A RAPID AND SENSITIVE *in situ* RNA HYBRIDISATION METHOD FOR OIL PALM TISSUES

OOI SIEW-ENG*; LEE FONG-CHIN and MEILINA ONG-ABDULLAH***

ABSTRACT

We report here an in situ *RNA (ribonucleic acid) hybridisation protocol with modified post-hybridisation procedures tested on tissues from two plants, oil palm and* Arabidopsis thaliana. *This protocol involves shorter post-hybridisation washes with SDS (sodium dodecyl sulphate) buffers, replacing the standard SSC (sodium chloride-sodium acetate-sodium citrate) and formamide buffers. This modified protocol was tested with a few genes, and contrary to the results from previously used protocols, clearer distinguishable signals were detected with antisense probes compared with the sense probes on hybridised tissue sections. The protocol also reduces the time taken by the previous standard protocol by approximately 6 hr and uses fewer reagents, thus saving time as well as costs.*

Keywords: DIG, *in situ* hybridisation, plants, SDS.

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INTRODUCTION

In situ ribonucleic acid (RNA) hybridisation is a widely used method that permits the localisation of target mRNA in a preserved tissue section. This information helps to elucidate clues towards understanding the mRNA distribution and function of a particular gene; thus, this method is widely utilised, especially in gene characterisation studies. Most *in situ* hybridisation methods either use non-isotopically or isotopically labelled riboprobes. Digoxigenin (DIG) compound is popularly used in the former type of labelling. For this, hybridised DIG-labelled riboprobes are normally detected with high affinity anti-digoxigenin (anti-DIG) antibodies that are conjugated to alkaline phosphatase that consequently allows for colorimetric visualisation of the anti-DIG antibody conjugate using NBT (nitro blue tetrazolium chloride)-BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidine salt) substrate solution. There is also the alternative method of using fluorescence-labelled probes. In addition, various *in situ* hybridisation protocols have been developed and reported over the years (Coen *et al*., 1990; Jackson, 1992).

In situ hybridisation, however, can be problematic when it comes to detecting low abundance mRNA or diffusely localised mRNA (McFadden, 1995). For oil palm samples, the previously used protocols based on Coen *et al*. (1990) and Jackson (1992) worked well only on certain transcripts. On some occasions, the difference between the sense and antisense hybridised sections was not distinct enough. Even after several optimisation attempts on hybridisation temperature, stringency washes, *etc*., the signals were still indistinguishable.

After many attempts at testing and optimising various *in situ* hybridisation protocols on several selected genes, we describe here a modified method of RNA *in situ* hybridisation based mostly on the standard protocol (Jackson, 1992), but with particular steps adapted from the method used by Muller and Sheen (2008) as well as a Northern hybridisation protocol (Church and Gilbert, 1984) routinely used in our laboratory. In this modified protocol, the tissue fixation and processing up to

* Malaysian Palm Oil Board, P. O. Box 10620, 50720 Kuala Lumpur, Malaysia. E-mail: meilina@mpob.gov.my

** Sime Darby Technology Centre Sdn Bhd, Block A, UPM-MTDC Centre III, Lebuh Silikon, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

the hybridisation steps are as described in Jackson (1992). The post-hybridisation step that involves washing of the hybridised tissue sections was modified as detailed in the Materials and Methods section. The subsequent steps are as described by Muller and Sheen (2008).

MATERIALS AND METHODS

Riboprobe Synthesis

Riboprobes were synthesised using the Ampliscribe™ T3 and T7 Flash™ Transcription Kit (Epicentre® Biotechnologies) with PCR products of interest containing T3 and T7 promoter regions, and replacing the nucleotides with 2 µl of 10X DIG RNA Labelling mix (Roche). Carbonate hydrolysis was then conducted on the DIG-labelled transcripts according to standard protocols, and the RNA was purified by ethanol precipitation overnight. The riboprobe was finally dissolved in 50% (v/v) formamide and stored at -20°C until needed. The concentration of riboprobe was estimated by performing dot-blot analysis according to the DIG Application Manual (Roche).

Fixation and Hybridisation

Samples were fixed and embedded according to standard protocols (Coen *et al*., 1990; Jackson, 1992). Pre-treatment was also based on standard protocols involving deparaffinization of the sections in xylene, followed by dehydration in a series of ethanol solutions. The slides were then immersed in 2X SSC solution for 15 min, followed by 30 min incubation in proteinase K solution [1 µg ml^{-1} proteinase K (Sigma) in 50 mM Tris-Cl pH 8.0, 5 mM EDTA] at 37°C, and the reaction was stopped by incubating in 2 mg ml^{-1} glycine in 1X phosphate-buffered saline (PBS), followed by rinsing twice in 1X PBS for 2 min each time. Subsequently, the sections were fixed in 4% (w/v) paraformaldehyde for 10 min, and rinsed twice in 1X PBS for 5 min each time. The sections were acetylated by incubating in 0.1 M triethanolamine, pH 8.0 and 0.5% (v/v) acetic anhydride for 5 min, rinsed twice in 1X PBS for 5 min each time. The slides were finally dehydrated in a series of ethanol solutions.

The treated slides were then applied with 100 µl hybridisation solution per slide comprising 80 µl of hybridisation buffer (10 µl 10X *in situ* salts, 40 µl deionised formamide, 20 µl 50% (w/v) dextran sulphate, 2 µl 50X Denhardt's solution, 1 µl tRNA (100 mg ml^{-1}) and 7 µl DEPC-treated water) and 20 µl of denatured probe in 50% formamide (25-50 ng/slide). Hybridisation was conducted overnight in a humidified chamber with 2X SSC and 50% formamide at 50°C.

Post-hybridisation

Standard Method (Jackson, 1992). The hybridised slides were washed twice in 0.2X SSC for 1 hr with gentle shaking at 55°C, followed by washing in 1X NTE (0.5 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA) twice for 5 min each time at 37°C. The slides were incubated in RNAse solution (20 mg ml^{-1} RNAse A in 1X NTE) with gentle shaking for 30 min (37°C). Subsequently, two washes in 1X NTE (37°C) for 5 min were conducted, followed by a 1-hr incubation in 0.2 X SSC with gentle shaking at 55°C . The slides were then rinsed in 1X PBS buffer for 5 min at room temperature, followed by incubation in 1% (w/v) blocking reagent in 1X TBS (100 mMTris-HCl pH 7.5, 150 mM NaCl)) for 45 min at room temperature. This was followed by incubation in Blocking solution B (1% (w/v) BSA (Bovine Serum Albumin, Sigma) and 0.3% (v/v) Triton X-100 in 1X TBS) for 45 min at room temperature.

The slides were covered with 100 µl 1:1250 diluted anti-Digoxigenin-AP (Roche) in Blocking solution B, and incubated in a humidified chamber for 2 hr at room temperature or overnight at 4°C. After incubation, the slides were washed four times in Blocking solution B for 10 min each at room temperature to remove the unbound antibody. The slides were then incubated in a buffer containing 100 mM Tris-Cl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl for 10 min at room temperature. This step was repeated. Finally, the slides were each stained with 150-200 µl detection buffer (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing diluted NBT/BCIP stock solution (Roche) at 1:50 ratio. This was performed in the dark for three to four days, or until colour developed.

Procedures according to Muller and Sheen (2008). After hybridisation, the slides were washed twice with 2X SSC in 50% formamide for 1 hr each time at 58°C. They were then incubated twice in 1X NTE buffer at 37°C for 5 min each. The slides were immersed in pre-heated (37°C) 1X TBS buffer (100 mM Tris-Cl, pH 7.5, 150 mM NaCl), and allowed to cool to room temperature. Anti-Digoxigenin-AP, diluted 1:2000 into 1% blocking reagent in 1X TBS was added, and incubation was carried out for 2 hr at room temperature. The slides were washed twice with 100 mM Tris pH 9.5, 100 mM NaCl for 10 min each. Staining in 150-200 µl detection buffer (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing diluted NBT/BCIP stock solution (Roche) at 1:50 ratio was performed in the dark overnight or until colour developed.

Modified method. After hybridisation, the slides were washed with 5% SDS in 40 mM sodium phosphate buffer followed by 1% SDS in 40 mM sodium phosphate buffer at 55°C with gentle

shaking for 15 min each wash. The slides were then rinsed twice with 1X NTE buffer (500 mM NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA) for 5 min each time at 37°C, and equilibrated in a pre-warmed (37°C) 1X TBS buffer (100 mM Tris-Cl, pH 7.5, 150 mM NaCl), then allowed to cool to room temperature. Subsequently, the antibody-binding step was carried out by covering the slides with 100 µl blocking solution [1% (w/v) Blocking reagent (Roche) in 1X TBS] containing diluted anti-Digoxigenin-AP Fab fragments (Roche) at 1:2000, and incubated for 2 hr at room temperature. Finally, the slides were washed twice in 100 mM Tris-Cl, pH 9.5, 100 mM NaCl for 10 min each prior to staining. Staining was performed overnight in 1:50 diluted NBT/BCIP stock solution (Roche) in a detection buffer (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) in the dark.

Enzymatic Termination Step

Enzymatic reaction was terminated by washing the slides twice in distilled water for 2 min each time, and dehydrated in a series of ethanol solutions for 30 s each, followed by rehydration through the same ethanol series in reverse, ending with a 2-min incubation in distilled water. The slides were air-dried and mounted in 50% (v/v) glycerol.

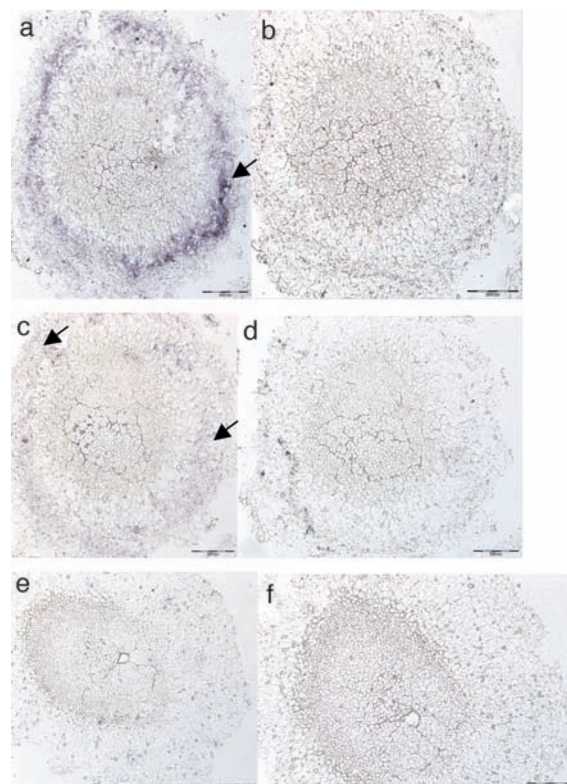

Figure 1. Comparison of OPSC10 in situ *hybridisations on oil palm suspension callus using the different protocols. a, b: modified protocol; c, d: hybridisation protocol by Muller and Sheen (2008); e, f: standard protocol. The a, c, e and b, d, f are antisense and sense hybridisations, respectively. Arrows indicate hybridisation signals. Horizontal bar = 200 um.*

RESULTS AND DISCUSSION

The genes tested for the comparison of all three protocols (Jackson, 1992; Muller and Sheen, 2008; and the modified protocol) included a constitutively expressing gene, an elongation factor 1-α (EL687602), and three selected genes of interest, namely, OPSC10 (AY254310), EgNAC1 (DQ267443) and EgSERK (unpublished). The elongation factor 1-α was included as it was considered a high abundance transcript and would be detected by most protocols. The ACC oxidase gene (OPSC10) was used as a positive control as it had worked previously with the standard protocol on oil palm suspension callus. Using the standard protocol, EgNAC1 and EgSERK were not detected in any of the tissues used although expression was detected through Northern analysis and real time PCR (data not shown).

Positive signals with the antisense riboprobes were clearly distinguishable compared with the hybridisation results of sense riboprobes (control) generated from OPSC10, a putative oil palm ACC oxidase gene. *In situ* hybridisation of OPSC10 had worked previously with the standard protocol and its results were reconfirmed in this study using the modified method. The modification rendered localised signals to be clearly recognised in antisense hybridisations compared with its corresponding sense hybridisations (*Figure 1*). The more intense localised signals detected using the modified protocol (*Figure 1a*) were able to clearly distinguish the regions where the riboprobes had bound strongly as compared with the signals resulting from the use of the protocol by Muller and Sheen (2008) (*Figure 1c*). On the other hand, the signals were very faint using the standard protocol (*Figure 1e*).

We have further tested the modified protocol with two other genes of interest, EgNAC1 and EgSERK, which were previously undetected using the standard protocol. The localisations of the elongation factor 1-α (ELF-1 α) (EL687602), EgNAC1 and EgSERK transcripts were clearly distinguishable in the sections hybridised with the antisense probes compared with their respective sense probes (*Figure 2*), suggesting that the modified method was more sensitive for our applications.

To verify the utility of the modified protocol on other plants, we tested it on *Arabidopsis*, a model plant extensively used and studied in plant molecular biology research. We tested the protocol successfully on various tissues from *Arabidopsis thaliana* using TUB5, a beta-tubulin (NM_101856), as well as when using a heterologous probe ELF-1α (EL687602) from oil palm (*Figure 3*).

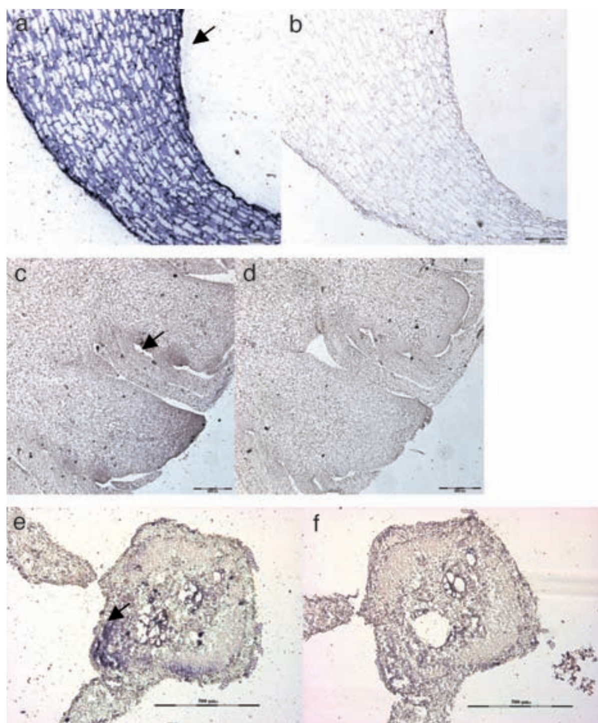

Figure 2. In situ *RNA hybridisation on other oil palm tissues with other probes. a, b: germinating* in vitro *embryoids hybridised with ELF-1α, bar = 200 um; c, d: young inflorescence hybridised with EgNAC1, bar = 200 um; e, f: embryogenic calli hybridised with EgSERK, bar = 500 um. The a, c, e are antisense and b, d, f are sense hybridisations. Arrows indicate hybridisation signals.*

The use of high concentrations of sodium dodecyl sulphate (SDS) in the washing buffer improves sensitivity (Church and Gilbert, 1984) and reduces background quite considerably. The method reported by Church and Gilbert (1984) was also found to improve the detection of NGF (nerve growth factor) mRNA by at least 10-fold compared with using a formamide buffer for Northern analysis (Shifman and Stein, 1995). Moreover, SDS is an effective blocking agent; thus, the RNase digestion step of unhybridised riboprobes could be eliminated from the *in situ* hybridisation protocol (Shain and Zuber, 1996). Whole-mount *in situ* hybridisation of *Xenopus laevis* embryos using high SDS buffer was also found to improve signal-to-noise ratio. The amount of time reported for this SDS-based protocol was reduced by half compared with other methods (Shain and Zuber, 1996). In this study, the modified protocol could also be completed in a shorter time compared with the previously used standard protocol, for which the post-hybridisation procedures included 2 to 3 hr of washing in SSC/ formamide buffers, RNAse treatment and blocking steps with buffers containing BSA. These post-hybridisation procedures took approximately 9 hr in total, while the post-hybridisation procedures in the method reported here could be accomplished within 3 hr.

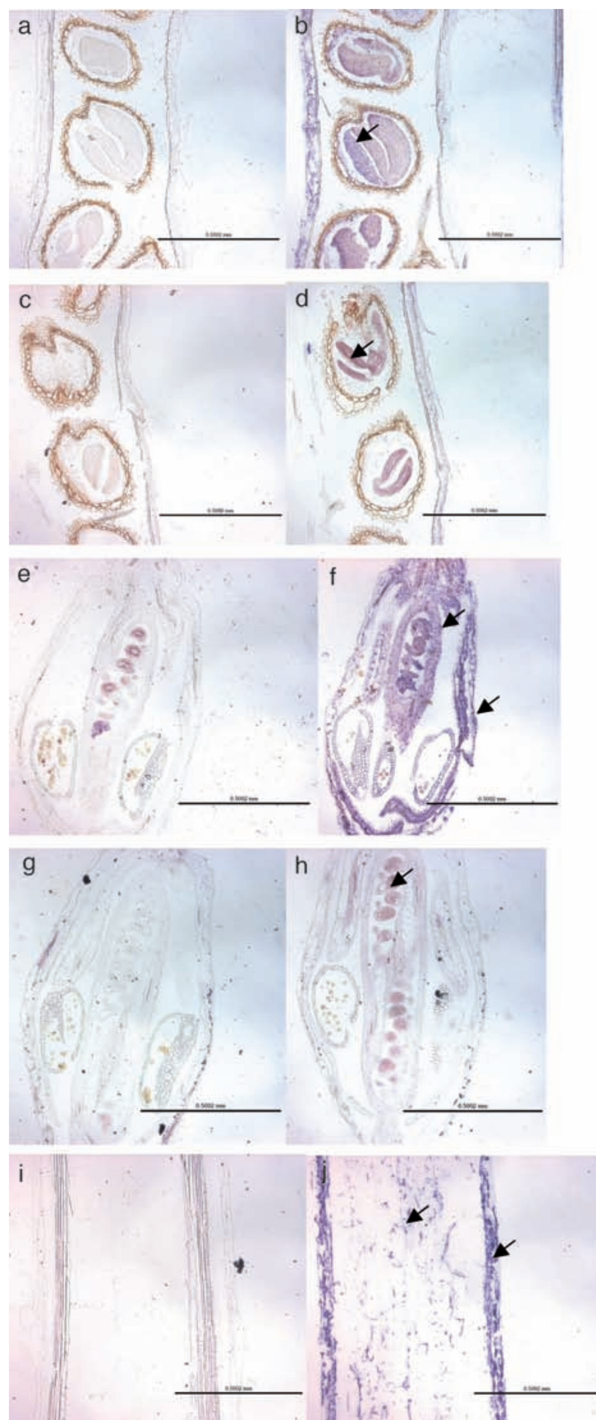

Figure 3. In situ *RNA hybridisation on various* Arabidopsis *tissues. Siliques hybridised with ELF-1α (a, b) and TUB5 (c, d); flowers hybridised with ELF-1α (e, f) and TUB5 (g, h); stem hybridised with ELF-1α (i, j). The a, c, e, g, i are sense and b, d, f, h, j are antisense hybridisations. Arrows indicate hybridisation signals. Bar = 500 um.*

CONCLUSION

We find that the modified RNA *in situ* hybridisation protocol reported in this study is shorter, simpler, uses fewer reagents and works well with reproducible results. This can be an alternative method to the rather lengthy standard method currently used for plants. Thus, costs and, more importantly, time

are reduced using this method. Our method may also be more sensitive as it reduces background staining.

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REFERENCES

CHURCH, G M and GILBERT, W (1984). Genomic sequencing. *Proc. of the National Academy of Sciences USA, 81*: 1991-1995.

COEN, E S; ROMERO, J M; DOYLE, S; ELLIOT, R; MURPHY, G and CARPENTER, R (1990). *Floricaula*: a homeotic gene required for flower development in *Antirrhinum majus*. *Cell, 63*: 1311-1322.

JACKSON, D P (1992). *In situ* hybridization in plants. *Molecular Plant Pathology: A Practical Approach* (Gurr, S J; McPherson, M J and Bowles, D eds.). Vol. 1. Oxford University Press, Oxford. p. 163-174.

McFADDEN, G I (1995). *In situ* hybridization. *Methods in Cell Biology* (Galbraith, D W; Bohnert, H J and Bourque, D P eds.). Vol. 49. Academic Press, San Diego. p.165-183.

MULLER, B and SHEEN, J (2008). Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature, 453*: 1094-1097.

SHAIN, D H and ZUBER, M X (1996). Sodium dodecyl sulfate (SDS)-based whole-mount *in situ* hybridization of Xenopus laevis embryos. *J. Biochem. Biophys. Methods, 31*: 185-188.

SHIFMAN, M I and STEIN, D G (1995). A reliable and sensitive method for non-radioactive Northern blot analysis of nerve growth factor mRNA from brain tissues. *J. Neuroscience Methods, 59*: 205-208.