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Analysis of *AtGUS1* and *AtGUS2* in *Arabidopsis* root apex by a highly sensitive TSA-MISH method

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ABSTRACT A new highly sensitive whole-mount *in situ* hybridization method, based on tyramide signal amplification (TSA-MISH) was developed and a combined GFP detection and TSA-MISH procedure was applied for the first time in plants, to precisely define the spatial pattern of *AtGUS1* and *AtGUS2* expression in the root apex. β-glucuronidases (GUSs) belonging to the glycosyl hydrolases (GHs) 79 family, are widely distributed in plants, but their functional role has not yet been fully investigated. In the model system *Arabidopsis Thaliana,* three different *AtGUS* genes have been identified which encode proteins with putative different fates. Endogenous GUS expression has been detected in different organs and tissues, but the cyto-histological domains of gene expression remain unclear. The results here reported show co-expression of *AtGUS1* and *AtGUS2* in different functional zones of the root apex (the cap central zone, the root cap meristem, the staminal cell niche and the cortical cell layers of the proximal meristem), while *AtGUS2* is exclusively expressed in the cap peripheral layer and in the epidermis in the elongation zone. Interestingly, both genes are not expressed in the stelar portion of the proximal meristem. A spatial (cortex *vs.* stele) and temporal (proximal meristem *vs.* transition zone) regulation of *AtGUS1* and *AtGUS2* expression is therefore active in the root apex. This expression pattern, although globally consistent with the involvement of GUS activity in both cell proliferation and elongation, clearly indicates that *AtGUS1* and *AtGUS2* could control distinct downstream process depending on the developmental context and the interaction with other players of root growth control. In the future, the newly developed approaches may well be very useful to dissect such interactions.

KEY WORDS: *Arabidopsis thaliana, multiprobe in situ hybridization, GUS, root, tyramide signal amplification (TSA)*

β-glucuronidases (GUS) are glycosyl hydrolases (GHs) which catalyse the hydrolysis of the glycosidic bond between glucuronic acid and other carbohydrates or molecules different from sugars, termed aglycones. GUSs have been identified in all the living organisms and according to their amino acid sequence, have been classified in three families: GH1 GH2 e GH79 (Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Bairoch, 1996).

GUS, belonging to GH79 family, are widely distributed in plants. GUS sequence was determined for the first time in *Scutellaria baicalensis* (Sasaki *et al.,* 2000) and three different *GUS* genes have been identified in *Arabidopsis Thaliana* and named *AtGUS1*, *AtGUS2* and *AtGUS3* (Fig. 1) (Woo *et al.,* 2007). Further analysis of plants genomes and transcriptomes confirmed the wide distribution of GH79 *GUSs* in plants (Honys and Twell, 2004; Pina *et al.,* 2005; Arul *et al.,* 2008; Konishi *et al.,* 2008; Matas *et al.,* 2011; Hafidh *et al.,* 2012). PCR and endogenous GUS activity analysis demonstrated their expression in all the different organs (Sood, 1980; Schulz and Weissenböck, 1987; Plegt and Bino, 1989; Hu *et al.,* 1990; Alwen *et al.,* 1992; Anhalt and Weissenböck, 1992; Wozniak and Owens, 1994; Morimoto *et al.,* 1995; Morimoto *et al.,* 1998; Muhitch, 1998; Sudan *et al.,* 2006; Schoenbeck *et al.,* 2007; Woo *et al.,* 2007).

The different roles proposed for GUSs in plants can be associated with changes in polysaccharide moieties (Sudan *et al.,* 2006; Eudes *et al.,* 2008) or to the release of signal molecules (Schulz and Weissenböck, 1987; Morimoto *et al.,* 1998; Wen *et al.,* 2004;

Abbreviations used in this paper: GH, glycosyl hydrolase; GUS, β-glucuronidases; MISH, multiprobe in situ hybridization; TSA, tyramide signal amplification.

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Woo *et al.,* 2005; Hirunuma *et al.,* 2011). GUS in plants has been demonstrated to be involved in modulating the glycosylation degree of arabinogalactanproteins (Eudes *et al.,* 2008) and in the changes in cell wall composition associated to cell elongation (Sudan *et al.,* 2006; Eudes *et al.,* 2008). Glucuronic acid and 4-O-methyl glucuronic acid residues are present in xylans, rhamnogalacturonans II and arabinogalactan proteins (Ishii and Matsunaga, 1996; Renard *et al.,* 1999; Zhong *et al.,* 2005; Koutaniemi *et al.,* 2012; Urbanowicz *et al.,* 2012); their removal by GUS is required for further hydrolysis of the polysaccharide moiety by other glycosyl hydrolases (Eudes *et al.,* 2008).

Concerning the role of GUS in modulating the presence of signal molecules, it works in opposition/coordination to β-glucuronosyltransferase (UGTs) enzymes, responsible of glucuronic acid addition to different molecules with their consequent physiological inactivation: one enzyme reverses the action of the other. This has been demonstrated in *S. baicalensis*, where the presence of baicalein has been found to be under the coordinate control of UGT and GUS activities (Morimoto *et al.,* 1998; Hirunuma *et al.,* 2011).

Interestingly, this UGT and GUS combined opposite function seems to operate in the regulation of cell division in the root cap meristem. It has been demonstrated that, in *Pisum sativum*, UGT (PsUGT1) plays a key role in cell cycle regulation by glycosylating, and therefore removing, a mitosis inhibitor, probably a flavonoid, which is instead released by GUS, with consequent cell cycle inhibition (Wen *et al.,* 2004; Woo *et al.,* 2005). This is supported by the observation that in several species (pea, alfalfa and *A.thaliana*) *uidA* expression under the *PsUGT1* promoter was lethal (Wen *et al.,* 2004). Moreover, inhibition of GUS activity through saccharolactone causes an enhanced production of border-like cells in the root apex of *A. thaliana*, indicating an increase in the mitotic activity of root cap meristem (Wen *et al.,* 2004).

Currently, despite the relevance of a time and space-dependent modulation of gene expression in the developmental processes, data on the cyto-histological domains of endogenous *GUS* expression in plants are inadequate, being mainly based on semiquantitative PCR analysis or GUS detection (Woo *et al.,* 2007). In particular, semiquantitative PCR analysis performed on whole organs showed that all the three *AtGUS* genes are expressed in the roots of *Arabidopsis Thaliana* although *AtGUS3* was expressed at a lower extent. *AtGUS* expression was also analysed in *pro AtGUS:: uidA* transgenic lines of *Arabidopsis Thaliana*. Histochemical detection revealed a similar expression pattern of *AtGUS1, AtGUS2* and *AtGUS3* in the root apex, but precise tissue localization is lacking.

To add information on these aspects we applied the whole mounting multiprobe *in situ* hybridization technique (MISH) set up in our laboratory (Bruno *et al.,* 2011), which represents a powerful techniques allowing the simultaneous localization at cellular level of different gene transcripts (Bruno *et al.,* 2011). Therefore, based on the predicted different fate of AtGUSs proteins (Woo *et al.,* 2007), we planned to investigate the expression pattern of *AtGUS1* (coding for secretory protein) *vs AtGUS2* (coding for membrane associated protein) in root apex.

Through this approach we observed that *AtGUS1* and *AtGUS2*

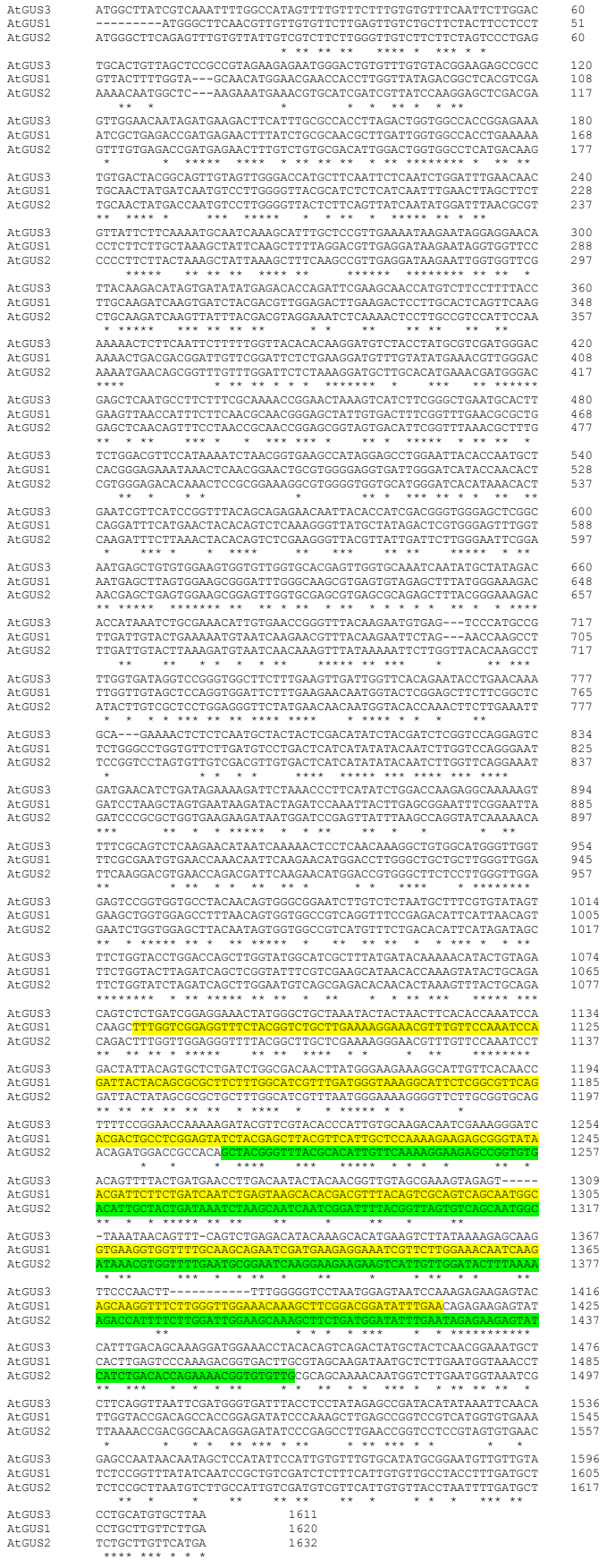

Fig. 1. Alignment of *AtGUS1* (AT5G61250), *AtGUS2* (AT5G07830) and *AtGUS3* (AT5G34940) nucleotide sequences from The *Arabidopsis* Information Resource (TAIR). *Yellow and green colours mark the region selected for synthesizing* AtGUS1 *and* ATGUS2 *riboprobes, respectively.*

transcripts largely co-localize but none of the transcripts could be detected in the stelar region of the proximal meristem, indicating that both genes are not expressed in that region. However, it could be not excluded that, in the applied hybridization conditions, gene expression in the proximal meristem stele could not be detectable since very low as compared to that of the other root zones. Therefore the aim of the present work was to develop an upgraded MISH method which, through the application of Tyramide Signal Amplification (TSA) approach (Molecular probes), enables to detect also very low levels of gene expression.

The new TSA-MISH method was first verified by localizing in both the root and shoot apex of *A. thaliana* seedlings the transcripts of *ELONGATA 3* gene (*ELO3*), whose spatial expression in dividing tissue is known. Thereafter it was used for the localization of *AtGUS1* and *AtGUS2* transcripts in the root apex. In addition a combined GFP labelling - mRNA hybridization approach was applied to transgenic lines of *A. thaliana* expressing GFP-linked SCARECOW (SCR) protein under its promoter (*pro SCR:GFP:SCR)* useful for a precise identification of cortex-stele boundaries.

Altogether these approaches allowed to clearly define the expression pattern of *AtGUS1* and *AtGUS2* in the root apex, thus providing additional informations useful for the definition of their putative functional role.

Results and Discussion

TSA-MISH versus MISH

The main difference of TSA-MISH versus MISH (Bruno *et al.,* 2011) deals with the detection phase. In both techniques appropriate different RNA-probes are constructed by using nucleotides modified with components specific for each probe. In the MISH, fluorescent antibodies are used to detect the specific probes. Therefore, signal intensity depends on the length of the probe, the percentage of modified nucleotides used in probe construction and the fluorescence efficiency of the compound used in probe detection. Instead, in TSA-MISH, peroxidase conjugated antibodies specifically react with the different modified nucleotides and a particular "fluorescent" reaction is used for their detection. Tyramide associated to a fluorofor (F-TYR) is the reaction substrate: the F-TYR oxidized product is a highly reactive radical which binds to nucleophilic residues, thus avoiding diffusion away from the detected probe (Fig. 2). Longer the incubation time, stronger the fluorescent signal associated to the probe. More probes, differently labelled, can be analysed together, by repeating the following steps: H_2O_2 treatment to inactivate the peroxidases - treatment with the anti-antibody specific for the probe – F-TSA peroxidase reaction (see method's work flow in Fig. 3).

Note that in the work flow (Fig. 3) it has been also highlighted that, when available, antibody versus the encoded protein can be added together with the antibody *vs* the relative mRNA-probe. Therefore a possible and very relevant extension of this technique is the concomitant detection of transcription (mRNA) and translation (proteins) products, useful to analyse the whole regulation of gene expression.

Validation of the TSA-MISH

The reliability of the method was tested by investigating the expression pattern of the *ELO3* gene in the shoot as well as in the root apex of young *A. thaliana* seedlings.

ELO3 is a component of a conserved histone acetyl transferase complex, that co-localizes with the elongating RNA POLYMERASE II (RNAP II) and targets genes for histone H3 acetylation (Nelissen *et al.,* 2010). Previously, tissue-specific expression pattern of *ELO3* was investigated through ISH and MISH techniques in both *A. thaliana seedling*s as well as during embryo development (Nelissen *et al.,* 2010; Bruno *et al.,* 2011; Himanen *et al.,* 2012) According to the role of ELONGATOR complex in cell proliferation, *ELO3* expression in seedling was confined to meristematic regions and proliferating cells.

The results obtained by using TSA-ISH (Fig. 4) fully matches that previously observed (Nelissen *et al.,* 2010; Bruno *et al.,* 2011). Indeed in both the present and the previous experiments (Nelissen *et al.,* 2010; Bruno *et al.,* 2011) *ELO3* transcripts are present in the shoot meristematic dome, in the emerging leaf primordia, in the blastozone of developing organs and provascular strands (Fig. 4A). In the primary root, a strong *ELO3* expression is evident in the whole proximal meristem, being detected in stem cell niche, as well as protoderm, cortex and in the vascular stele (Fig. 4B).

These results clearly show that the upgraded procedure does not affect neither the penetrability of the probe into the tissues, being detected also in the stele, nor the efficiency of probes and antibodies, thus demonstrating the reliability of TSA-based method.

Expression pattern of AtGUS1 and AtGUS2 genes in root apex trough MISH vs TSA-MISH

AtGUS1 and *AtGUS2* putatively encode protein with different fate: secretory and membrane-associated respectively (Woo *et*

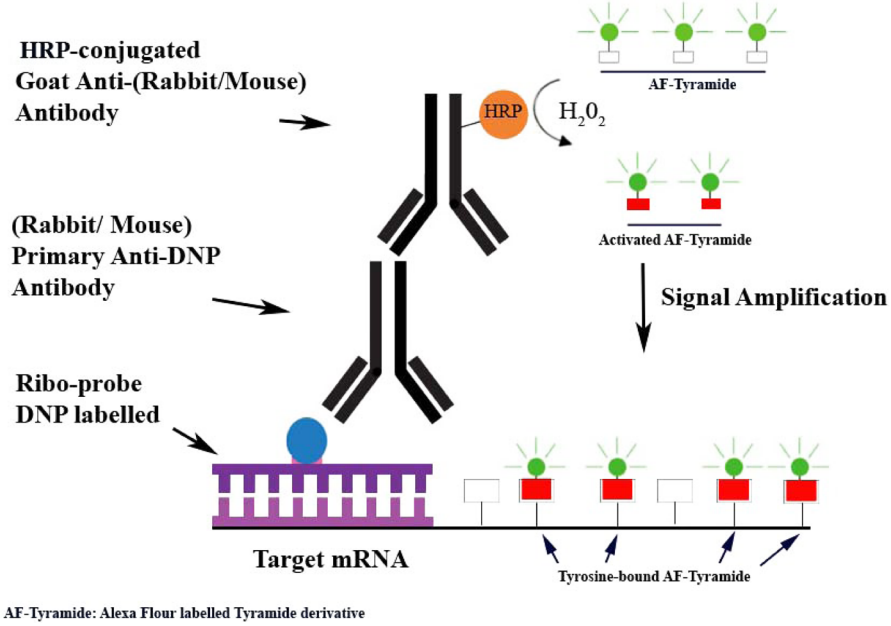

Fig. 2. Schematic representation of TSA detection applied to MISH. *HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide.*

al., 2007). Since patterning and organization of *Arabidopsis* root meristem into different functional zones are well defined processes (Dolan *et al.,* 1993), we selected to perform our study on root apex as a suitable system for defining the relationship between spatial *GUSs* expression pattern and cell commitment.

The expression pattern obtained through MISH approach shows that spatial accumulation of *AtGUS1* and *AtGUS2* transcripts in the root apex overlaps (Fig. 5 A,B,C). In particular the presence of both transcripts is clearly evident in the cap central zone (columella), in the root cap meristem, in the staminal cell niche, in the epidermis and in the cortical cell layers of proximal meristem, whereas *AtGUS1* and *AtGUS2* expression is not detectable in the stele. Faint *AtGUS2* expression is observable in the cap outermost layer (Fig. 5B).

A higher hybridization signal in a lower background is observed when TSA-MISH is applied to detect *AtGUS1* and *AtGUS2* expression pattern in the root apex (Fig. 5 A',B',C'), thus demonstrating the high sensitivity of the new approach. Interestingly, the high sensitivity of the new detection method clearly evidenced a differential expression of the two genes: i.e. only *AtGUS2* is expressed in the cap external layer (Fig. 5 B'). Moreover, in the transition zone *AtGUS1* transcripts are not any more detectable along the epidermis, highlighting a temporal regulation of its expression (proximal meristem *vs* elongation zone) (Fig. 5 A'). Despite this higher sensitivity, the signal associated to *AtGUS1* and *AtGUS2* transcript is again not detectable in the stele of the proximal meristem, confirming the results obtained by MISH. (Fig. 5 B',C'). Longer incubation times during the peroxidase reaction, which enhances the fluorescent signal, do not change such expression pattern (data not shown). This result further supports the absence of *AtGUS1* and *AtGUS2* expression in the stele.

Finally, we decided to analyse *AtGUS1* and *AtGUS2* expression in *proSCR:GFP:SCR (SGS)* transgenic lines of *A. thaliana.* Namely, SCARECROW (SCR) is a member of the GRAS transcription factor family (Lee *et al.,* 2008) and its expression is strictly confined to the pericycle cell line (Pysh *et al.,* 1999). Therefore, in the root of *SGS* plants. GFP fluorescent signal precisely marks the cortex-stele boundary allowing an accurate identification of *AtGUS1* and *AtGUS2* expression domains. However simultaneous GFP fluorescence detection and gene transcript localization was not possible so far (Darby *et al.,* 2006) because the heat treatment necessary for MISH, irreversibly denatures GFPs and quenches their fluorescence (Bokman and Ward, 1981; Ward, 1981; Ward and Bokman, 1982). To overcome such problem new antibodies have been recently raised against heat denatured GFP and used in a combined GFP labelling - mRNA hybridization study on neuron of mice (Nakamura *et al.,* 2008). We successfully applied this technique for the first time in plants, simultaneously localizing GFP and *AtGUS1* and *AtGUS2* transcripts in *SGS* roots. By highlighting pericycle cell line through GFP-signal we clearly confirmed

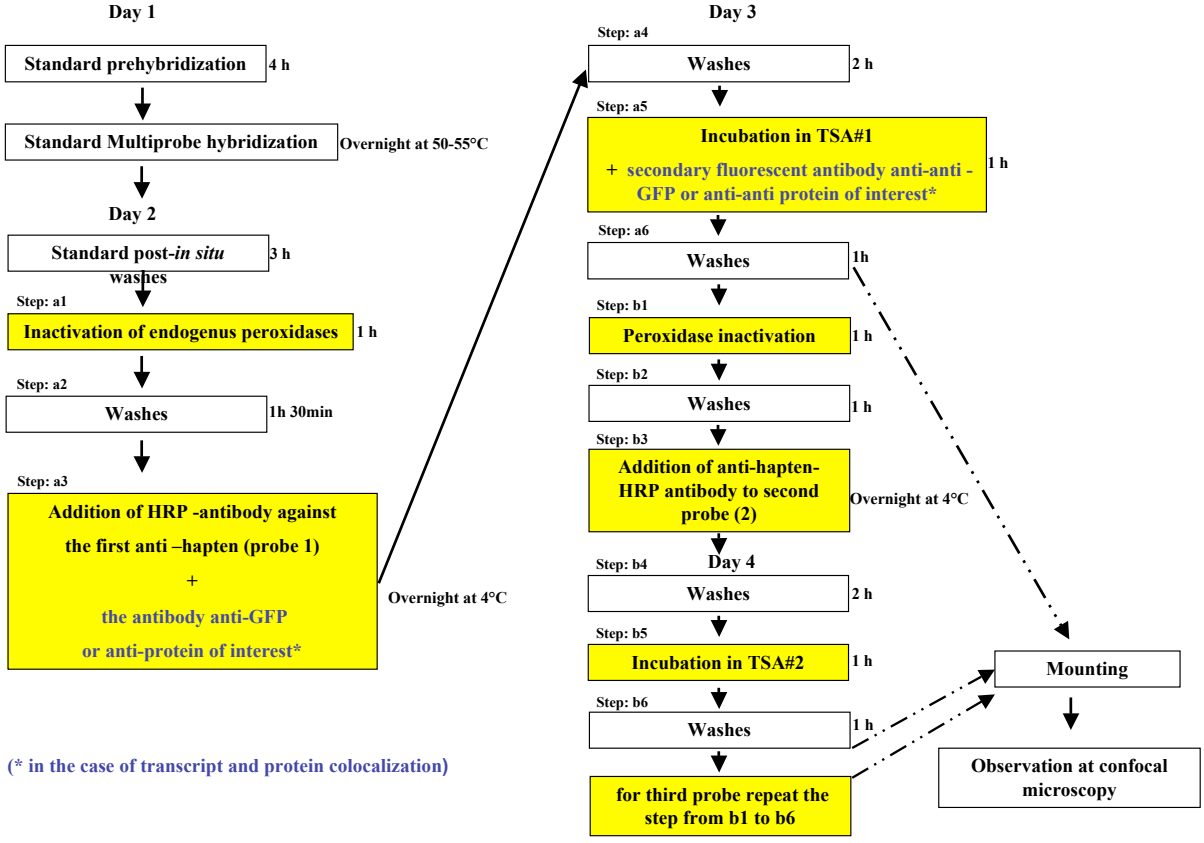

Fig. 3. Workflow of Multiprobe *in situ* hybridization by TSA (TSA-MISH). *Yellow boxes highlight relevant points for the development of procedure with respect to MISH. Approximate times required for each step are indicated. Day 1 is primarily dedicated to steps common to standard MISH. Day 2 and 3 are dedicated to endogenous peroxidase inactivation and detection of the first anti-hapten. Subsequent couple of days are dedicated to the detection of other anti-hapten.*

that in the proximal meristem the expression of *AtGUS1* (Fig. 6 B,C) and *AtGUS2* (data not shown) is excluded from the stele, detailing a spatial (cortex versus stele) regulation of *AtGUS1,2* expression.

Moreover, these results (Fig. 6 A,C) confirmed the absence of that *AtGUS1* expression in the epidermis of elongation zone and evidenced that at a major distance from the cup-meristem junction *AtGUS1* and *AtGUS2* (data not shown) are expressed also in the stele, thus indicating that also in this region gene expression is temporally regulated (Fig. 6 A,C).

The presence of *AtGUS1* and *AtGUS2* transcripts in the stem cell niche, encompassing quiescent centre and surrounding initial cells which slowly progress into cell cycle (Clowes, 1954; Scheres, 2007), as well as their simultaneous absence in the stelar proximal meristem, appear consistent with the proposed UGT and GUS opposite effect on the regulation of cell division. Indeed, it has been demonstrated that, in *Pisum sativum* UGT1, binding to glucuronic acid, sequesters a mitotic inhibitor, probably a flavonoid, whose release by GUS causes cell cycle inhibition (Wen *et al.,* 2004; Woo *et al.,* 2007). *AtGUS1* and *AtGUS2* expression in the root transition zone, where cells stop dividing and start to elongate and differentiate, might be related to GUS involvement in the cell wall modifications required for cell differentiation (Sudan *et al.,* 2006; Eudes *et al.,* 2008).

However, the action of *AtGUS1* and *AtGUS2* in root patterning and growth appears to be more complex. Indeed, *AtGUS1* and *AtGUS2* transcripts strongly accumulate in the cortical region of the proximal meristem (Moubayidin *et al.,* 2013) despite the

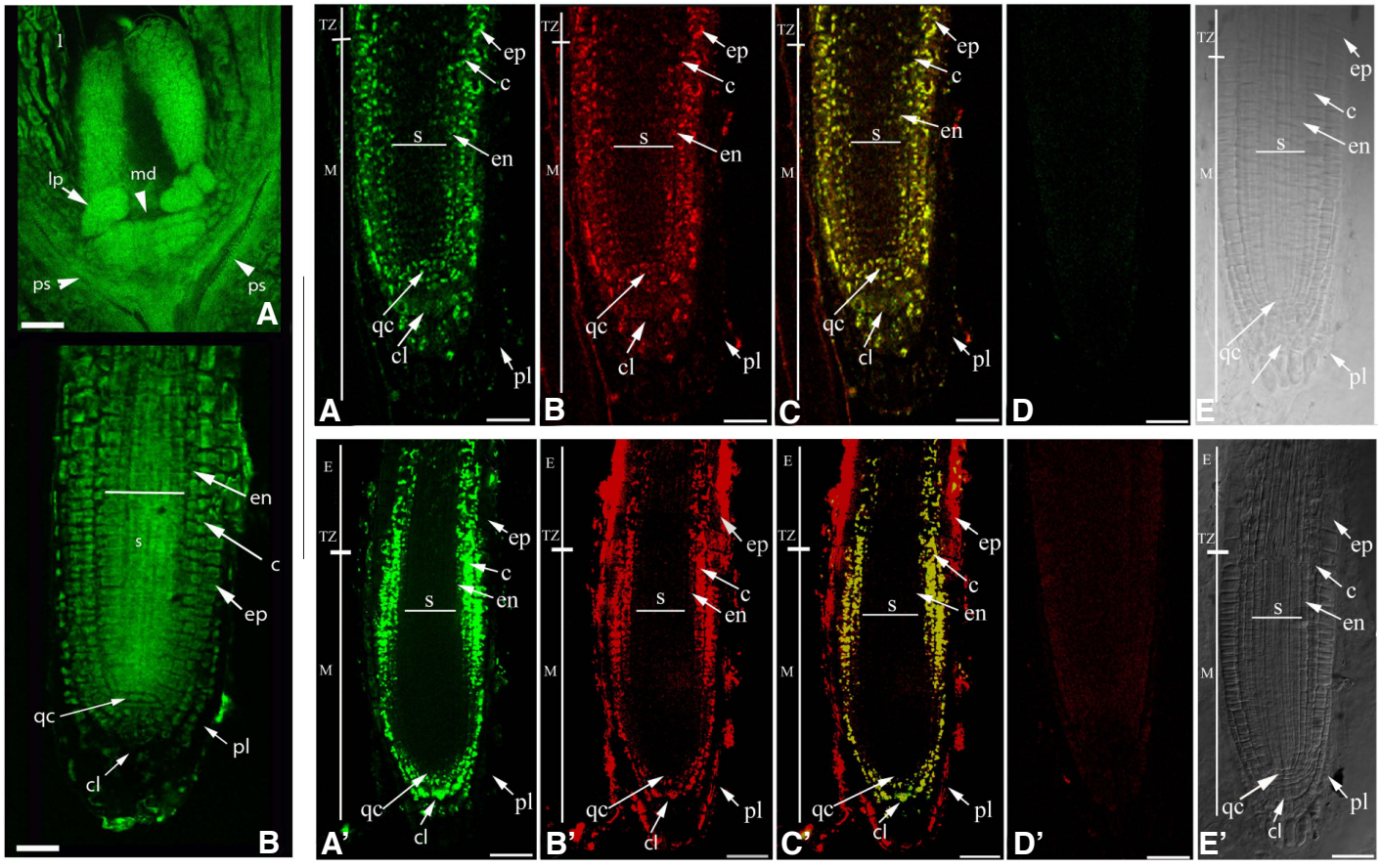

Fig. 4 (Left). *ELO3 in situ* hybridization by TSA-ISH (A) in the shoot and (B) in the root apical meristems. *The samples have been treated with* ELO3 *Bio-riboprobe, mouse monoclonal anti-Bio primary antibody and HRP-goat anti-mouse IgG secondary antibody; detection by AF488-tyramide treatment. KEY: AF, Alexa Fluor dye; Bio, biotin; cl, columella; c, cortex; en, endodermis; ep, epidermis; HRP, horseradish peroxidase; lp, leaf primordium; md, shoot meristematic dome; pl, cap peripheral layers; ps, provascular strand; qc, quiescent center; s, stele; Images by fluorescence were acquired through Leica inverted TCS SP8 confocal scanning laser microscope, with a 40X oil immersion objective. Scale bars 35 μm.*

Fig. 5 (Right). *AtGUS1* and *AtGUS2* multi-probe *in situ* hybridization (A-D) by MISH and (A'- D') by TSA- MISH (A, A') AtGUS1 *expression;* **(B, B')** AtGUS2 *expression;* **(C, C')** *merging of* AtGUS1 *and* AtGUS2 *expression;* **(D)** AtGUS1 *and* **(D')** AtGUS2 *sense control;* **(E, E')** *root images by transmission light. Green: samples treated with* AtGUS1 *Bio-riboprobe and mouse anti-Bio followed* **(A)** *by AF488-Donkey anti-mouse IgG (MISH) or* **(A')** *by HRP-goat anti-mouse IgG plus AF488 Tyramide treatment (TSA-MISH). Red: samples treated with* AtGUS2 *Dig-riboprobe and sheep anti-Dig, followed* **(B)** *by AF555 Donkey anti-sheep (MISH) or* **(B')** *HRP-goat anti-sheep IgG plus AF555 Tyramide treatment (TSA-MISH). KEY: AF, Alexa Fluor dye; Bio, biotin; cl, columella; c, cortex; Dig, digoxigenin; E, elongation zone; en, endodermis; ep, epidermis; HRP, horseradish peroxidase; M, meristematic zone; pl, cap peripheral layers; qc, quiescent center; s, stele; TZ, transition zone; Images were acquired through Leica inverted TCS SP8 confocal scanning laser microscope, with a 40X oil immersion objective.* **(A-D)** *and* **(A'-D')** *image by fluorescence;* **(E, E')** *images by transmission light. Scale bars (A-E) 50 μm;* (A'-E') *45 μm.*

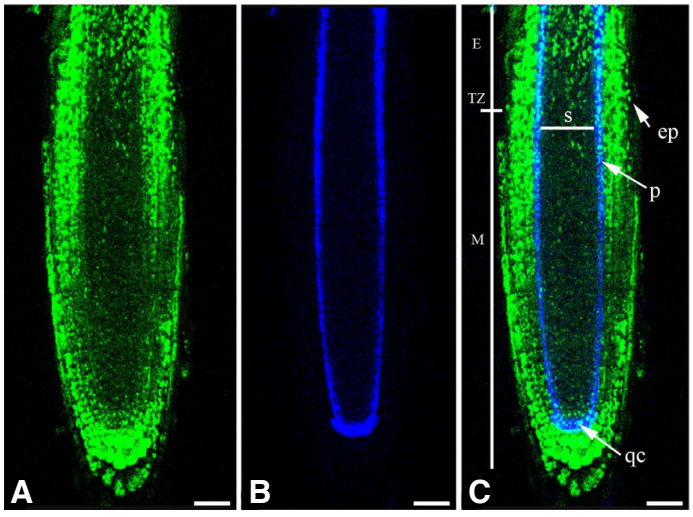

Fig. 6. *AtGUS1* expression in the root apex of *proSCR:GFP:SCR (SGS) Arabidopsis* transgenic plants. (A) AtGUS1 *Expression by TSA-MISH;* **(B)** *SCR expression by GFP detection;* **(C)** *merging of* **(A)** *and* **(B)**. *Green: sample treated with* AtGUS1 *Bio-riboprobe and mouse anti-Bio followed by HRP-goat anti-mouse IgG plus AF488 Tyramide treatment. Blue: samples treated with rabbit anti-heat denaturated GFP plus AF647 Donkey anti-rabbit IgG. KEY: AF, Alexa Fluor dye; Bio, biotin; E, elongation zone; HRP, horseradish peroxidase; M, meristematic zone; p, pericycle; qc, quiescent center; s, stele; TZ, transition zone; Images by fluorescence were acquired through Leica inverted TCS SP8 confocal scanning laser microscope. Scale bars, 35 μm.*

presence of proliferating cells. However, as indicated also by the predicted different fate of AtGUSs proteins, GUS expression can be associated with different roles and it is largely known that complex genetic network and hormonal interactions regulate root apex establishment and maintenance through the homeostasis between cell proliferation and differentiation, thus assuring correct root growth (Blilou *et al.,* 2005; Dello Ioio *et al.,* 2007; Moubayidin *et al.,* 2013). Further investigations are required, particularly on *AtUGT85A versus AtGUS* expression and on the simultaneous investigation of other compounds acting in the control of root growth, like auxins and cytokinins. Thus the method developed in the present paper, which allows the colocalization of transcripts and different antigenic molecules, will be very helpful to dissect such interactions.

Conclusion

In the present work a highly sensitive *in situ* hybridization method (TSA-MISH) has been developed. Through this method and taking advantage of a combined TSA-MISH and GFP detection, it was possible to accurately co-localize *AtGUS1* and *AtGUS2* transcripts in the same root apex. TSA-MISH was useful to confirm that both genes are effectively not expressed in the stelar proximal meristem. Moreover, the new procedure (TSA-MISH) allowed to detect differences in *AtGUS1* and *AtGUS2* expression, not observed by the traditional MISH, evidencing a spatial (cortex *versus* stele) and temporal (meristem *versus* transition zone) regulation of *AtGUS1* and *AtGUS2* expression. In particular from these observations, *AtGUS1* appeared to be spatially regulated in the cap being expressed in the columella but not in peripheral layers, and temporally in the epidermis being present in the proximal meristem and disappearing in the elongation zone. Moreover both *AtGUS1* and *AtGUS2* appeared temporally regulated in the stele, being expressed in the elongation zone and silenced in proximal meristem.

In conclusion, the TSA-MISH technique expands upon the previously one established in Bruno L *et al.,* (2011) and provides the following advantages: a) brilliant hybridization signal with reduced background; b) amplification of signal with low-abundance genes expression which could be underestimated or missed by using MISH; c) reliable concomitant detection of genes with very different levels of expression; namely, in the last case, it could be hard to establish hybridization conditions adapted to avoid either over- or under-estimation of "highy expressed" and "lowly expressed" genes, respectively. Similar difficulty could occur facing single gene expressed at different level in the different organs and tissues. All these aspects are highly relevant when information on the cyto-histological domains of transcript accumulation needs to be acquired.

Finally, a very relevant extension of TSA-MISH technique is the concomitant detection of transcription (mRNA) and translation (proteins) products, in order to analyse the whole regulation of gene expression. In fact, as above mentioned, antibodies *versus* the encoded protein together with the antibody *vs* the relative mRNA-probe can be simultaneously detected. Moreover, a combined TSA-MISH and GFP detection has been set up which can be applied for a concomitant detection of mRNA and its encoded protein in GFP-lines transformed for the protein of interest, to overcome the un-availability of protein specific antibody.

Materials and Methods

Plant material fixation and dehydration

Seeds of *Arabidopsis Thaliana* (L.) Heynh. ecotype Columbia (Col) were surface sterilized by incubation in absolute 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 4°C for 48 h to ensure uniform germination, and then moved to a growth chamber at 21°C, under 16 h light (150 μmol m^{-2} s^{-1}) and 8 h dark and 60% relative humidity. Five to eight day-old seedlings were fixed in 4% (w/v) paraformaldehyde, 15% (v/v) DMSO and 0.1% Tween -20 in PBS 10%(w/v), 1M NaOH, 1XPBS (10XPBS: 1,3 M NaCl, 70 mM Na_2HPO_4, 30mM KH_2PO_4 pH 7) and chlorophyll was removed as previously reported (Bruno *et al.*, 2011). Fixed material was stored in absolute ethanol overnight at -20°C.

Synthesizing labeled RNA probe

The following primer sequences *AtGUS1* (AT5G61250) FW 5'-TTTGGTCGGAGGTTTCTACG-3'and BW 5'-TTCAAATATCCGTCCGAAGC-3'; *AtGUS2* (AT5G07830) FW 5'-GCTACGGGTTTACGCACATT-3' and BW 5'-CAACACACCGTTTTCTGGTG-3; *ELO3* (At5g50320) FW 5'-TGAAGATACACGCCAGGACA-3' and BW 5'-CACCAGAAATCACACCGATT-3', were used in a PCR reaction to amplify gene-specific fragments (GSTs) 342, 250 and 300 bp long, respectively (Fig. 1).

Sense and antisense strands were synthesized for each gene by T7 and SP6 RNA polymerase promoter included in the appropriate primer.

Labeled RNA probes were synthesized using *in vitro* transcription in the presence of Digoxigenin-11-UTP (*AtGUS2* probe), Biotin-16-UTP (*AtGUS1*, *ELO3* probe) and processed as previously published (Bruno *et al.,* 2011).

In situ *hybridization by MISH*

Fixed whole 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 20 μg ml^{-1} proteinase K (Roche) for 15 min. Digestion was stopped by incubating the samples in 1X PBS plus 2 mg ml^{-1} glycine for 5 min and then washing them twice in PBT for 10 min. Samples were refixed in fixative solution for 10 min at RT, washed twice in PBT for 10 min and once in the hybridization solution consisting in 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 10 min, 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 hybridization solution, supplemented with 10 μg ml^{-1} of Salmon sperm DNA, containing denatured (80°C for 2 min) *AtGUS1* and *AtGUS2* labeled RNA probes at 20–100 ng ml^{-1} final concentration. After hybridization the samples were subjected to the following washing: 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 50°C; twice for 20 min in 2x SSC, 0.1% (v/v) Tween-20 at 50°C; once for 20 min in 0.2x SSC, 0.1% (v/v) Tween-20 at 50°C; three times for 10 min in PBT at RT; once for 30 min in PBT plus 1% BSA (Roche) (PBT+BSA). Thereafter samples were incubated for 2 h at RT under gentle shaking with a mixture of primary antibodies (sheep anti-digoxigenin, Roche; mouse anti-biotin, INVITROGEN) diluted 1:100 in PBT+BSA. After washing (10 min x3 in PBT and 30 min x1 in PBT+BSA) samples were incubated overnight at RT in the dark with a mixture of the secondary antibodies (Alexa Fluor dyes 555 Donkey Anti-Sheep, INVITROGEN; Alexa Fluor dyes 488 Donkey Anti-Mouse, INVITROGEN) diluted 1:100 in PBT plus BSA. After washing twice for 15 min in PBT under gentle shaking in the dark the samples were whole mounted with antifade reagent (Fluka) on a microscope slide for viewing.

In situ *hybridization by TSA-MISH*

Standard prehybridization and multiprobe hybridization were performed as above described. Following hybridization, 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 50°C, twice for 20 min in 2x SSC, 0.1% (v/v) Tween- 20 at 50°C and once for 20 min in 0.2X SSC, 0.1% (v/v) Tween-20 at 50°C. Sample were then washed three times in PBT for 10 min. and incubated for 60 min. at RT in 0.2X SSC with 1% (v/v) H_20_2 to inactivate endogenous peroxidase activity and washed twice for 30 min in PBT at RT. Thereafter samples were preincubated in PBT+BSA for 30 min at RT and incubated overnight at 4°C under gentle shaking in PBT+BSA in the presence of mouse anti-biotin and rabbit anti-digoxigenin monoclonal antibodies (INVITROGEN) diluted 1:500 (*AtGUS1* and *AtGUS2* detection, respectively). After washing four times for 20 min in PBT and once for 30 min in PBT plus 1% BSA (Roche) at RT, the samples were incubated with HRP-goat anti-mouse IgG for 60 min at RT without shaking. After washing for 30 min in PBS 1X at RT, the samples were incubated for 60 min at RT in the presence of Alexa 488-conjugated tyramide, in the INVITROGEN amplification reagent working solution. This step allowed to detect the presence of biotin, i.e. of *GUS1* transripts. After washing at RT in darkness (30 min X 2 in PBT) samples were incubated for 60 min at RT in 4% v/v H_20_2 to inactivate the peroxidase conjugated to the anti-mouse HRP-antibody.

Following H_2O_2 treatment, the above reported procedure has been repeated using HRP-goat anti-rabbit IgG (anti-dig) (INVITROGEN) diluted 1:100 (*AtGUS2* detection). After washing for 30 min in PBS 1X at RT, the samples were incubated for 60 min at RT in the presence of Alexa 555-conjugated tyramide in the amplification reagent working solution, as described in INVITROGEN protocol. This step allowed to detect the presence of digoxigenin, i.e. of *AtGUS2* transripts.

Samples were then washed twice for 30 min in PBT at RT and whole mounted with antifade reagent (Fluka) on a microscope slide for viewing.

The same procedure above reported for *AtGUS1* has been performed for *ELO3* single probe.

Combined GFP detection and TSA-MISH

The above described TSA-MISH procedure for *AtGUS1* detection has been used. Rabbit anti - denatured GFP antibody (gentle gift from professor Takeshi Kaneko), at a final concentration of 100 ng/ml, was concomitantly added with the HRP-goat anti mouse. At the end of the TSA-MISH procedure for *AtGUS1* detection, i.e. following the 4% v/v H_20_2 tretament, samples were washed three times for 10 min in PBT at RT and once for 30 min in PBT plus 1% BSA (Roche) (PBT+BSA). Thereafter they were incubated for 2h at RT under gentle shaking with Donkey Anti-rabbit conjugated to Alexa Fluor dye 647 (INVITROGEN), diluted 1:500. After washing twice for 15 min in PBT under gentle shaking in the dark, the samples were whole mounted with antifade reagent (Fluka) on a microscope slide for viewing.

Confocal visualization

Samples were imaged using a Leica inverted TCS SP8 confocal scanning laser microscope. Simultaneous detection of Alexa Fluor dyes was performed by combining the settings indicated in the sequential scanning facility of the microscope.

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