Whole mount in situ localization of miRNAs and target mRNA transcripts in plants

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Abstract

The functional characterization of miRNAs often involves understanding of their spatiotemporal expression, which mostly relies on reporter-based or in situ hybridization studies. The available in situ localization methods follow separate protocols for pre-hybridization, hybridization, post-hybridization, and detection steps for both miRNA and mRNA transcripts in plants. In this study, we present a single method which can be used for whole mount in situ localization of both miRNAs and mRNAs in different plant tissues. Our modified method provides enhanced sensitivity for the localization of miRNA and their target transcripts. Consequently, a less laborious, time-saving, economic and efficient method has been proposed by the modification of pre-hybridization, hybridization, post-hybridization and detection steps.

Keywords In situ · miRNA · Gene expression · *Arabidopsis* · Root development

Introduction

MicroRNAs (miRNAs) are ~20–24 nucleotide small non-coding RNAs, which regulate the expression of their targets, either by transcriptional cleavage of mRNA or translational inhibition (Chen 2012). In plants, miRNAs regulate various biological processes related to growth, development and stress responses (Singh et al. 2018). Recent technological advances made in genomics approaches have been able to identify a significant number of miRNAs in plants, however, the biological functions of many of them remain poorly studied. Elucidating the spatiotemporal expression pattern of a miRNA and its target transcripts is an important step for getting a preliminary insight into its biological function (Chen

2012). Transgenic reporter-based method, involving visualization of green fluorescent protein (GFP), yellow fluorescent protein (YFP), and β -glucuronidase (GUS) driven by promoters of miRNA/mRNA genes, is widely used to determine the tissue-specific expression pattern of transcripts in plants (Chiu et al. 1996). Use of these transgenic reporter-based methods is limited due to time-consuming steps of transgenic plant generation, which further require standardization for different species. However, in situ hybridization can be used to localize specific ribonucleic acids (RNAs) in a fixed plant tissue using antisense probes.

Most of the in situ localization methods and histological studies require embedding and sectioning of plant tissue samples for hybridization of labelled antisense RNA/miRNA probes (Javelle and Timmermans 2012). Sectioning of some plant tissues, such as root, is very challenging in some plant species, like *Arabidopsis*. Sometimes, oblique sections used in the experiment may produce results lacking uniformity.

Whole mount in situ hybridization/localization method is used to avoid laborious sectioning steps, but here, the penetration of various antibodies and other important reagents may be hindered by plant cell wall in the whole mount method. To address these limitations, attempts are being made to modify the already available in situ protocols which can be used for the localization of miRNA and their targets (mRNA) in the intact plant tissues (de Engler

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et al. 1998; Friml et al. 2003; Traas 2008). The already available methods require independent and separate protocols for miRNA and target mRNA localization during in situ hybridization (Begheldo et al. 2013; Bleckmann and Dresselhaus 2016; Hejatko et al. 2006; Ghosh Dastidar et al. 2016; Hernandez-Castellano et al. 2017). Here, we demonstrate a single protocol, which can be used to localize both miRNAs and their target transcripts in *Arabidopsis* tissues. This protocol has been tested and verified for various miRNAs and their target transcripts in *Arabidopsis*. We expect our protocol to work well with plant samples with higher tissue complexity facilitating precise localization of the selected miRNA and their target transcripts in the plant tissues.

Materials and methods

Reagents

Diethyl pyrocarbonate (DEPC) (Sigma, Cat. no. 77017)
 Histoclear (Sigma, Cat. no. H2779)
 Ethanol (Himedia, Cat. no. MB106)
 Pronase (Sigma, Cat. no. P5147)
 Glycine (Sigma, Cat. no. G8898)
 Deionized formamide (Sigma, Cat. no. F9037)
 BSA (Himedia, Cat. no. MB083)
 Tween 20 (Sigma, Cat. no. P1379)
 Anti-DIG-AP, Fab fragments from sheep (Roche, Cat. no. 1093274)
 NBT/BCIP stock solution (Roche, Cat. no. 1681451)
 Sodium citrate (Sigma, Cat. no. PHR1416)
 Sodium phosphate dibasic (Sigma, Cat. no. RES20908-A7)
 Monosodium phosphate (Sigma, Cat. no. S8282)
 RNase-free H₂O (Sigma, Cat. no. W4502)
 Parafilm
 Trizma base (Sigma, Cat. no. T1503)
 Magnesium chloride (Merck, Cat. no. M8266)
 Sodium chloride (Sigma, Cat. no. S7653)
 Heparin sodium salt (Sigma, Cat. no. L0380000H3393)
 Levamisole hydrochloride (Sigma, Cat. no. L0380000)
 Hydrochloric acid (Merck, Cat. no. 100317)
 Sodium hydroxide (Sigma, Cat. no. S8045)
 Paraformaldehyde (Sigma, Cat. no. P6148)
 Methanol (Himedia, Cat. no. MB113)
 Salmon sperm DNA (Invitrogen, Cat. no. AM9680)
 Digoxigenin (DIG) RNA labelling mix (Roche, Cat. no. 11277073)
 Terminal transferase-RTT-RO (Roche, Cat. no. 03333566001)
 Digoxigenin-11-ddUTP (Roche, Cat. no. 11363905910).

Equipments

DNA electrophoresis unit, pH meter, weighing balance, heating block, water bath, centrifuge, micro-centrifuge, slides, cover slips (24 × 60 mm), Zeiss microscope, fume hood, humidity chamber, dry heating block.

Probe selection and labelling

For miRNA/small RNA

In situ locked nucleic acid (LNA) probes labeled on both 5' and 3' ends are generally used to increase the sensitivity of detection. We used LNA oligo probes having a sequence complementary to desired miRNAs for their localization (Grunweller and Hartmann 2007; Javelle and Timmermans 2012; Vester and Wengel 2004). Labeling of LNA probes for whole mount in situ is discussed in Table 1.

For mRNA transcripts

For carrying out the hybridization reaction, antisense probes labeled with DIG are commonly used. DIG-labeled probes can be generated by cloning the desired transcripts in the vector such as pGEM-T, having the T7 or SP6 polymerase site. The positive clone is further linearized by selecting the restriction enzyme which can create a 5' overhang or a blunt end. Alternatively, a PCR-based method in which the sequence of interest is transcribed in vitro by RNA polymerase using labeled DIG-UTP can be used. The detailed protocol of probe preparation and labeling is being discussed by Hejatko et al. (2006), Table 2.

For the detection of mRNA transcripts, we used antisense probes of the transcripts of interest and sense probes as negative control. For miRNA localization, we used end labeled LNA probes. Human U6 or no probes were used as negative control.

Table 1 Labeling of the LNA probe (miRNA)

Components	Volume
5 × buffer	4 µL
25 mM CoCl ₂	4 µL
LNA (200 pM)	1 (200 pM)
1 mM DIG-ddUTP	1 µL
Recombinant terminal transferase	1 (2.5 U)
DEPC water (volume up to)	20 µL

Final concentration of the LNA probe/slide = 10 pM/µL

Table 2 In vitro transcription and labeling of the mRNA probe

Components	Volume
750 ng of DNA template	x μ L
RNase-free water	x μ L
100 mM DTT	2 μ L
10 $\times$ transcription buffer	2.5 μ L
DIG-UTP mix	2.5 μ L
RNase inhibitor (20 U/ μ L)	1 μ L (2.5 U)
T7 polymerase (10 U/ μ L)	1 μ L

Total volume = 25 μ L

Labelling of LNA probes (miRNA)

Localization of the desired miRNA in the plant tissue can be done using LNA probes which have a sequence complementary to that of the mature miRNA. In LNA probes, every third base is locked which offers specificity to the probe during the hybridization reaction (Vester and Wengel 2004; Kloosterman et al. 2006). Labelling of the LNA probe is described in Table 1. For setting up the hybridization reaction, 5 pM of DIG-labelled probe was used per 100 μ L of the hybridization reaction. The hybridization temperature varied from the probe to probe which needs an earlier optimization. In the current study, we have used DIG-labeled LNA probe for *miR166*, *miR167* and DIG-labelled *U6* (Javelle and Timmermans 2012; Lou et al. 2015) (Table S1).

The reaction mix (Table 1) was incubated at 37 °C for 1 h. The reaction was stopped by adding 4 μ L of 0.1 M EDTA (pH 8.0). Deionized formamide (24 μ L) was added to the probes and storage was done at –20 °C. As standardized, a concentration of 500 pM/10 mL of the probe was used during hybridization reaction.

Labelling of mRNA probes for target transcript

mRNA localization in the desired plant tissue can be done using antisense probes labelled at the 3' end. Labelling of the mRNA probes was done as reported previously (Hejatko et al. 2006). For setting up the hybridization reaction, 80–800 ng of mRNA probe was used per 100 μ L of reaction mixture. Hybridization temperature may vary from probe to probe which needs to be optimized earlier. In the current study, DIG-labeled antisense probes of *PHABULOSA* (*PHB*), *SHOOT MERISTEMLESS* (*STM*) and *PLETHORA1* (*PLT1*) were used (Aida et al. 2004; Singh et al. 2014; Endrizzi et al. 1996) along with a sense negative control which in the current study worked best at a hybridization temperature of 55 °C. The probe labeling components are listed in Table 2.

The above reaction mix was incubated at 37 °C, and 0.5 μ L of the reaction mix was run on a standard agarose gel.

Fig. 1 Schematic representation of the whole mount in situ hybridization in plant tissues

If expected bands were observed on the gel, 2 μ L of RNase-free DNase I was added into the remaining reaction mix and was incubated at 37 °C for 30 min. Transcripts were hydrolyzed by adding an equal volume of 2 $\times$ carbonate buffer (80 mM sodium bicarbonate, 120 mM sodium carbonate) and incubated at 37 °C for ‘n’ min, wherein, n = original length of probe (kb) – desired length (~0.2 kb)/0.11 $\times$ original length of probe (kb) $\times$ desired length (kb).

10% acetic acid was added at an amount equal to 1/10th volume of the carbonate buffer and the resultant mix was chilled on ice. Standard ethanol-based precipitation was done, followed by dissolving the probe in 50–80 μ L of 50% formamide, and stored at –80 °C. Labeled RNA probes of required concentration (80–800 ng/slide) were used depending on the weak/strong expression of the gene.

Whole mount in situ method

To avoid RNase contamination, tips, gloves, working boxes, glass slides, forceps, and other apparatus were made RNase free. The plastic wares were treated with DEPC, and the glassware and metal forceps were baked at 180 °C for 12 h. Generally, the entire procedure of the whole mount in situ hybridization in plants takes 4–5 days for completion. The schematic representation of the whole mount in situ localization in plants is summarized in Fig. 1.

Tissue fixation (day 1)

In the current method, we have used 5-day-old *Arabidopsis* seedlings for miRNA and mRNA transcript localization.

Arabidopsis seedlings were fixed by keeping in fixative (Table 3) for 45 min at room temperature, followed by a change of solution with 100% methanol, two times, for 5 min each. Subsequently, the solution was exchanged with 100% ethanol three times, after every 5 min. After the third wash, 100% ethanol was added and the samples were kept in -20°C overnight.

Pre-hybridization and hybridization (day 2)

The fixed *Arabidopsis* seedlings were allowed to come to room temperature. Fresh 100% ethanol was added and the vials were incubated for 5 min at room temperature. After 100% ethanol wash, 50% histoclear and 50% ethanol mix were added to the tube and incubation was carried out for 30 min at room temperature. Thereafter, seedlings were washed with 100% ethanol twice, for 10 min each. The tissue was further rehydrated in decreasing gradients of ethanol prepared in $1\times$ phosphate buffered saline (PBS) which were; 75% ethanol for 10 min, 50% ethanol for 10 min and 25% ethanol for 10 min. Subsequently, the tissue was washed with $1\times$ PBS for 5 min at room temperature and 4% para-formaldehyde for 20 min at room temperature. This was followed by washing with $1\times$ PBS with Tween-20 (PBT), twice, for 10 min at room temperature and treatment with pronase (40 mg/mL in $1\times$ PBS) for 15 min at 37°C . The reaction was stopped by using $1\times$ glycine PBS (pH 7.4) for 5 min at room temperature. Samples were washed using PBT solution, twice, for 10 min and kept in the pre-hybridization buffer for 1 h at 42°C . Salmon sperm DNA (10 mg/mL) was denatured at 98°C for 5 min. To the hybridization mix, 1.5 mL of the denatured salmon sperm DNA was added and kept at 42°C for 1 h. This was followed by the addition of denatured LNA probe and subsequent transfer to 42°C overnight with gentle shaking (5 pM/100 μL). The amount of the probe required for hybridization was calculated

empirically. The hybridization mix was added to the *Arabidopsis* seedlings.

Washing and antibody addition (day 3)

The sample was incubated in washing buffer (Table 3) at 42°C which involved gentle shaking for 10 min, 60 min and 20 min, with a fresh change of washing buffer after every time interval. This was followed by washing with $2\times$ SSC containing 0.1% Tween-20 for 20 min at 42°C and subsequently with $0.2\times$ SSC containing 0.1% Tween-20 for 20 min (twice) at 42°C , and finally washing with PBT, three times, for 20 min. The samples were pre-incubated in antibody buffer at RT for 90 min with gentle shaking. Antibody solution comprising of antibody buffer:anti-digoxigenin-AP antibody (1:2000) was added and samples were incubated overnight in the dark at room temperature.

Washing and detection (day 4)

The samples were washed eight times with PBT for 15 min at room temperature with a fresh change of PBT each time. Plant samples were washed with detection buffer for 5 min followed by the addition of 20 μL NBT/BCIP mix/1 mL of detection buffer. Thereafter, the samples were incubated in dark from 10 min to several hours with regular monitoring for the development of signal. After the development of signal, the reaction was stopped by adding 10% glycerol. Various reagents used to carry out in situ hybridization are listed in Table 3.

Signal was observed from 6 to 24 h after the addition of substrate. For the detection of miRNA, NBT/BCIP was used as a substrate. Signal was observed after 5–30 min after the addition of the substrate, the reaction was stopped by adding 10% glycerol..

Table 3 List of the solutions used in the whole mount in situ hybridization in plants

Solution name	Components	Final volume (mL)
Fixative	4% PFA, 15% DMSO, 0.1% Tween 20 in PBS (pH 7.4)	100
PBT	$1\times$ PBS + 0.1% Tween 20	100
Glycine PBS	0.2% glycine + $1\times$ PBS, pH 7.4	50
Pre-hybridization mix	50% formamide, $5\times$ SSC, 0.1 mg/mL heparin sodium salt, 0.1% Tween 20, 1 mg/1 mL of Salmon sperm DNA; final volume was adjusted by DEPC-treated water	15
Washing buffer	50% formamide, $2\times$ SSC, 0.1% Tween 20; the final volume was adjusted by DEPC-treated water	50
$20\times$ SSC	Sodium chloride (3 M), Sodium citrate (0.3 M), adjust pH to 7.2	100
Antibody buffer	1.5% BSA in $1\times$ PBT	20
Detection buffer	0.1 M Tris pH 9.5, 0.1 M sodium chloride, 50 mM magnesium chloride, 0.1% Tween 20, 2 mM of levamisole hydrochloride	50

Results and discussion

The already available protocols for in situ hybridization are based on the use of separate methods for pre-hybridization, hybridization, and detection of miRNA and target mRNA localization in plants (Hejatko et al. 2006; Javelle and Timmermans 2012). With some modifications, here we propose a single whole mount in situ localization protocol which can be used for the localization of both miRNAs and their target transcripts in *Arabidopsis* tissues. We have confirmed the expression pattern of *miR166* and *miR167* in *Arabidopsis* root meristem (Fig. 2a, b). We have also checked the expression of *miR166* and its target *PHB* to check the complementary expression pattern of the miRNA and its target (Figs. 2b, 3a). In the present study, the expression of mature *miR166* was localized to the root tip of *Arabidopsis*, mostly in endodermis (Fig. 2b). The expression of *PHB* was localized to the stele region of root tip and the sense probe did not show binding (Fig. 3a, b). Opposite expression pattern of *miR166* and its target *PHB* (a member of *class III HD-ZIP*) in *Arabidopsis* root further confirmed the sensitivity of the protocol. We have also checked the expression pattern of the shoot meristem maker gene *STM* in the shoot meristem (Fig. 3c, d) and *PLT1* (Fig. 3g, h) in the root meristem to check the specificity of the probe. *PLT1* is known to express in the root meristem, whereas *STM* is known to express in the shoot meristem.

Whole mount in situ is visualized as the development of deep purple-brown color in particular cells/tissues of the

plant samples. For confirming the authenticity of developed signal, the signal generated for *PHB* was compared to signal generated by sense probe and for *miR166* the signal was compared to negative control. The signal normally visible as deep purple-brown color in tissue samples was observed under a microscope. In situ localization is an efficient method for the localization of the miRNAs and their target transcripts in a particular tissue. The current method works well for both candidate miRNAs and their target transcripts in plants; however, there is always a scope to improve the functionality of the method which relies on empirical observations. As a case, if the signal background is high in the plant tissue, the amount of the probe for setting the hybridization reaction could be reduced. The intensity of the signal can be improved by increasing the hybridization temperature and the washing temperature for different tissues. Failure of the whole mount in situ reaction can be attributed to degradation of the probe or an inefficiently labelled probe. In the present study, NBT/BCIP was used as a substrate during signal detection which gives a colored product due to the alkaline phosphatase activity at the optimum pH of 9.5. It is always recommended to change the staining solution until the signal develops. The background can be limited by avoiding longer incubations and requires regular monitoring during signal development. Whole mount in situ signals should be compared to negative and positive controls to eliminate the possibility of getting false positive results. Since our method makes use of single protocol for miRNA and target mRNA transcripts localization, it is less time consuming and cost-effective. This protocol has been tested and verified for various miRNAs and their target transcripts in *Arabidopsis*. We expect our protocol to work well with plant samples of higher tissue complexity, with some empirical modifications, thus, offering a precise localization of the selected miRNAs and their target transcripts in the plant tissues.

Conclusion

Separate methods are reported for the localization of mRNA transcripts and miRNAs in plants till date; however, the current method represents a common protocol which can be used to localize both mRNA transcripts and miRNAs in intact plant tissue. We have significantly improved our method in key steps such as tissue fixation, hybridization, washing and detection. We have tested our method in various tissues of *Arabidopsis* which included shoot meristem, root meristem and lateral root. To check the sensitivity of our method, we have used suitable controls such as U6 as a negative control or no probe control for localization of miRNAs. For mRNA localization, we have used sense probe as a negative control which has

Fig. 2 Whole mount in situ hybridization of different miRNAs in a 5-day-old *Arabidopsis* root. **a** *miR167* localization in root meristem, **b** *miR166* localization in root meristem, **c** U6 as negative control, **d** negative control without probe. Scale bar indicates 50 μ m

Fig. 3 mRNA transcript localization in various tissues of *Arabidopsis*. **a** *PHB* (antisense) in root, **b** *PHB* (sense probe, negative control), **c** *STM* (antisense) in shoot meristem, **d** *STM* (sense) in shoot meristem, **e** *PHB* (antisense) in lateral root, **f** *PHB* (sense) in lateral root, **g**

PLT1 (antisense) in root meristem, **h** *PLT1*(sense) in root meristem. Scale bar for **a**, **b**, **e**, **f**, **g**, **h** indicates 0.005 mm and for **c** and **d** indicates 0.02 mm

shown no signal. The number of fixation and washing steps has been reduced, leading to the lesser damage to the plant tissue. Thus, our work represents the development of an efficient and rapid method for the localization of miRNA and their target mRNA transcripts using a single protocol for both. With empirical research observations, the method can be further utilized for the localization of miRNA/target transcripts in a broader range of tissues and, therefore, would surely be helpful in assisting the researchers working on spatiotemporal expression analysis of miRNAs and their target transcripts.

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Author contributions AS, VG, and AS conceived, designed, and performed all of the experiments and related data analysis. SS made the probe constructs. VG, AS, and SC wrote the paper. SV helped in the imaging of the slides. The study was supervised by AS, who provided scientific insight and guidance.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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