasmid contains T7 and SP6 RNA polymerase promoter sequences for *in vitro* transcription. Additionally, several unique recognition sites for restriction enzymes creating blunt end or 5′-overhangs for plasmid linearization are available. In the presented examples (Figs. 1 and 3) we cloned the coding sequence of *EGG CELL 1.1* (*EC1.1*) (AT1G76750; 477 bp) and *SUBTILASE 4.13* (*SBT4.13*) (AT5G59120; 2200 bp) like described above. Alternatively, the T3-, T7- or SP6-specific promoter sequence is added to a gene-specific region by PCR-mediated ligation. In both cases, the purified DNA is transcribed by the RNA polymerases to produce DIG, biotin or fluorescein labeled "run off" transcripts (DIG RNA Labeling Kit: Roche, cat. No. 11175025910; Biotin RNA Labeling Mix: Roche, cat. No. 11685597910; Fluorescein RNA Labeling Mix: Roche, cat. No. 11685619910). DIG-11-UTP, Biotin-16-UTP or Fluorescein-12-UTP is incorporated into the probe by RNA polymerases at a rate of approximately every 20–25th nucleotide. The DNA template is removed from the synthesized probe by DNaseI treatment and the probe purified by LiCl based precipitation. The washed pellet can be dissolved in RNase-free water and stored at −80 °C. The specific reaction setup should be done according to the respective company manual. Long probes (>500 nt) can have a negative effect on hybridization efficiencies or tissue penetration depth. The size can be reduced by hydrolysis to generate an optimal probe size of ~300–500 nt. The *SBT4.13* probe was hydrolyzed by adding 1 volume of freshly prepared Alkaline Hydrolysis Buffer and incubation was performed at 60 °C for a calculated time (t) (t [min] = (probe length [kb] − desired probe length [kb])/(0.11 * probe length [kb] * desired probe length [kb])). Hydrolysis can be stopped by adding 0.5% (v/v) acetic acid and 0.1 M sodium acetate to neutralize the solution. An additional precipitation step is essential. The washed pellet can be dissolved in RNase-free water and stored at −80 °C.

Table 1
Buffer composition.

Buffers	
Alkaline Hydrolysis Buffer	0,2 M Sodium Carbonate (0.08 M $NaHCO_3$ + 0.12 M Na_2CO_3); 1 mM EDTA
Fixative	4% (w/v) Formaldehyde (freshly prepared from paraformaldehyde), 15% (v/v) DMSO and 0.1% (v/v) Tween®20 in PBS Prepare the solution as follows: dissolve paraformaldehyde as a 10% (w/v) solution in water by short heating (⩾60 °C) and add few drops of 1 N NaOH until the solution becomes clear. The pH has to be below pH 9. Afterwards add the remaining solutions
10X PBS	1.3 M NaCl, 70 mM Na_2HPO_4, 30 mM NaH_2PO_4 (pH 7.4); DEPC-treated
PBS-T	PBS + 0.1% (v/v) Tween®20
20X SSC	3 M NaCl, 300 mM sodium citrate (pH 7.0); DEPC-treated
Pre-Hybridization Mix	50% (v/v) formamide, 5x SSC, 0.1% (v/v) Tween®20; 10% (v/v) heparin
Hybridization Mix	Pre-Hybridization Mix + denatured and fragmentized (~500 bp) salmon sperm DNA (1 mg/ml); labeled probe (denatured for 10 min at 65 °C)
Wash Solution 1	50% (v/v) formamide, 2x SSC, 0.1% (v/v) Tween®20
Wash Solution 2	2x SSC, 0.1% (v/v) Tween®20
Wash Solution 3	0.2x SSC, 0.1% (v/v) Tween®20
AP Buffer	0.1 M Tris–HCl (pH9.5), 0.1 M NaCl, 50 mM $MgCl_2$ (freshly added), 0.1% (v/v) Tween®20
AP Staining Solution	AP Buffer + 2 mM levamisole, 110 μg/ml NBT and 90 μg/ml BCIP
Clearing Solution	2,5 g Chloral hydrate in 1 ml 30% (v/v) glycerol
TNT Buffer	0.1 M TRIS–HCl (pH 7.5), 0.15 M NaCl, 0.05% Tween®20
TSA Staining Solution:	Always prepare fresh: dilute TSA-Plus Stock Solution 1:50 in 1x Amplification Diluent (TSA-Plus-Cyanine3-Kit: PerkinElmer, cat. No. NEL744001KT)

2.2. Sample preparation and fixation

To achieve high sensitivity and low background, the used tissue should be as clean as possible. Tissue preparation has to be conducted as fast as possible without touching, for example, ovules directly. Any kind of sample drying, lesion or squeezing will lead to a strong background and unspecific staining. For sample preparation, carpels from unfertilized or fertilized pistils are removed using cannulas (Fig. 1A). The ovules which are still connected to the transmitting tract are transferred to sieves, placed in glass petri dishes filled with an emulsion of fixative:n-Heptan (1:1) and incubated on a shaker for 30–60 min. Penetration depth of the fixative can be increased by vacuum infiltration. Available nylon mesh-based sieves are not resistant to all liquids, which will be used during the experiments. To minimize tissue loss, we use a stainless steel mesh (mesh width 25 μm) glued to reaction tubes slices by heat. Afterwards, the tissue is dehydrated and the chlorophyll removed by incubation in methanol (3× for 5 min) followed by ethanol treatment (2× for 5 min). The fixed/dehydrated tissues can be stored at −20 °C for weeks.

2.3. Hydrogen peroxide-mediated degradation of endogenous peroxidase (only required for the HRPO/TSA-system)

Endogenous peroxidases oxidate and thus activate the dye-linked tyramides, which would lead to a high fluorescing background. To degrade these peroxidases, the tissue is incubated for 1 h in freshly prepared 3% (v/v) H_2O_2/methanol followed by two ethanol washing steps for 5 min each as described above.

2.4. Tissue permeabilization/dewaxing

Waxes and lipids have to be removed by incubation in a strong solvent like a HistoClear:ethanol (1:1) mixture for 30 min, followed by two ethanol washing steps for 5 min. HistoClear contains D-limonene; therefore only resistant plastic or baked glass ware should be used. Afterwards, a separation of the sieves into well plates is possible if required.