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Multi-probe *in situ* hybridization to whole mount *Arabidopsis* seedlings

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ABSTRACT ***In situ*** **RNA-RNA hybridization (ISH) is a molecular method for localization of gene transcripts at the cellular level and is widely used to provide spatial and temporal information regarding gene expression. However, standard protocols are complex and laborious to implement, restricting analysis to one or a few genes at any one time, each one observed on separate ISH preparations. Multi-probe whole-mount *in situ* hybridization is a powerful technique to compare the expression patterns of two or more genes simultaneously in the same tissue or organ. We describe for the first time in plants, the detection of three different mRNAs in a single fixed whole mount *Arabidopsis* seedling. A combination of bright fluorescent secondary antibodies was used for the detection of riboprobes differentially labeled by digoxigenin, biotin and fluorescein. The 3-D detection of each of the multiple fluorescent hybridization signals or in combination was obtained through confocal laser-scanning microscopy. The reliability of the method was tested in the root, using the *PINFORMED (PIN)* genes with non-overlapping temporal and spatial expression patterns. In the shoot, a class-I *KNOTTED* -like homeobox gene from *Arabidopsis* (*KNAT1)* with expression restricted to the shoot apical meristem was used in combination with *ELONGATOR3 (ELO3)* gene. In addition, the expression patterns of ELONGATOR complex gene (*ELO2, ELO3)* and *HISTONE MONOUBIQUITINATION1 (HUB1)* genes were analyzed in both shoot and root and a partial overlapping was observed. The whole procedure takes only 6 days.**

KEY WORDS: *whole mount, in situ hybridization, confocal microscopy, Arabidopsis thaliana*

Introduction

In multicellular organisms, the detection of the temporal and spatial expression of genes provides information on the putative sites of activity of their encoded proteins and provides novel insight into gene function in the processes of growth and development. In addition, marker gene expression is very helpful for tissue typing in mutants.

In situ RNA-RNA hybridization (ISH) is a powerful technique that enables the localization of gene transcripts at the cellular level. Riboprobes are synthesized, labeled and hybridized to mRNA derived from complementary genes, and visualized with different approaches depending on probe labeling. The ISH technique is complementary to Northern blotting and RT-PCR (reverse transcriptase-polymerase chain reaction) in which the RNA extraction procedure invariably results in the loss of spatial information. ISH also complements DNA microarrays, a genome-wide expression profiling technique, that is generally used at seedling or organ level (Chuaqui *et al.* 2004; Wellmer *et al.* 2004). Microarray resolution at the cellular level is also possible if combined with sorting of fluorescent cells derived from transgenic reporter lines. However, it requires advanced equipment and is expensive (Birnbaum *et al.* 2005). Spatial gene expression is also investigated through the analysis of promoter-reporter gene fusion expression. However, a limitation to this technique is that it

Abbreviations used in this paper: ELO, ELONGATOR gene; HUB, HISTONE MONOUBIQUITINATION gene; ISH, in situ hybridization; PIN, PINFORMED gene.

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enables the measurement of gene promoter activity, which does not necessarily correspond to the *in vivo* expression of the investigated gene (Taylor 1997). Indeed, regulatory elements that are usually located in the promoter and are responsible for cell-specificity or inducibility, sometimes are located in introns or coding parts of the gene. Other limitations are: i) the long half-life of their products which prevent monitoring of rapid changes in the transcription; ii) the diffusion of reporter gene products to neighboring cells resulting in artifacts (Drews *et al.*1992); iii) the necessity to develop transgenic lines, which is time–consuming, expensive and requires specific expertise.

On this basis, ISH is an essential technique to investigate gene expression at a cellular level. Methods for ISH, first developed in the 1980s, used radioactively labeled antisense RNA probes to detect expression of genes on histological sections prepared from wax embedded tissues and processed according to autoradiographic techniques (Harrison *et al.*1974). Improvements that led to safer and more accurate assays involved: i) the introduction of hapten-labeled probes that allowed the use of immunohistochemical procedures for probe detection; ii) the use of fluorophore-labeled antibodies for the detection of nucleic acids and iii) the direct (i.e. without antibodies) hybridization of fluorescently labeled nucleic acids which resolved the problems related to antibody detection through enzymatic assay *per se* (Bauman *et al.* 1980). The ISH methodologies associated with it have undergone continuous refinement (Levsky and Singer 2003). ISH performed on sections has been widely used in many model organisms, but it is time-consuming and requires much expertise.

Since the 1990s, whole mount *in situ* technology has eliminated the need for embedding procedures and has made the analysis of gene expression patterns rapid (Hejátko *et al.* 2006; Piette *et al.* 2008; Traas 2008; Vize *et al.* 2009). Originally, the whole mount *in situ* technology was introduced and optimized for transcript localization in animals (Kosman *et al.* 2004). In plants, the challenge was to overcome problems imposed by the cell wall, which can limit efficient penetration of the probe and hence hamper the hybridization outcome. Recently, whole mount *in situ* hybridization methods were optimized and work effectively in plants, using only one digoxigenin-labeled probe for a single whole mount (Hejátko *et al.* 2006; Traas 2008). However, there is a need for the simultaneous visualization of transcripts of several genes to discern whether they act in the same or different domains or tissue during plant development.

In 2004, a multiplex *in situ* hybridization method was implemented to detect simultaneously, a large number of different fluorescently labeled antisense RNA probes in a single whole-mount *Drosophila* embryo (Kosman *et al.* 2004). In particular, this technique enabled, at high resolution, complete visualization and temporal expression of two to seven genes during embryo development in normal conditions and upon experimental treatment and genetic manipulation (Kosman *et al.* 2004). Recently, it was successfully adapted to mouse and *Xenopus* embryos (Piette *et al.* 2008; Vize *et al.* 2009). Hitherto, this has not been achieved in plant whole mounts.

Our aim was to apply multi-probe mRNA *in situ* hybridization, for the first time, to *Arabidopsis* seedlings. In particular, we used different labeled probes, detected through fluorescent antibodies, to investigate simultaneously the spatial expression of three different genes in 5–8 day old whole mount seedlings. Whole mount multi-probe ISH was combined with confocal laser-scanning microscopy, in order to obtain maximal 3D information on gene expression in whole tissues such as the primary root, hypocotyls and shoot apex. Merging of the fluorescent signals allowed us to determine whether genes were expressed in the same or in different cells, tissues or domains. Here we present a detailed protocol, recommended controls and troubleshooting advice. The method is verified in the root using *PIN* genes with complementary expression, and in the shoot using *KNAT1* that exhibits spatial expression restricted to the shoot apical meristem (SAM). Novel expression information is presented for the *ELO* and *HUB1* genes.

Experimental Protocols

Plant material fixation and dehydration

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Landsberg erecta (Ler) were surface sterilized by incubation in 100% ethanol for 2 min and 1.75% hypochlorite solution (NaClO) for 12 min. After thorough washing with sterile distilled water (3 X 5 min), the seeds were sown on Petri dishes containing germination medium (GS), 1% sucrose (Valvekens *et al.* 1988) and 0.7% plant cell culture agar (Sigma-Aldrich). The plated seeds were left at 4ºC for 48 h to ensure uniform germination, and then moved to a growth chamber at 21ºC, under 16 h (150 μmol m^{-2} s^{-1}) light and 8 h dark and 60% relative humidity. Five-to eight day-old seedlings were fixed in 15 ml Falcon tubes containing the fixative solution consisting of 4% (w/v) paraformaldehyde, in 1X PBS (10X PBS: 1.3 M NaCl, 70 mM Na_2HPO_4* $2H_2O$ and 30 mM KH_2PO_4, pH to 7.4 with 1 M KCl), under vacuum until the samples were drawn to the bottom of the tube (~ 20 min). Samples were then transferred to plastic tubes or glass vials containing fresh fixative. Each vial was capped and taped on its side to an orbital platform shaker, and shook gently for 1 h at 60-80 r.p.m at 4ºC. The fixative was then drained out, and in order to remove chlorophyll, samples were washed in methanol 2 X 5 min and 3 X 5 min in 100% ethanol while gently shaking, at 4°C Material was stored in 100% ethanol overnight at -20°C.

Synthesizing labeled RNA probe

Short and gene-specific fragments (GSTs) of *ELO2* (At5g13680), *ELO3* (At5g50320), *HUB1* (At2g44950), *PIN1* (At1g73590) *PIN4* (At2g01420); were cloned in the pGEM-TEasy vector (Promega). The following primer sequences
ELO2 FW 5'- GAAAGCGAGAGCTGAAGTCG-3' and
BW 5'- AGGCATCTGAATCTCGTGCT-3';
ELO3 FW 5'-TGAAGATACACGCCAGGACA-3' and
BW 5'-CACCAGAAATCACACCGATT-3';
HUB1 FW 5'- CATGCTCAGCAAGTTTTGGA-3' and
BW 5'- TCCCAAAATGGTTCCTCAAA-3';
PIN1 FW 5'- TCGAATCTAACCAACGCTGA-3' and
BW 5'- TCTTCCGTTTCCGTCTTGTC-3';
PIN4 FW 5'-CGAATCTTACCGGAGCTGAG and
BW 5'- GAAGCTCCTTAGCGTCATGG-3'; were used in a PCR reaction to amplify a GST. Plasmids containing DNA templates were linearized by *Spe*I and *Nco*I endonucleases.

Labeled RNA probes were synthesized using *in vitro* transcription in the presence of Digoxigenin-11-UTP, Biotin-16-UTP or Fluoroscein-12-UTP by RNA polymerase T7 or SP6 (DIG, Biotin, FITC RNA labeling Mix, Roche).

KNAT1 probe (At4g08150): PCR-based generation of template for RNA probe synthesis using KNAT1 all FW 5'-CAACAGCACCACTCCTCAAA-3' and KNAT1 all BW 5'-TTGTAATGCAACTCCCACCA-3'; T7 and SP6 RNA polymerase promoter should be included in the appropriate primer. Labeled RNA probes were synthesized using *in vitro* transcription and processed according to Hejátko (2006) and Traas (2008).

Sample treatment and hybridization

Fixed whole mount seedlings were permeabilized in a 1:1 mixture of ethanol and xylene for 30 min, washed twice in ethanol for 5 min and progressively rehydrated in 75% ethanol (v/v in water), 50% and 25% ethanol (v/v in 1X PBS) for 10 min each. Samples were refixed in fixative solution, for 20 min at room temperature (RT), washed twice in PBT (1X PBS plus 0.1% (v/v) Tween-20) for 10 min and then incubated with 125 μg ml^{-1} proteinase K (Roche) for 15 min. Digestion was stopped by incubating the samples in 1X PBS plus 0.2% glycine for 5 min and then washing them twice in PBT for 10 min. Samples were refixed in fixative solution for 20 min at RT, washed twice in PBT for 10 min and once in the hybridization solution 50% (v/v) formamide in 5X SSC (20X SSC: 3 M NaCl, 300 mM sodium citrate, pH 7.0 with 1 M HCl), 0.1% (v/v) Tween-20 and 0.1 mg ml^{-1} of heparin (Sigma) for 10min, and then preincubated in the same solution for 1 h at 50°C.

The hybridization step was performed overnight at 50°C by incubating samples in supplemented hybridization solution (modified by adding 10 μg ml^{-1} of Salmon sperm DNA to the hybridization solution) containing a cocktail of denatured (80°C for 2 min) labeled RNA probes (20–100 ng per ml of the hybridization solution).

The optimal hybridization temperature was 50°C, which ensured sufficient hybridization specificity. However, with less abundant transcript or less specific probes, 45°C or 55°C, respectively gave optimal results.

Post-hybridization and fluorescent detection

Samples were washed: three times (10 min, 60 min and 20 min) in a solution of 50% (v/v) formamide, 2X SSC and 0.1% (v/v) Tween-20 at 55ºC; once for 20 min in 2x SSC, 0.1% (v/v) Tween-20 at 55ºC; twice for 20 min in 0.2x SSC, 0.1% (v/v) Tween-20 at 55ºC; three times for 10 min in PBT at RT; once for 30 min in PBT plus 1% BSA (Roche). Thereafter samples were incubated with a mixture of the selected primary antibodies (Sheep anti-digoxigenin, Roche; Mouse anti-biotin, Roche; Rabbit anti-fluorescein, Molecular Probes) diluted (1:100) in (PBT+BSA), for 2 h at RT under gentle shaking. Subsequently, samples were washed three times for 10 min in PBT, once for 30 min in PBT plus BSA and then incubated with a mixture of the secondary antibodies (Alexa Fluor dyes 555 Donkey Anti-Sheep, INVITROGEN; Alexa Fluor dyes 488 Donkey Anti-Mouse, INVITROGEN; Alexa Fluor dyes 647 Donkey Anti-Rabbit, INVITROGEN) diluted (1:100) in PBT plus BSA overnight at RT in the dark. After incubation samples were washed twice for 15 min in PBT under gentle shaking in the dark. In all phases of the multi-probe methodology, care was taken to ensure a complete immersion of samples in the applied solutions (use 0.5 ml solution in a 2 ml eppendorf tube).

Samples were mounted with antifade reagent (Fluka) on a microscope slide for viewing. Specific antifade-based mounting medium permits storage of the specimen at -20°C for several months with only slight diminution of signal.

Confocal visualization

Samples were imaged using a Leica TCS SP2 (Spectral Confocal and Multiphoton System) confocal scanning laser microscope. Simultaneous detection of Alexa Fluor dyes (AF) 488, AF555 and AF647 was performed by combining the settings indicated in the sequential scanning facility of the microscope, as instructed by the manufacturer. The dye conjugates were excited at 488 nm, 555 nm and 647 nm, respectively by an Ar/He/Ne laser. The fluorescence emission for 488 nm was collected at 517 nm, for 555 nm at 569 nm and for 647 nm at 671 nm using a Leica 10x0.3 NA HC PL fluotar lens. Under these conditions autoflorescence was not detected. Serial optical sections of the root were collected with a mechanical focus increment of 3$\pm$0.2 μm to a 75 μm depth. Image processing and 2D reconstruction of serial sections was performed with Leica LCS software (Leica Microsystems, Germany). For the shoot apical meristem, single

TABLE 1

TROUBLESHOOTING

Problem	Possible cause	Solution
Loss of morphology and tissue breaking	Fixation problem	Respect timing of fixation and always prepare fresh fixative
	Agitation too strong	Decrease the speed of agitation
Low or no hybridization signal	Probe degradation	Ensure that solution are RNAse free Ensure that solution are at proper pH Confirm that hybridization/washing conditions are appropriate with single fluorescence *in situ* hybridization
	Poor RNA synthesis	Substitute the SP6 promoter with a T3 or T7 promoter
	RNase contamination	Work carefully, wear gloves and RNase free chemicals.
	Poor permeation of riboprobes or antibodies in the plant tissue	Use appropriate positive controls (riboprobes for constitutive genes or for a gene whose expression pattern has been studied before)
	Different riboprobes might require different hybridization condition	Use riboprobes of similar length and GC content
	Tissues exposed to too much light	Protect the solution from the light
High background	Hybridization conditions not optimized	Hybridize sense-strand to the same sample as a control, which should yield very low to no signal Increase hybridization temperature Increase pos-thybridization wash temperature Decrease SSC concentration in washing steps
	Gene not expressed in the tissues	Check available expression profiling databases and/or use RT-PCR to identify the tissue(s) and condition(s), when your gene is expressed

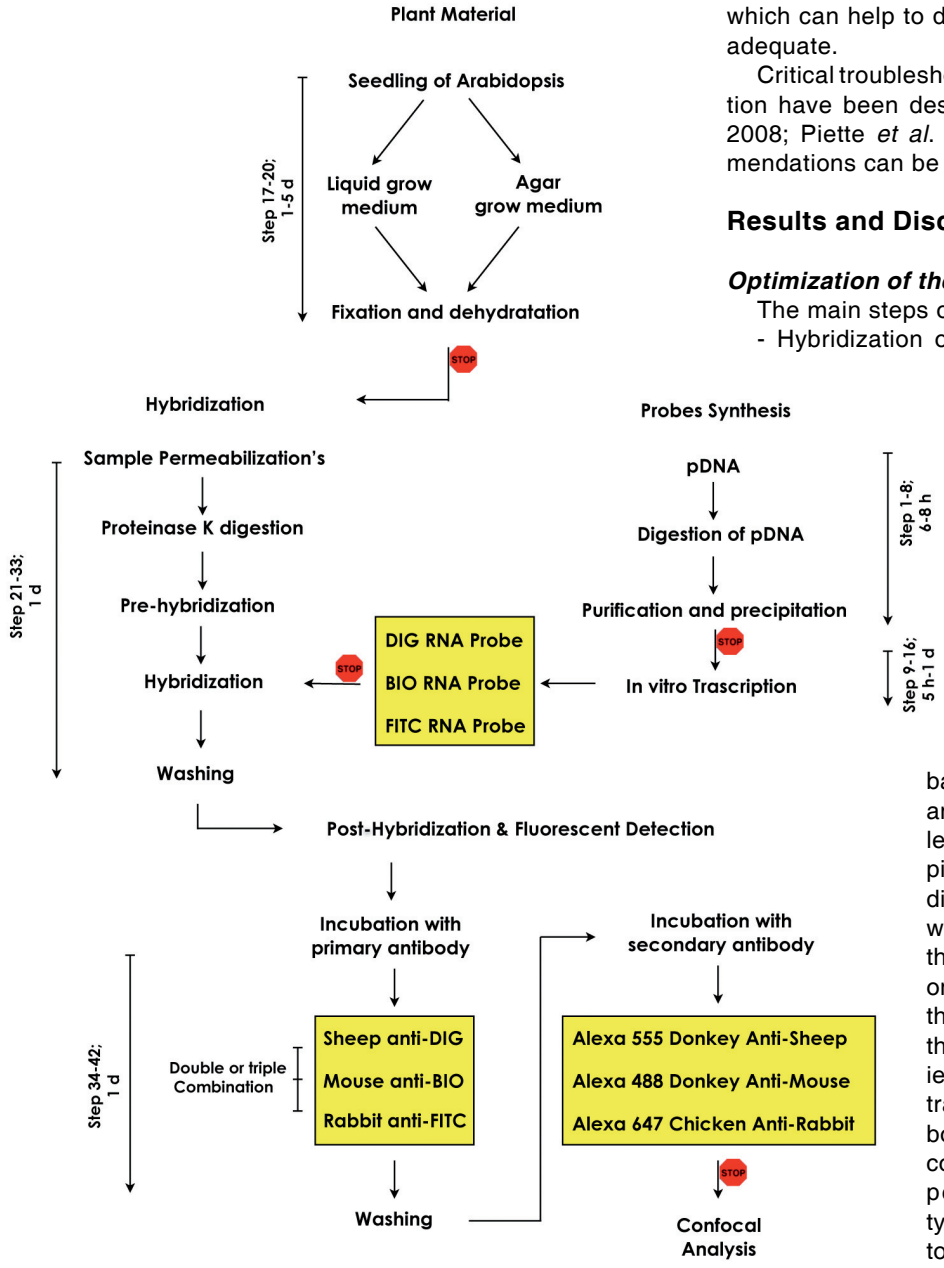

Fig. 1. Multi-color whole-mount *in situ* hybridization workflow. *Yellow boxes highlight relevant points for the development of procedure with respect to single whole Mount ISH. Times required for each step are indicated. KEY: STOP indicates a pause in the flow; DIG, digoxigenin; BIO, biotin; FITC, fluorescein; AF, Alexa Fluor dyes, (Molecular Probes).*

optical section and 2D reconstruction of serial sections were performed with Leica LCS software (Leica Microsystems).

General comments

Before attempting to carry out the multi-probe procedure, optimal hybridization and washing conditions should be determined for each probe separately. This can be achieved with the use of single-label ISH, using the protocol detailed above (Hejátko *et al.* 2006), and confirmed independently by Quantitative RT-PCR (Q-PCR). These analyses allow an assessment of general patterns of expression and transcript abundance, which can help to determine whether multiprobe patterns are adequate.

Critical troubleshooting steps for optimizing probe hybridization have been described in detail elsewhere (Pinaud *et al.* 2008; Piette *et al.* 2008). Additional troubleshooting recommendations can be found in Table 1.

Results and Discussion

Optimization of the multiplex whole mount ISH procedure

The main steps of the procedures are described below:

- Hybridization of combinations of two/three differentially labeled RNA probes were done which were synthesized using *in vitro* transcription in the presence of either Digoxigenin-11-UTP, or Biotin-16-UTP or Fluoroscein-12-UTP to differentially label the probes. This chemical modified bases serve as haptens that can be detected using hapten–specific antibodies followed by fluorescent detection.

-In the detection phase, a cocktail of primary antibodies specifically directed against these probes was utilized. This step was followed by incubation with fluorescently labeled secondary antibodies, accurately selected for their wavelength excitation, in order to avoid overlapping of signal from different probes. Note that direct detection of fluorescein-labeled probe was excluded due to its weak sensitivity, and thus also in this case both primary and secondary antibodies were applied. Moreover, the best results were obtained by extending the incubation time with secondary antibodies to overnight and increasing the concentration of both primary and secondary antibodies (for details see experimental protocols). In the case of gene poorly expressed a peroxidase-mediated precipitation of tyramide linked fluorophores can be applied to amplify hybridization signal.

It is absolutely necessary that every multiprobe whole mount mRNA *in situ* hybridization experiment includes controls to determine the specificity of expression signal. For positive control, the best way is to use simultaneously, during the multiprobe procedure, an antisense probe of a housekeeping gene with a known staining pattern. For negative controls, the best way is to test simultaneously sense probes of analyzed genes, differentially labeled as for multiprobe procedure. Another good negative control relies on the omission of secondary fluorophores-linked antibody.

An overview of the steps- from sample fixation to triple probe synthesis, simultaneous hybridization and multiplex detection- is provided in Fig. 1. Yellow boxes highlight relevant differences in the procedure with respect to single whole mount ISH.

Verification of the multi-probe ISH in the primary root

In order to test the reliability of the method we investigated the expression pattern of two members of the *PINFORMED (PIN)* gene family in *Arabidopsis thaliana* root.

In *Arabidopsis,* eight *PIN* genes have been identified and the biological functions of five of them have been characterized (Morris *et al.* 2004; Paponov *et al.* 2005). *PIN* genes encode plasma membrane-associated proteins (PIN), called auxin efflux facilitators, which control polar flow of the phytohormone auxin and are characterized by cell type specific polar localization. The polar cell-to-cell flow of auxin underlies multiple developmental processes in plants (Vanneste and Friml 2009).

For our analysis we selected *PIN1* and *4* genes, known to be expressed at low levels, as judged by single whole mounts, and to exhibit distinct expression patterns in root tissues (Blilou *et al.* 2005). Under our experimental conditions, *PIN1* localized in the stele and more weakly in the cortex and in the epidermis (Fig. 2 A, E, C, G). *PIN4* was instead detected in the quiescent centre and cells surrounding it and in provascular cells of stele (Fig. 2 B, F, C, G). Thus, the results reported here confirm in the same individual the previous findings obtained by Blilou (2005) and Vieten (2005).

Verification of the multi-probe ISH in the shoot of apical meristem

In order to confirm the reliability of the method in the SAM we investigated the expression pattern of *KNAT1*, a member of the class-I *KNOTTED*-like homeobox *(KNOX)* gene family exhibiting a well defined expression domain, in combination with *ELO3* gene.

The plant class 1 *KNOX* genes encode homeodomain-(HD) containing transcription factors (TFs). They are differentially required for SAM establishment and function and constitute a pathway that controls meristem cell fate (Reiser *et al.* 2000; Hake *et al.* 2004). In simple-leafed species, these genes are typically expressed in the SAM, but their down-regulation is required both at the leaf initiation site of the SAM (P0), the leaf primordium and throughout leaf development (Hay and Tsiantis, 2006). In *Arabidopsis* class 1 *KNOX* genes include *KNAT1*, also named *BREVIPEDICELLUS (BP)*, *KNAT2*, and *KNAT6* (Reiser *et al.* 2000).

Regarding ELO3, it is a component of a conserved histone acetyl transferase (HAT) complex, consisting of six subunits, that co localizes with the elongating RNAP II in plants and targets auxin-related genes for histone H3 acetylation. *elo* mutants were originally identified as leaf mutants but also have auxin-related phenotypes (Nelissen *et al.* 2005; 2010). Recently, ISH has been applied to investigate tissue or domain specific expression pattern of *ELO* genes in *Arabidopsis* plants by using single probes (Nelissen *et al.* 2010). Results showed that *ELO* genes were expressed predominantly in the meristematic tissues (shoot apical meristem, provascular strands of young seedlings, meristematic and elongation zone of the primary and lateral root tip) suggesting that Elongator complex formation is restricted to actively dividing tissues (Nelissen *et al.* 2010).

Using multi-probe *in situ* hybridization, we observed that in the aerial organ of young seedling (i.e. SAM and developing leaves) the spatial expression of *KNAT1* was restricted to the SAM and absent in the developing leaves (Fig.3 B, E, C, F). Thus, the result reported here fully matches that previously obtained by Lincoln (1994). Whereas, *ELO3* expression was present in the SAM, in the emerging leaf primordia and provascular strands (Fig. 3 A, D, C, F), thus confirming expression pattern previously obtained by Nelissen (2010). By merging the two expression patterns, in the same sample an overlapping of *KNAT1* and *ELO3* fluorescent signals, tightly confined to the SAM, was clearly observed (Fig. 3 C, F).

ELO and HUB1 gene expression patterns partially overlap in the shoot and root

The next set of genes were *ELO2, ELO3* and *HUB1,* all involved in activating transcription through histone modification. In particular, *HUB1* encodes the functional homolog of yeast and human histone H2B monoubiquitinating BRE1 RING E3 ligases, and has a role in plants in the regulation of the cell cycle during early organ growth (Fleury *et al.* 2007). However, the *HUB1* spatial expression pattern has not been investigated by ISH before in plants, but RT-PCR and pHUB1-GUS analyses showed

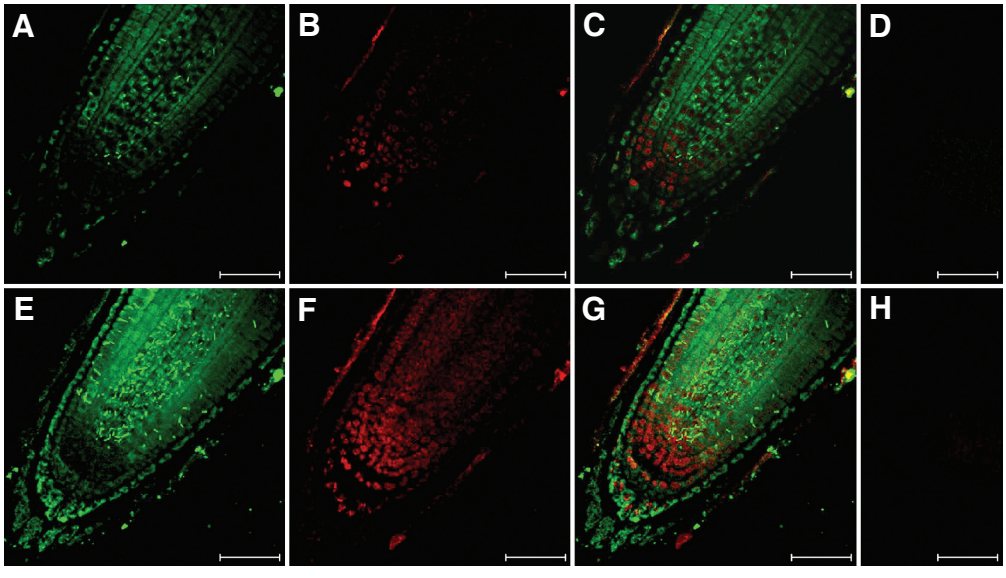

Fig. 2. Double multi-color fluorescent *in situ* hybridization in *Arabidopsis* seedling root tips (A-H). *Images were acquired on a Leica SP2 confocal microscope with a 40X oil immersion objective. Green:* **(A)** PIN1 *BIO riboprobe, mouse anti-BIO and AF488 donkey anti-mouse. Red:* **(B)** PIN4 *DIG riboprobe, sheep anti-DIG and AF555 donkey anti-sheep;* **(C)** *merge and* **(D)** PIN1 *sense control. 2D Maximum projection from a z-stack assembly* **(E-G)**, *images were acquired on a Leica SP2 confocal microscope with a 40X oil immersion objective. Green:* **(E)** PIN1 *BIO riboprobe, mouse anti-BIO and AF488 donkey anti-mouse. Red:* **(F)** PIN4 *DIG riboprobe, sheep anti-DIG and AF555 donkey anti-sheep;* **(G)** *merge and* **(H)** PIN4 *sense control. KEY: DIG, digoxigenin; BIO, biotin; FITC, fluorescein; AF, Alexa Fluor dyes, (Molecular Probes). Set. Scale bars, 75 μm (A-H).*

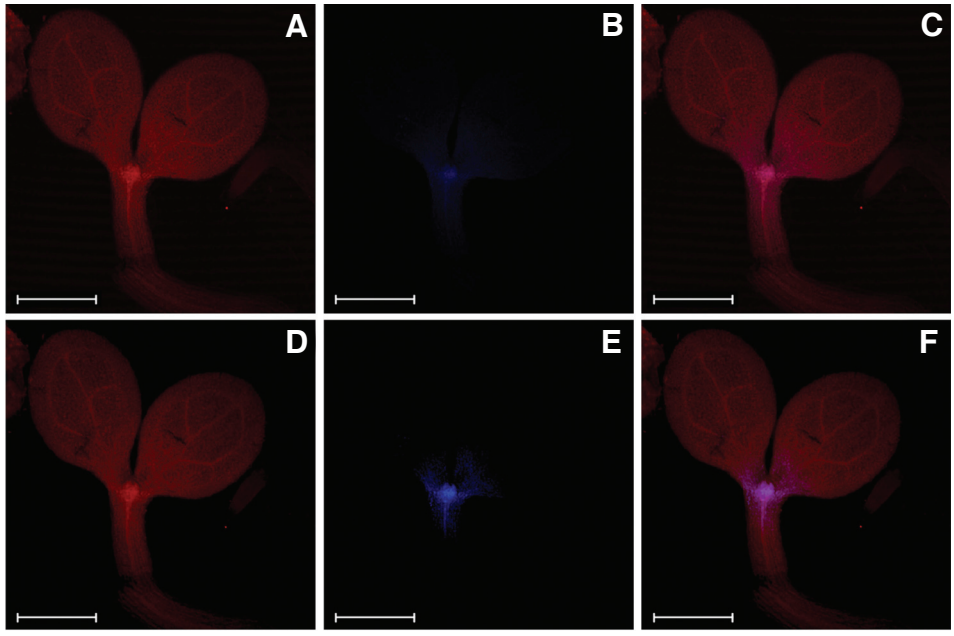

Fig. 3. Double multi-color fluorescent *in situ* hybridization in *Arabidopsis* seedling shoot apices (A-F). *Images were acquired on a Leica SP2 confocal microscope with a 10X objective. Red:* **(A,D)** ELO3 *DIG riboprobe, sheep anti-DIG and AF555 donkey anti-sheep. Blue:* **(B,E)** KNAT1 *FITC riboprobe, rabbit anti-FITC and AF647 chicken anti-rabbit;* **(C,F)** *merge 2D Maximum projection from a z-stack assembly* **(D-F)**, *images were acquired on a Leica SP2 confocal microscope with a 40X oil immersion objective. Red:* **(D)** ELO3 *DIG riboprobe, sheep anti-DIG and AF555 donkey anti-sheep. Blue:* **(E)** KNAT1 *FITC riboprobe, rabbit anti-FITC and AF647 chicken anti-rabbit;* **(F)** *merge. KEY: DIG, digoxigenin; FITC, fluorescein; AF, Alexa Fluor dyes, (Molecular Probes). Set. Scale bars, 300 μm (A-F).*

respectively *HUB1* gene expression or promoter activity in roots, stems, leaves and flowers (Liu *et al.* 2007).

Using the Multi-probe whole mount, we observed a clear overlapping of *ELO2* and *ELO3* fluorescent signals in the SAM, emerging leaf primordia and provascular strands of young seedlings (Fig. 4 A, B, D), in the meristematic and elongation zone of the primary root tip while probe signals were absent in the differentiation zone (Fig. 4 E, F, H). Thus, we demonstrate in the same sample and in the entire organ, that two *ELO* genes have identical developmental expression patterns as previously stated by comparing gene expression in different samples (Nelissen *et al.* 2010).

Concerning *HUB1* gene, we observed that in the aerial organ of young seedling (i.e. SAM and developing leaves) its spatial expression overlapped with that of *ELO* genes (Fig. 4 C, D). In the primary root, *HUB1* transcripts mainly accumulated in the protodermal cell layer of the meristematic and elongation zone in contrast to *ELO* transcripts that were abundant in the whole meristematic dome, as well as along protoderm, cortex and, even if to a less extent, in the vascular stele of elongation zone (Fig. 4 G, H). Thus, *HUB1* expression pattern overlaps fully with those of *ELO* genes in the shoot, but it differs in the primary root suggesting a differential tissue specific role for the *HUB1* and *ELO* genes in the *Arabidopsis* root.

Conclusions

We applied for the first time a functional and reproducible procedure for multi-probe whole mount mRNA *in situ* hybridization in *Arabidopsis* seedlings, based on the method described by Hejátko (2006). The success of this procedure depends on the specific labeled probes used for the hybridization, and the choice of appropriate primary antibodies from different host species, as well as spectrally separable fluorescent secondary antibodies for visualization in a multichannel confocal laser microscopy.

In conclusion the method described here provides a fast and efficient tool to simultaneously define the *in situ* expression pattern of several genes at high resolution in plants.

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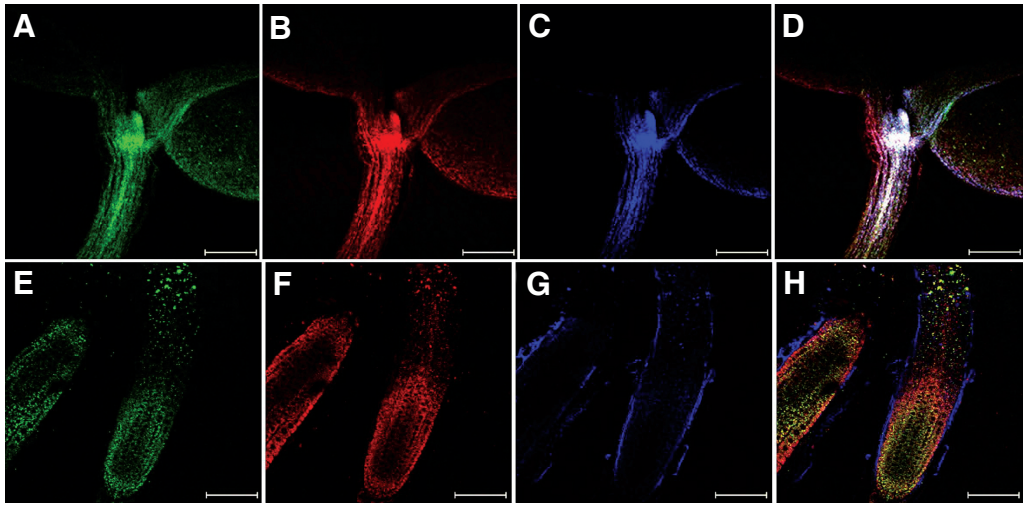

Fig. 4. Triple-label whole-mount fluorescent *in situ* hybridization in a seedling shoot apical meristem (A-D), and in a seedling root apical meristem (E-H). *Images were acquired on a Leica SP2 confocal microscope with a 20X objective for* **(A-D)** *and a 40X oil immersion objective for* **(E-H)**. *Green:* **(A,E)** ELO2 *BIO riboprobe, mouse anti-BIO and AF488 donkey anti-mouse; Red:* **(B,F)** ELO3 *DIG riboprobe, sheep anti-DIG and AF555 donkey anti-sheep; Blue:* **(C,G)** HUB *FITC riboprobe, rabbit anti-FITC and AF647 chicken anti-rabbit; Merge* **(D,H)**. *Key: DIG, digoxigenin; BIO, biotin; FITC, fluorescein; AF, Alexa Fluor dyes, (Molecular Probes). Set. Scale bars, 345 μm* **(A-D)**, *75 μm* **(E-H)**.

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