

Chapter 12

In situ Analysis of Gene Expression in Plants

Sinéad Drea, Paul Derbyshire, Rachil Koumproglou, Liam Dolan,
John H. Doonan, and Peter Shaw

Summary

In the post-genomic era, it is necessary to adapt methods for gene expression and functional analyses to more high-throughput levels of processing. mRNA in situ hybridization (ISH) remains a powerful tool for obtaining information regarding a gene's temporal and spatial expression pattern and can therefore be used as a starting point to define the function of a gene or a whole set of genes. We have deconstructed 'traditional' ISH techniques described for a range of organisms and developed protocols for ISH that adapt and integrate a degree of automation to standardized and shortened protocols. We have adapted this technique as a high-throughput means of gene expression analysis on wax-embedded plant tissues and also on whole-mount tissues. We have used wax-embedded wheat grains and *Arabidopsis* floral meristems and whole-mount *Arabidopsis* roots as test systems and show that it is capable of highly parallel processing.

Key words: High-throughput, Spatial patterns of gene expression, In situ hybridization.

1. Introduction

In situ hybridization (ISH) is one of the methods of choice for determining the spatial expression pattern of a given gene. High resolution protocols provide cellular and even subcellular resolution. One of the most significant advantages inherent in the technique is that it is applicable to any species whether or not these species are amenable to other methods of functional analyses such as stable transformation. For this reason it has proved to be invaluable in the evo-devo (evolution of development) field for instance where, in the absence of direct functional data in diverse species, it can provide detailed gene expression patterns across

the evolutionary spectrum that can be informative for studies of comparative development (1).

ISH effectively complements Northern blotting, RT-PCR (reverse transcriptase-polymerase chain reaction) and microarrays where the extraction of the RNA invariably results in the loss of spatial information. Microarrays allow many genes to be studied in parallel and are currently the most powerful tool to study gene expression. However, the microarray outputs need to be verified by independent methods, such as ISH (2, 3). To match the level of output, ISH must be made more efficient and less time-consuming. A number of variations on the traditional in situ protocols have been reported, including whole-mount ISH (4), in situ PCR (5, 6) and the use of vibratome sectioned tissues (7), but the main shortcoming of ISH is undoubtedly the low-throughput nature of the technique. Efforts to make the ISH technique into a highly parallel, systematic process have been successful in flies and primitive chordates (8–10). Attempts have been made to address this issue in plants using the whole-mount ISH (WISH) and in situ PCR techniques (11, 12). However, though the potential is noted, the actual throughput is undetermined.

The high-throughput protocols used in animal embryos involve whole-mount methods that are more feasible for these systems (8–10). The challenge in plants is the sheer size of the tissues required for analysis and this not only compromises the penetration of probe and hybridization but makes microscopic examination more difficult and therefore more time-consuming, though we have effectively used it as a means of gene expression in the small and more easily penetrable *Arabidopsis* root. The other option for cellular localization of transcripts is promoter fusions to reporter genes and subsequent transformation. This approach has recognized shortcomings as elements controlling a genes expression are know to be located not only in the traditional promoter region upstream of the coding region, but intergenically and at unconventional distances from the gene (13). The resources required for mass transformation and the fact that not all plant species are amenable limits the application of this approach to well-studied model species.

Two of the most significant developments in tissue and cell type-specific gene expression involve fluorescence-activated cell-sorting (FACS) and laser capture micro-dissection (LCM). These techniques overcome the limitations of non-specific manual tissue manipulation for RNA extraction and bring together the ability to isolate cell-specific material for use in genome-wide transcriptional profiling. The former method has been applied very elegantly to obtain what is a useful reference for gene expression patterns in *Arabidopsis* root cell types (14). However, the approach is dependent on the availability of transformable lines with cell specific GFP expression, on the protoplasting of plant

material and on the existence of microarray facilities for the species being analyzed. LCM allows the isolation of RNA samples from individual cell types (15) but requires specialized microscopy facilities and the RNA isolated needs to be amplified prior to its application on microarrays.

Sequence and statistics based methods such as SAGE (serial analysis of gene expression) and MPSS (massively parallel signature sequencing) and direct statistical profiling of EST (expressed sequence tags) are certainly very high-throughput in terms of scale and constitute useful reference databases; and MPSS has proven a particularly useful tool for the analysis of small RNAs (16–18). SAGE and MPSS rely on the matching of a short sequence to cognate genes in order to be identified and are therefore most useful for species with well-characterized genomes.

In this description of ISH on plant material, we will draw on three ISH projects conducted and/or underway in our group and which involve(d): gene expression analysis on early wheat grain development (19), gene expression patterns in Arabidopsis flower meristems (20, R. Koumproglou, unpublished) and finally in Arabidopsis roots. These projects involved the optimization of the protocol for different tissue types; different sources of probe templates; using both wax-sectioned (flower meristems and wheat grains) and whole-mount (roots) approaches; and, for the wax-sectioned material, using different automated slide processors. We begin each section with probe-making as this part of the protocol was virtually identical in each project.

2. Materials

2.1. Probe Making

1. A 10× stock of NTP-mix for in vitro transcription consisted of 1 µl each of ATP, CTP, GTP, 0.65 µl UTP (100 mM stocks from Roche), 3.5 µl of Dig-UTP (10 mM stock from Roche) and 2.85 µl sterile water for a 10 µl stock.
2. RNA polymerases (Roche or Promega) and used as recommended.
3. RNase inhibitor available from Roche or Promega and used as recommended.
4. QIAquick PCR purification kit (QIAGEN) used according to the manufacturer's instructions.
5. Montage Clean-up Kit (Millipore) was used for PCR purification in 96-well format.
6. 200 mM Carbonate buffer, pH 10.2 (80 mM NaHCO₃, 120 mM Na₂CO₃) for hydrolysis of probes.
7. Nitrocellulose (Amersham).

2.2. Plant Tissue Preparation

2.2.1. Whole-Mount Seedling Tissue

1. Vortex bleach (Procter & Gamble Ltd)
2. Parafilm[®] laboratory film (Pechiney Plastic Packaging, Menasha, USA)
3. Standard growth medium contained 1× Murashige and Skoog (MS) basal salts (micro and macro elements) (Duchefa), 1% (w/v) sucrose and 0.5% (w/v) Phytigel[™] dissolved in de-ionized water with pH adjusted to 5.7 with KOH, followed by autoclaving for 20 min. Medium was cooled to 50–60°C and ~20 ml poured 9 cm petri plates (Bibby Sterilin Ltd) and allowed to solidify.
4. Paraformaldehyde (Sigma) 4% (w/v) solution in PBS (1.3 M NaCl, 70 mM Na₂HPO₄, 30 mM NaH₂PO₄, pH 7 – made up as 10× stock and diluted in sterile water before use) and prepared fresh for each use.
5. Alternative fixative, FAA: 3.7% formaldehyde (a 37% stock solution is available from Sigma), 50% ethanol and 5% acetic acid.

2.3. Wax-Embedded Tissue

6. Tissue-Tek[®] Vacuum Infiltration Processor (Sakura; distributed by Bayer, UK) for processing material before embedding.
7. Sectioning of wax-embedded material was done using a Leica Microtome (RM2125RT). Silicone isolators used for precise positioning of sections on slides were obtained from Grace Biolabs. For post-situ embedding and sectioning of root material we used Technovit 7100[®] (Kulzer GmbH, Germany) resin and an Ultracut-E microtome (Reichert-Jung, Austria) with a glass knife.

2.4. Pre-treatment, Hybridization, Washing and Staining of Slides/Tissues

1. Automated ISH on wax-sectioned tissue was performed using the VP2000 (Vysis) and the InSituPro (Intavis) slide processors.
2. Buffers used in pre-treatment of slides prior to hybridization (*see Note 1*): PBS (diluted from a 10× stock solution containing 1.3 M NaCl, 70 mM Na₂HPO₄, 30 mM NaH₂PO₄, pH 7).
3. Proteinase K (Roche) was made up as a 25 mg/ml stock in sterile water and used at 2–3 or 10 µg/ml in Tris buffer (100 mM Tris–HCl, 50 mM EDTA, pH 7.5).
4. Acetic anhydride (Sigma) was used at 0.5% in 0.1 M triethanolamine (Sigma).
5. Glycine was used at 0.2% in PBS.
6. Hybridization solution (HS) (Salts [300 mM NaCl, 10 mM Tris–HCl pH 6.8, 10 mM NaPO₄, 5 mM EDTA] 50% deionized formamide, 5% dextran sulphate, 0.5 mg/ml tRNA, 1× Denhardts, 0.1 mg/ml Salmon testis DNA) and maintained stably at –20°C until hybridization.

7. Hybridization chambers were obtained from Grace Biolabs.
8. Solutions used for washing prior to staining: 2× SSC and 1× SSC (20× SSC stock: 3 M NaCl, 0.3 M NaCitrata) made up in 50% formamide.
9. TBS (10 mM Tris-HCl, 250 mM NaCl, pH 7 – made up as 10× stock and diluted in water before use).
10. AP-buffer (100 mM Tris-HCl, 100 mM NaCl pH 9.5; 50 mM MgCl₂).
11. NBT (0.1 mg/ml) and BCIP (0.075 mg/ml) from Promega.
12. Anti-digoxigenin-alkaline phosphatase (anti-Dig-AP) antibody and blocking reagent (Roche).
13. Ethanol (diluted in water if required).
14. Triton or Tween surfactants (Sigma).
15. Calcofluor (Fluorescent Brightener 28 from Sigma) used at 0.1% in water.
16. Entellen (Merck).

2.5. Microscopy

1. A Nikon E800 microscope using a digital camera under brightfield conditions for wheat sections and with UV filter for the calcofluor-counterstained *Arabidopsis* sections.
2. A Nikon Coolpix 950 digital camera attached to a Leica WILD M10 binocular microscope was used to capture low magnification images of roots after ISH. White light from above and white paper underneath the plates improved the signal contrast.

3. Methods

3.1. Probe Making

1. Primers are designed in order to append a T7 RNAP site to the 3' end of the gene sequence to be labelled (*see Note 2*). We use a standard PCR cycle, for example, 94°C 3 min, then 30 cycles of 94°C 45 s, 63°C 45s and 72°C 1.5 min, final extension of 72°C for 6 min. For 96-well plates PCR-product purification was done using the Montage Clean-up Kit (Millipore). Individual PCR templates can be cleaned using the available commercial kits, for example, from Qiagen.
2. In vitro transcription was performed with ~500 ng of PCR template in 10 µl reactions for 2 h at 37°C in the presence of Dig-UTP nucleotides (*see Note 3*).
3. Hydrolysis was carried out immediately in 100 mM carbonate buffer pH 10.2 at 60°C for a standard 30 min (*see Note 4*),

and products precipitated in 2.5 M ammonium acetate and 3 volumes absolute ethanol for 1 h at 4°C.

4. Plates were centrifuged at $\sim 2,300\times g$ for 30 min (or tubes for 10 min at $\sim 7,400\times g$ in a microfuge at 4°C) and pellets resuspended in 30 μ l TE (10 mM Tris-HCl, 1 mM EDTA) buffer.
5. Dilutions (100 times) were made in water and 1 μ l of each spotted on nitrocellulose for dot-blot: 30 min in blocking solution (Sigma), 30 min in anti-Dig-AP, 5 min wash in TBS, 5 min in AP-buffer and developed as described above until signal was sufficient (*see Note 5*). All probes were then diluted 100 times in HS and maintained stably at -20°C until hybridization. Probes diluted in HS were denatured for 2 min at 85°C before application to slides or seedlings (*see Note 6*).

3.2. Plant Tissue Preparation for In situ Hybridization

3.2.1. Whole-Mount Tissues

1. Seeds of *Arabidopsis thaliana* L. Heynh, ecotype Columbia-0 (Col-0) were sterilized in 5% (v/v) bleach for 5 min, and washed $\times 3$ in sterile distilled water (sdH₂O).
2. Seeds were dropped individually onto the surface of the growing medium in horizontal lines at a density of 5–10 seeds per centimetre.
3. Plates were then sealed with Parafilm were placed in darkness at 4°C for 48 h to stimulate and synchronize germination.
4. Following cold treatment, plates were transferred to a growth room maintained at 25°C and incubated in a near vertical position, under fluorescent lamps emitting $\sim 70 \mu\text{mol}/\text{m}^2/\text{s}$ in a continuous white light regime.

3.2.2. Tissues for Wax-Sectioning

1. For *Arabidopsis* flower meristems we used ecotype Columbia grown under long day conditions in the greenhouse because this produced larger meristems (and therefore more sections containing the central meristematic zone) than other conditions that were tested. Wheat plants (variety Savannah) were grown under controlled environment conditions (16°C, 16 h light) and ears tagged daily at anthesis.
2. Wheat grains harvested at 3, 6 and 9 days after anthesis (DAA) were trimmed and *Arabidopsis* floral meristems were removed just after bolting. All tissues were fixed in paraformaldehyde or FAA (6 h 35°C in the Tissue-Tek Vacuum Infiltration Processor – VIP).
3. Tissue-Tek VIP cycle further included the following steps: 70% ethanol 1 h 35°C, 80% ethanol 1.5 h 35°C, 90% ethanol 2 h 35°C, 100% ethanol 1 h 35°C, 100% ethanol 1.5 h 35°C (repeat 2 h), xylene 0.5 h 35°C (repeat 1 h and again 1.5 h), wax 1 h 60°C (repeat same then for 2 h twice). All steps are

performed under vacuum and the plant tissue is contained in plastic cassettes (also Tissue Tek).

4. Cages containing the samples were then transferred to the Tissue-Tek Embedding Console and embedded in the desired orientation – we used longitudinal sections for flower meristems and transverse sections for wheat grains.
5. 14 μm sections were found to be most suitable for wheat grains but a standard 8 μm was used for Arabidopsis tissues. Sections were allowed to dry onto slides overnight at 42°C (*see Note 7*). For wheat grains and for use with the VP2000 slide processor, the arrangement and number of tissue sections on the slide was made uniform using adherent, but removable, silicone isolators. This allowed the parallel screening of multiple probes on the same slide containing up to eight sections, each section in an isolated well. For using the Intavis Processor Arabidopsis meristems were sectioned right through and all sections from one meristem positioned on one slide (~30 sections).

3.3. Pre-treatment, Hybridization, Washing and Staining of Slides/Tissues

3.3.1. For Whole-Mount Tissues

The description of this method and an example of results obtained is summarized by the schematic in **Fig. 1**.

1. 4-day-old seedlings were fixed in 4% paraformaldehyde while still on MS/agar plates by applying a weak vacuum to ensure penetration of the fixative.
2. The seedlings were then transferred with tweezers in clusters of 40–50 into Tissue-Tek mess biopsy cassettes (Sakura). A brief vacuum infiltration was applied with each change of the following solutions, to submerge the cassettes:
3. Dehydration for 1 h each in 30, 65, 100, 65 and 30% (v/v) ethanol; PBS 30 min, acetic anhydride/TEA 30 min; PBS $\times 2$ 15 min each.
4. Seedlings (10–12) were tweezer-transferred from cassettes into 1.5 ml microfuge tubes containing 100 μl probe-HS and incubated at 50°C 16 h.
5. Following the hybridization reaction, seedlings were tweezer-transferred into a 48 well mesh-bottom plate (1 reaction per well), covered with a lid and the plate placed in a plastic box [100 mm (w) \times 200 mm (l) \times 50 mm (h)] containing 100 ml of appropriate washing solution. Material was subjected to three washes in 2 \times SSC/50% (v/v) formamide and one wash in 1 \times SSC/50% (v/v) formamide 30 min each at 50°C; 1 \times SSC 5 min and PBS 10 min at room temperature (r.t.).
6. Material was prepared for antibody labelling by washing in TBS for 10 min; TBS + 0.5% (w/v) blocking reagent 1 h, and TBS/1% (w/v) BSA/0.3% (v/v) Triton X-100 1 h.

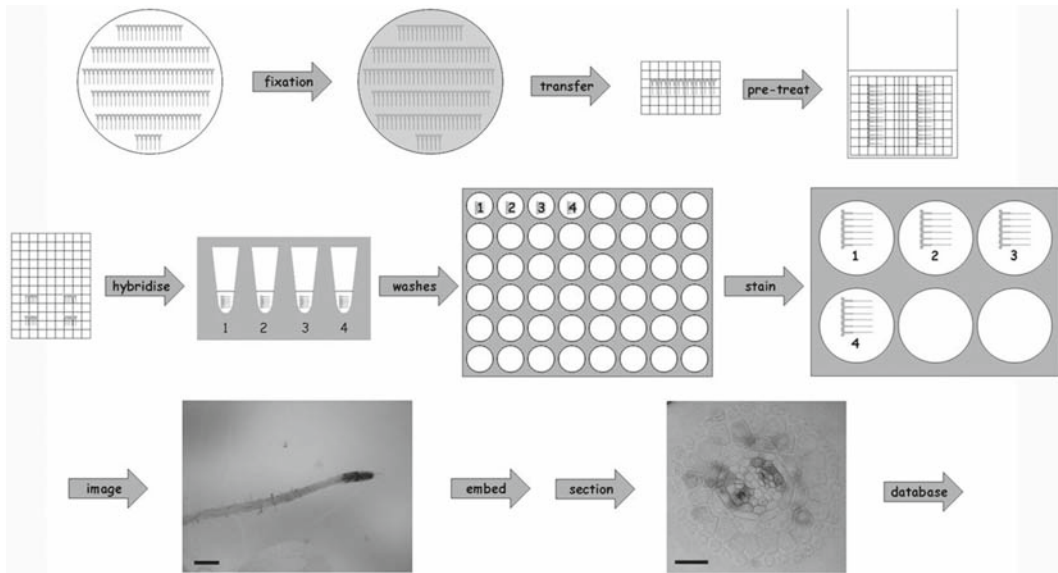


Fig. 1. Flow diagram describing whole-mount in situ hybridization on *Arabidopsis* roots (**Subheading 3.3.1**). Seedlings are fixed in plates and transferred into mesh biopsy cassettes followed by pre-treatment washes in a beaker. Groups of seedlings are then transferred into microfuge tubes and incubated in probe/hybridization solution overnight. Groups of seedlings are placed into individual wells of a mesh bottom 48-well plate and subjected to post-hybridization washes, then collected into separate wells of a 6 well plate and stained. Low magnification images are collected to show general spatial expression patterns, and selected roots embedded in resin and sectioned in the zones of expression, giving cell-specific resolution. All images are then collected into a database. Images of results using a probe for *Histone4* are shown. Scale bars; root whole-mount = 300 μm , section = 25 μm .

7. Anti-Dig-AP was diluted (1/3000) in the TBS/BSA/Triton buffer and used for seedling incubation at r.t. (1 h) then 4°C 16 h.
8. Seedlings were given three 20 min washes at r.t. in the same buffer (without antibody), followed by one wash in TBS for 20 min, and one wash in AP-Buffer 10 min.
9. Seedlings were tweezer-transferred into six well plates and colour detection with NBT/BCIP was carried out in complete darkness for 2–4 h and then stopped in water. Expression profiles were broadly separated into four categories; absent (-), weak (+), moderate (++) and strong (+++).

3.3.2. Using the VP2000 and Multiple Probes per Slide

The use of corresponding isolators and chambers for section organization and hybridization is shown in **Fig. 2**. This arrangement was used to maximize efficiency and economy when working with probes in 96-well format and has been described (20).

1. Silicone isolators were removed from the slides when dry and the slides loaded in the slide rack for the VP2000 processor. The rack-capacity is 50 slides.
2. The slides are put through the following program: xylene (*see Note 8*) 20 min \times 2, (with agitation for final minute of

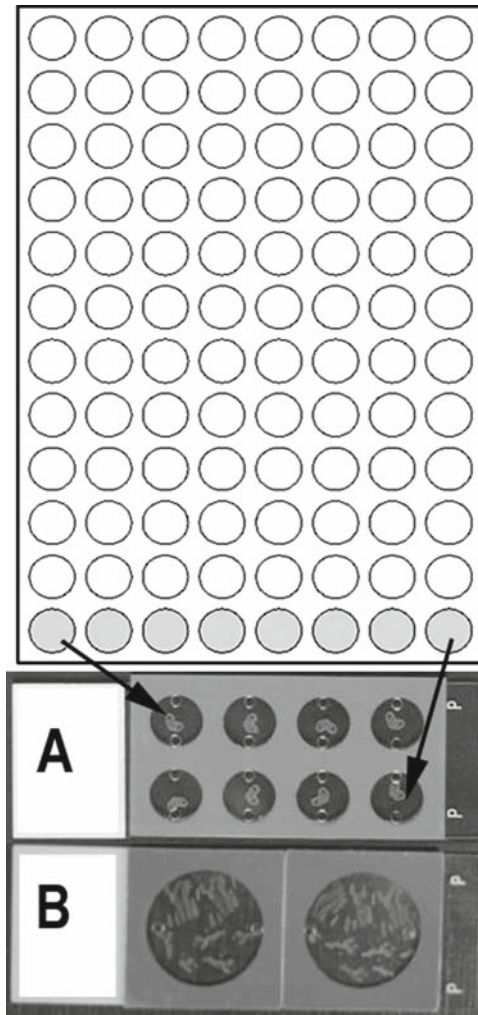


Fig. 2. Silicone isolators and hybridization chambers as used to arrange and hybridize various probes to wax-sectioned wheat grains when used in conjunction with a 96-well probe preparation format, as described in **Subheading 3.3.2.** and in Drea et al. (20) (A). Hybridization chambers applied to slides and probes added from 96-well plate (B). Alternative format using larger hybridization chambers.

second treatment); 100% ethanol 10 min (with agitation for final minute), then through a 95%, 85%, 50%, 30% ethanol series for 2 min each (*see Note 9*); PBS 3–4 min $\times 2$; Proteinase K 30 min at 37°C; glycine 2 min; PBS 3–4 min; acetic anhydride 10 min (with agitation); PBS 3–4 min, then back through the ethanol series. Slides were completely dry at this stage and ready for hybridization.

3. Hybridization chambers were applied securely to the slides (after pre-treatment) and probes (diluted in HS) were applied

to one well (two sections) for the three stages individually. Coverslips were placed on the chambers to prevent evaporation and hybridization was performed overnight in a 50°C incubator.

4. Chambers are removed and slides arranged in the VP2000 for washing program: 15 min in 2× SSC/50% formamide (*see Note 10*) at 40°C, 40 min in same at 50°C, 20 min in 1× SSC/50% formamide at 50°C (all steps with constant agitation), 5 min in 1× SSC at room temperature, 5 min in TBS at room temp (*see Note 11*).
5. Then slides are transferred into trays/boxes [eight slides fit in a box 100 mm (w) × 200 mm (l) × 50 mm (h)] for staining: 1% blocking solution in TBS 1 h, TBS containing 1/3,000 dilution of anti-Dig-AP and 0.05% Tween-20 for 1 h, 4× 10 min washes in TBS, 5 min in AP-buffer.
6. Develop in AP-buffer containing NBT and BCIP (*see Note 12*). Slides were then washed several times in water to stop the reaction followed by sequential washes in 70% and 100% ethanol to remove excess stain (the duration of the ethanol washes depends on the level of colour development and should be monitored by eye). Slide and then allowed to dry and permanently mounted in Entellan.

3.3.3. Using the Intavis InSituPro

Using the Intavis Processor allows automation of the protocol from hydration after de-waxing to signal-detection stage:

1. Slides were de-waxed in xylene manually before loading in the processor (the capacity is 60 slides) for the following program: 5 min in 100% ethanol ×4; 2 min each ×2 in 95%, 85%, 50%, 30% ethanol; 5 min ×2 in PBS; 15 min in Proteinase K (10 µg/ml); 5 min ×2 in glycine; 5 min ×2 in PBS; 20 min in 4% paraformaldehyde (*see Note 13*); 5 min ×2 PBS; 14 h hybridization at 50°C; 10 min ×10 in 2× SSC/50% formamide at 50°C; 5 min in 2× SSC/50% formamide at 37°C; 10 min ×2 in 1× SSC; 5 min in PBS; 5 min ×2 in TBS; 30 min ×2 in blocking buffer; 1 h in anti-Dig-AP antibody (1/3,000 in 1× TBS, 1% BSA, 0.3% Triton); 10 min ×10 in TBS.
2. Slides were transferred to boxes and processed as in **Sub-heading 3.3.2.** (step 5 and 6).

3.4. Microscopy

3.4.1. For Whole-Mount ISH

Low magnification images of root in situ were captured with a dissecting microscope and attached digital camera. To get detailed image data in cross section, samples were embedded in plastic resin and sectioned with an ultramicrotome (*see Note 14*). Images of these sections were captured with a digital camera attached to a light microscope using DIC optics.

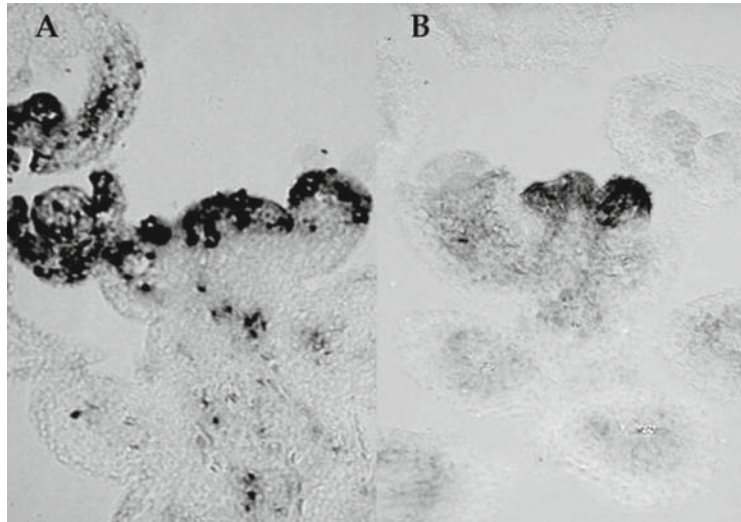


Fig. 3. Results of ISH on wax-sectioned Arabidopsis flowers using the Intavis Pro (**Sub-heading 3.4.2.**) (A). *HistoneH4* expression in floral meristem (B). *STM* expression in floral meristem.

3.4.2. For Wax-Sectioned ISH

Images of the sections were captured on a digital camera attached to a light microscope with fluorescence for slides counterstained with calcofluor. An example of the results obtained is shown in **Fig. 3**.

3.5. Data Processing

In our experience, the rate limiting steps in conducting in situs on a high-throughput scale are the image capture and data-processing stages. Arranging sections in a reproducible order on the slides makes manual image capture more routine as the same conditions and settings can be used for all samples. In other fields, there are more advanced attempts to automate and computerize the image capture and interpretation of expression patterns (21–23). For wheat grain work we recorded all details of probes (sequences, slide positions, folders where corresponding images were stored, etc.) in excel spreadsheets and used these to construct a database of the results (19). Gene expression studies in other systems have also produced web-accessible and searchable databases (8–10).

Notes

1. We did not find it necessary to use DEPC (diethylpyrocarbonate; Sigma)-treated water for pre-treatment of slides or probe making. We used fresh sterile water for dilutions and

autoclaved all buffers and solutions (for procedures prior to, and during, hybridization when RNase-precautions are most important). The buffers and solutions were set aside exclusively for in situ work so as to minimize chance of contamination. Likewise, we do not pretreat all the boxes/dishes by baking for each experiment but set aside a set of apparatus for RNA work exclusively.

2. T3 RNAP can also be used for transcription by appending the appropriate recognition site, but we do not recommend SP6 RNAP, especially for large numbers of probes, as we have found it to be less efficient and less reliable. In many protocols, sense probes (transcribed from the 5' end) are used as negative controls but when conducting many in situs simultaneously we usually include just one or few sense probes as negative controls. Gene sequences may be amplified from genomic (exon regions) or cDNA directly but each gene will require individually-designed primers. We usually begin with many genes inserted in a common vector, for example, cDNA library (19) or the SALK cDNA collection for Arabidopsis etc. This allows one to design a set of common primers targeted to the surrounding vector sequence and amplify templates for all genes simultaneously. With regard to probe specificity: UTR sequences are often used as templates so as to minimize the chance of cross-hybridization between similar genes. If using cDNA library clones as templates, since these are often made using polyadenylated tails as anchors, they will already contain 3'UTRs and should therefore be specific for the gene in question (19).
3. We usually check 0.5 μ l of the transcription reaction on a 1% agarose gel (run at 50–70 V) to determine if transcription proceeded efficiently – the single-strand RNA should run as a smaller band below the double-stranded DNA template.
4. There is a formula to determine the amount of time required to hydrolyze the RNA to the desired size: $t = (L_i - L_f) / K \times L_i \times L_f$, where t = time in minutes, K = rate constant (=0.11 kb/min), L_i = initial length (kb) and L_f = final length. When making probes in 96-well format we used a standard 30 min for all probes. Some labs have found that it is not essential to reduce the size of the probe by hydrolysis and have obtained stronger signals with longer probes.
5. We use the dot-blot as a means of qualitatively determining the success of Dig-labelling rather than as a means of quantification.
6. Most protocols require separate denaturation of the probe in 50% deionized formamide prior to dilution in HS but we have found it unnecessary.

7. Many labs recommend processing wax sections immediately after they are adhered to the slides. When dried onto slides, we have routinely stored the slides covered in a box at r.t. or at 4°C for days or weeks prior to dewaxing, pre-treatment and hybridization.
8. There are alternatives to xylene (often less toxic and more easily to dispose of safely) for dewaxing slides such as Histo-clear (Raymond Lamb Inc., UK) and CitriSolv (Fisher Scientific).
9. It is not always necessary to use a very elaborate ethanol dehydration sequence and a broader series can be designed as long as it does not affect the quality of the tissue.
10. It is possible to use a simple 0.2× SSC solution for washing slides also and the use of formamide can be avoided if desired.
11. We found that RNase treatment during washing steps did not significantly affect the results of the in situs.
12. We recommend monitoring the development of slides (when using colour-based detection systems) by eye or using a dissecting microscope and always using a well-characterized positive control (like HistoneH4) as an indication of how efficiently the experiment proceeded. As in all high-throughput techniques, there may be false negatives under standardized conditions. For instance, when working on individual or small numbers of probes we have found that using a larger template (>500 bp) may produce a stronger signal (*see* **Notes 2 and 4**)
13. Proteinase K treatment can sometimes result in some tissue damage and sometimes it is necessary to include a re-fixation step in the pre-treatment protocol.
14. Because of the delicate nature of the Arabidopsis root, we have found that embedding in a plastic resin preserves the histology of the layers optimally.

References

1. Kramer, E.M. and Irish, V.F. (1999) Evolution of genetic mechanisms controlling petal development. *Nature* 399, 144–148.
2. Chuaqui, R.F., Bonner, R.F., Best, C.J., Gillespie, J.W., Flaig, M.J., Hewitt, S.M., Phillips, J.L., Krizman, D.B., Tangrea, M.A., Ahram, M., Linehan, W.M., Knezevic, V., and Emmert-Buck, M.R. (2004) Post-analysis follow-up and validation of microarray experiments. *Nat. Genetics* 32, 509–514.
3. Wellmer, F., Riechmann, J.L., Alves-Ferreira, M., and Meyerowitz, E.M. (2004) Genome-wide analysis of spatial gene expression in Arabidopsis flowers. *Plant Cell* 16, 1314–1326.
4. de Almeida Engler, J., Van Montagu, M., and Engler, G. (1998) Whole-mount in situ hybridization in plants. *Methods Mol. Biol.* 82, 373–384.
5. Johansen, B. (1997) In Situ PCR on Plant Material with Sub-cellular Resolution. *Ann. Bot.* 80, 697–700.

6. Pesquet, E., Barbier, O., Ranocha, P., Jauneau, A., and Goffner, D. (2004) Multiple gene detection by in situ RT-PCR in isolated plant cells and tissues. *Plant J.* 39, 947–959.
7. Borlido, J., Pereira, S., Ferreira, R., Coelho, N., Duarte, P., and Pissarra, J. (2002) Simple and Fast In Situ Hybridization. *Plant Mol. Biol. Rep.* 20, 219–229.
8. Tomancak, P., Beaton, A., Weiszmann, R., Kwan, E., Shu, S., Lewis, S.E., Richards, S., Ashburner, M., Hartenstein, V., Celniker, S.E., and Rubin, G.M. (2002) Systematic determination of patterns of gene expression during *Drosophila* embryogenesis. *Genome Biol.* 3, RESEARCH0088-8.
9. Satou, Y., Takatori, N., Fujiwara, S., Nishikata, T., Saiga, H., Kusakabe, T., Shin-i, T., Kohara, Y., and Satoh, N. (2002) *Ciona intestinalis* cDNA projects: expressed sequence tag analyses and gene expression profiles during embryogenesis. *Gene* 287, 83–96.
10. Quiring, R., Wittbrodt, B., Henrich, T., Ramialison, M., Burgtorf, C., Lehrach, H., and Wittbrodt, J. (2004) Large-scale expression screening by automated whole-mount in situ hybridization. *Mech. Dev.* 121, 971–976.
11. Koltai, H. and McKenzie Bird, D. (2000) High throughput cellular localization of specific plant mRNAs by liquid-phase in situ reverse transcription-polymerase chain reaction of tissue sections. *Plant Physiol.* 123, 1203–1212.
12. Friml, J., Benkova, E., Mayer, U., Palme, K., and Muster, G. (2003) Automated whole mount localisation techniques for plant seedlings. *Plant J.* 34, 115–124.
13. Taylor, C. (1997) Promoter fusion analysis: an insufficient measure of gene expression. *Plant Cell* 9, 273–275.
14. Birnbaum, K., Shasha, D.E., Wang, J.Y., Jung, J.W., Lambert, G.M., Galbraith, D.W., and Benfey, P.N. (2003) A gene expression map of the *Arabidopsis* root. *Science* 302, 1956–1960.
15. Kerk, N.M., Ceserani, T., Tausta, S.L., Sussex, I.M., and Nelson, T.M. (2003) Laser capture microdissection of cells from plant tissues. *Plant Physiol.* 132, 27–35.
16. Oghihara, Y., Mochida, K., Nemoto, Y., Murai, K., Yamazaki, Y., Shin, I.T., and Kohara, Y. (2003) Correlated clustering and virtual display of gene expression patterns in the wheat life cycle by large-scale statistical analyses of expressed sequence tags. *Plant J.* 33, 1001–1011.
17. Gowda, M., Jantasuriyarat, C., Dean, R.A., and Wang, G.L. (2004) Robust-LongSAGE (RL-SAGE): a substantially improved LongSAGE method for gene discovery and transcriptome analysis. *Plant Physiol.* 134, 890–897.
18. Nakano, M., Nobuta, K., Vemaraju, K., Tej, S.S., Skogen, J.W., and Meyers, B.C. (2006) Plant MPSS databases: signature-based transcriptional resources for analyses of mRNA and small RNA. *Nucleic Acids Res.* 34, D731–D735.
19. Drea, S., Leader, D.J., Arnold, B.C., Shaw, P., Dolan, L., and Doonan, J.H. (2005) Systematic spatial analysis of gene expression during wheat caryopsis development. *Plant Cell* 17, 2172–2185.
20. Drea, S., Corsar, J., Crawford, B., Shaw, P., Dolan, L., and Doonan, J.H. (2005) A streamlined method for systematic, high resolution in situ analysis of mRNA distribution in plants. *Plant Methods* 1, 8.
21. Camp, R.L., Chung, G.G., and Rimm, D.L. (2002) Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat. Med.* 8, 1323–1327.
22. Brey, E.M., Lalani, Z., Johnston, C., Wong, M., McIntire, L.V., Duke, P.J., and Patrick, C.W., Jr. (2003) Automated selection of DAB-labeled tissue for immunohistochemical quantification. *J. Histochem. Cytochem.* 51, 575–584.
23. Fernandez, D.C., Bhargava, R., Hewitt, S.M., and Levin, I.W. (2005) Infrared spectroscopic imaging for histopathologic recognition. *Nat. Biotechnol.* 23, 469–474.