Embedding root and nodule tissue in plastic (BMM)

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Chapter 3.2

EMBEDDING ROOT AND NODULE TISSUE IN PLASTIC (BMM)

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Immunolocalisation and in situ *hybridisation allow the detection of proteins and RNA respectively, in individual cells of different tissue types. Hybridisation on serial 5-8 µm sections is most frequently performed with tissue embedded in paraffin wax but it is often difficult to obtain high-resolution sections from soft tissue with this embedding process. To overcome this, alternative localisation protocols have been developed utilising plastic resins. We have used plastic embedded tissue from* Lotus japonicus *roots and young nodules successfully in immunolocalisation experiments and developed a protocol that can also be adapted for* in situ *RNA localisation studies. The different parameters tested are described, as well as the use of alkaline phosphatase- or fluorescently-conjugated secondary antibodies.*

INTRODUCTION

Functional analyses of genes require essential knowledge of their expression pattern at the cellular level and it is often valuable to compare the spatiotemporal distribution of both the transcriptional and translational products, since in some cases the RNA expression pattern of a given gene does not overlap with that of the encoded protein (Lucas, 1995).

In situ hybridisation and immunocytochemistry give the possibility to detect RNA and proteins respectively, in the individual cells of different tissues. Although, thin sections of tissue embedded in paraffin wax are routinely used for hybridisation studies, it has always been difficult to obtain high-resolution sections from soft tissues embedded in paraffin. As a result, alternative methodologies making use of plastic polymers suitable for embedding have been developed (Baskin et al., 1992; Kronenberger et al., 1993; Ruzin 1999; Scarpella et al., 2000).

In *Lotus japonicus,* the use of paraffin works well with nodule tissue, but root sections maintaining a reasonable preservation of the cellular structure are difficult to obtain. A wealth of information is rapidly accumulating with respect to the plant genes/proteins involved in the early recognition steps during the symbiosis between rhizobia and legumes (Schauser et al., 1999; Stracke et al., 2002, Radutoiu et al., 2003, Madsen et al., 2003). With this in mind, it would be valuable to adapt existing RNA and protein localisation protocols to allow for higher resolution root sections in order to follow the induction of such genes/proteins, for example, in root hair and epidermal cells where signal molecules are perceived.

We have successfully employed plastic embedding for immunolocalisation of *Lj*SBP1 on *L. japonicus* root tissue (Flemetakis et al., 2002), as well as for different superoxide dismutases (EC 1.15.1.1) "SODs" in young nodules. The particular protocol used in these experiments is described in detail in the following paragraphs and has been used for root and nodule tissue prepared for *in situ* RNA localisation studies. The use of plastic in such studies has been described for Arabidopsis (Kronenberger et al., 1993) and rice (Scarpella et al., 2000) but it has proven to be difficult to implement in *L. japonicus*, in terms of high signal intensity when compared to paraffin sections processed in parallel. Some modifications of both embedding and hybridisation procedures have been tried, but still further optimisation is needed for plastic embedded tissue to become the preferred starting material for *in situ* RNA hybridisation studies in our hands.

The detection method used in most of our immunolocalisation experiments and in all the *in situ* RNA hybridisation studies so far, is based on the 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phophatase (BCIP) detection of alkaline phosphatase-conjugated anti-rabbit secondary and anti-DIG primary antibodies (Jackson et al., 1994). In some of the immunolocalisation experiments a fluorescently-labelled secondary antibody has been used instead, with subsequent monitoring of the signal by confocal microscopy, providing a useful alternative for a better resolution at the cellular level and it will also be tested for detection of transcripts. The use of different fluorescently-labelled (secondary) antibodies for detection of signals in the hybridisation procedures would furthermore enable simultaneous localisation of two proteins or transcripts in the same tissue.

MATERIALS AND PROCEDURE

Immunolocalisation

Fixation

Root and young nodule tissue was cut and placed in fixation buffer and then mild vacuum was applied for 30-60 minutes, until the tissue sank. The tissue was incubated overnight at 4°C, slowly rotating. Fixation buffers used were made from a fresh stock of paraformaldehyde diluted to a concentration of 4% in 1x PBS or 0.1M Na_2HPO_4 buffer with or without 0.25% glutaraldehyde, while for some experiments 2% paraformaldehyde and 2.5% glutaraldehyde in 1x PBS was used instead. All fixation buffers contained 10 mM DTT.

Dehydration

The tissue was dehydrated in ethanol series including 10 mM DTT: 70%, 80%, 90%, 96%, 2 times 100%, 30 minutes each step, slowly rotating at room temperature.

Infiltration and embedding

Tissue was embedded in the acrylic medium BMM, which, in contrast to most other plastics, can subsequently be removed from the obtained sections using acetone.

BMM

BMM is a mixture of 4 Butyl Methacrylate, 1 Methyl Methacrylate (Merck), 10 mM DTT and either as photo-catalyst for polymerisation: 0.5% (w/v) benzoin ethyl ether/benzoin methyl ether (both work equally well), combined with UV polymerisation at -20°C to 4°C, or a thermal-catalyst: 1% benzoyl peroxide, combined with heat (60°C) polymerisation (see Comments). After mixing, the BMM solution is bubbled with N_2 in order to displace dissolved oxygen, since oxygen will inhibit polymerisation.

Following dehydration, the tissue was gradually infiltrated with BMM in a series of: 3:1, 1:1, and finally 1:3 of 100% EtOH:BMM, left slowly rotating at 4°C for more than 1½ hours at each step, and finally at 4^0C overnight in 100% BMM. The following day the BMM solution was replaced with fresh and incubated another 2 hours at 4°C, before embedding.

Embedding was performed in BEEM capsules (Agar Scientific, UK), the important features of the capsules are that they are airtight and UV transparent.

UV-polymerisation

The UV lamp(s) (6-8 W, long-wave, Philips, The Netherlands) were placed 15 cm (for root tissue) or 20-25 cm (for nodule tissue) below the capsule holders (see Comments).

Sectioning

Sections of 5-8 μm were made with a Leica RM 2165 rotary microtome and floated on drops of sterile water on BIOBOND (EMS catalogue #71304) coated slides. Slides were allowed to dry on a hot plate for a few hours to allow the sections to adhere to the slides.

Immunolocalisation

Blocking, hybridisation, and washing was all done in the same *immuno-buffer* consisting of: 20 mM Tris-HCl pH=8.2, 0.9% NaCl, 0.01% BSA-$_c$[TM] (Aurion) (0.1% for hybridisation) and 0.02% fish skin gelatine (Sigma G7765). In some of the experiments the sections went through a denaturation step before the actual localisation protocol, a treatment that seemed not to be essential for the particular proteins tested, but might have an impact in general depending on the protein in question. Denaturation was performed in 0.4% SDS, 3 mM β-mercaptoethanol, 12

mM Tris-HCl pH=6.8.

The equilibration buffer for alkaline phosphatase/ NBT-BCIP detection was 0.2 M Tris-HCl pH 9.5, 10 mM $MgCl_2$ and it was also used to dilute NBT and BCIP before addition to the slides. 10 mg NBT was dissolved in 0.1 ml dimethylformamide and 0.1 ml buffer. 5 mg BCIP was dissolved in 0.1 ml dimethylformamide before mixing with 30 ml buffer. Alternative ready-made NBT-BCIP tablets (Roche) were dissolved in water and added to the slides.

Cover slips were mounted in *gelvatol* (20 g polyvinyl alcohol dissolved in 40 ml glycerine and 80 ml PBS (pH= 6 to 7), stirring overnight at 4°C, after centrifugation the supernatant was used as mounting medium). In the case of fluorescent signal, we used gelvatol containing the anti-fading agent DABCO (Sigma), 100 mg/ml.

Immunolocalisation protocol

1. The embedding medium (BMM) was removed from the sections by two rounds of 15 minute acetone treatment (see Comments)

2. Slides were washed in 1x PBS

3. Denaturation of proteins were performed in the buffer given above, incubation 20 minutes at room temperature (this step is optional).

4. Slides were blocked in immuno-buffer 3 times for 10 minutes at room temp.

5. Primary antibody was diluted in immuno-buffer and applied slides resting on a racked hybridisation container with water in the bottom, to keep the humidity high. Slides were incubated with primary antibody overnight at 4°C or at room temperature for a minimum of two hours.

6. Slides were washed 5 times for 10 minutes in immuno-buffer at room temperature

7. Secondary antibody, diluted in immuno-buffer was applied to the slides and incubated at room temperature for more than two hours in the dark (darkness is essential, when using fluorescently-labelled secondary antibodies).

8. Slides were washed 5 times for 10 minutes in immuno-buffer

Detection

For fluorescently-labelled secondary antibodies, slides were rinsed in H_2O, mounted in gelvatol containing DABCO, and examined with a confocal microscope.

For alkaline phosphatase/NBT-BCIP detection, slides were equilibrated 3 times for 5 minutes in equilibration buffer, before adding the NBT/BCIP substrate. The slides were incubated at room temperature, in the dark, until colour appeared and checked regularly under a light microscope. The colour reaction was stopped by rinsing several times in H_2O and coverslips were mounted with gelvatol before inspection.

In situ RNA hybridisation

Fixation

Root and young nodule tissue was cut and placed in fixation buffer and a mild vacuum was applied for 1-2 times for 30 minutes, until the tissue sank. The tissue was incubated overnight at 4°C, slowly rotating. Fixation buffers used were made from a fresh stock of paraformaldehyde diluted to a concentration of 4% in 1x PBS or 0.1M Na_2HPO_4 buffer with 0.25% glutaraldehyde.

Dehydration

The tissue was dehydrated in ethanol series containing DTT (see Comments): buffer, 10%, 30%, 50%, 70%, 90%, 3 times 100%, 30 minutes each step, slowly rotating at room temperature.

Infiltration and embedding

Following dehydration, infiltration and embedding was performed as described above (see Comments).

Sectioning

Sections of 5-8 µm were made with a Leica RM 2165 rotary microtome and floated on drops of RNase-free water on Super Frost R Plus slides (Menzel Glaser). Slides were allowed to dry on a hot plate for a few hours to allow the sections to adhere to the slides.

In situ RNA hybridisations

Hybridisations were carried out according to the protocol we use as standard on paraffin sections, essentially as described in Papadopoulou et al., 1996. In one comparative experiment, we tested also the protocol used for maize and rice (Langdale, 1994; Scarpella et al., 2000), but the different protocols did not show any significant differences in signal detection.

Glass ware was baked 4 hours at 180°C, hybridisation chambers and plastic slide holders were treated with 0.4 M NaOH and solutions were treated with 0.1% (v/v) DEPC prior to use, to be RNase-free.

Probe transcription and labelling

The RNA probes were DIG labelled, using a DIG RNA labelling mix from Roche, and a T7 transcription kit from Ambion. Approximately 1 µg of linearised cDNA template was mixed with 2 µl of DIG RNA labelling mix, 2 µl transcription buffer and 2 µl T7 RNA polymerase in a total reaction volume of 20 µl. Transcription was allowed to proceed for more than 60 minutes at 37°C, 1 µl DNaseI (Ambion) was added and incubated at 37°C for more than 30 minutes, to remove the cDNA template. The reaction was stopped and the product precipitated twice, according to the Ambion protocol, in order to remove un-incorporated nucleotides. The product yield was estimated on an agarose gel and approximately 0.5-1 µg DIG-RNA was

used for hybridisation, after hydrolysis, per slide (see Comments). Hydrolysis of the transcripts was performed according to Papadopoulou et al. (1996), to yield probes of approximately 150 bp.

In situ RNA hybridisation protocol

1. The embedding medium (BMM) was removed from the sections by two rounds of 15 minutes acetone treatment (see Comments). Sections were left to air dry.

2. Sections were hydrated in an ethanol series: 100%, 90%, 70%, 50%, 30%, 10%, 3 times H_2O, 1 minute each step

3. Proteinase K treatment was performed in 100 mM Tris-HCl pH=7.5, 50 mM EDTA, 1 mg/ml proteinase K, for 30 minutes at 37°C with gentle shaking (see Comments).

4. Slides were rinsed 3 times in H_2O.

5. To remove positive charges, slides were acetylated in 0.1 M triethanolamine, 0.25% (v/v) acetic anhydrite. Triethanolamine was dissolved in H_2O, acetic anhydrite added just before the slides and the solution was stirred until foam appeared, where after the slides were soaked in the solution and incubated 10 minutes at room temperature.

6. Slides were rinsed for 10 minutes in 2x SSC.

7. Slides were dehydrated in an ethanol series: 10%, 30%, 50%, 70%, 90%, 2 times 100%, 1 minute each step, and air dried in vacuum for at least one hour.

8. Meanwhile, the hydrolysed and precipitated probes were resuspended in 10 μl H_2O. 10 μl deionised formamide was then added, mixed with probe, denatured at 68°C for 10 minutes, cooled on ice, and mixed with 80 μl hybridisation mixture. The hybridisation solution was heated at 60°C for 3 minutes before it was added to the slide and a cover slip was applied. Slides were resting on a rack, with 2x SSC soaked paper below to keep the hybridisation chamber moist overnight.

9. Hybridisation was performed at 42°C overnight (12-16 hours).

10. Post-hybridisation, the cover slips were gently let to glide off in a washing buffer of 4x SSC containing 5 mM DTT, slides were washed 4 times for 10 minutes at room temperature.

11. RNase treatment was performed in 500 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH=7.5, 50 μg/ml RNaseA, 30 minutes at 37°C, with subsequent washes in RNase buffer for 4 times for 15-20 minutes at 37°C.

12. Slides were washed in 2x SSC, 30 minutes at room temperature, prior to blocking, antibody incubation and detection.

13. Slides were incubated in B1 for 5 minutes at room temperature.

14. Blocking was performed first in B1 + blocking reagent and then in B2 + BSA, at least 30 minutes in each buffer at room temperature.

15. AP conjugated anti-DIG-antibody was diluted in B2+0.1% BSA, applied to slides and incubated for at least 2 hours at room temperature, in the dark.

16. Slides were washed in B2, 30 minutes and in B1 3 times for 20 minutes at room temperature before detection.

Hybridisation mixture

- 50% deionised formamide, 300 mM NaCl, 10 mM Tris-HCl pH=7.0, 1 mM EDTA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA, 10% Dextran sulphate, 70 mM DTT

Buffers

- B1: 100 mM Tris-HCl pH=7.5, 150 mM NaCl
- B2: 100 mM Tris-HCl pH=7.5, 150 mM NaCl, 0.3% Tween-20

Blocking buffers

- B1 + 0.5% (w/v) blocking reagent (Roche)
- B2 + 1% (w/v) BSA (0.1% BSA for antibody incubation)

Detection

Equilibration and detection was performed as described above for immunolocalisation. The colour reaction was stopped by rinsing the slides several times in H_2O. The slides were then dehydrated in an ethanol series, allowed to air dry and mounted with Eukit (Agar Scientific, UK) before inspection.

COMMENTS

For the *in situ* RNA hybridisation studies, we tested both UV polymerisation and heat polymerisation, in the embedding step. Using UV polymerisation, nodules do not polymerise properly in the central tissue. Better polymerisation of the nodules was obtained using heat polymerisation, but it turned out that with this procedure no signal could be obtained, whereas parallel experiments on UV polymerised nodules, gave signal, although weak. So it seems that heat polymerisation is incompatible with the protocols tested for *in situ* RNA hybridisation.

The optimal distance from the UV source, and thereby the speed of polymerisation should be tested for each specific tissue. We found that increasing the distance for nodule tissue/decreasing the speed of polymerisation, allowed the central tissue of the nodule to be polymerised in a bigger percentage of the samples.

For thicker sections, the slides may have to be incubated longer in acetone to remove the plastic completely. This does not seem to interfere with the following reactions.

For the dehydration, DTT was included in the EtOH steps, in some experiments 10 mM was added in each step, as used in immunolocalisation studies; in other experiments only 1 mM DTT was added in the steps until 100% EtOH, where 10 mM DTT was then added, as described in Kronenberger et al. (1993). No significant difference was observed.

The amount of probe found to be needed for obtaining only a weak signal for *in situ*

RNA hybridisation on BMM embedded tissue was approximately 10 times higher than for paraffin embedded tissue.

We have tried treating the sections longer with proteinase K (up to 1 hour), to see if that would allow better penetration of the probe, but we did not observe any difference in the final signal.
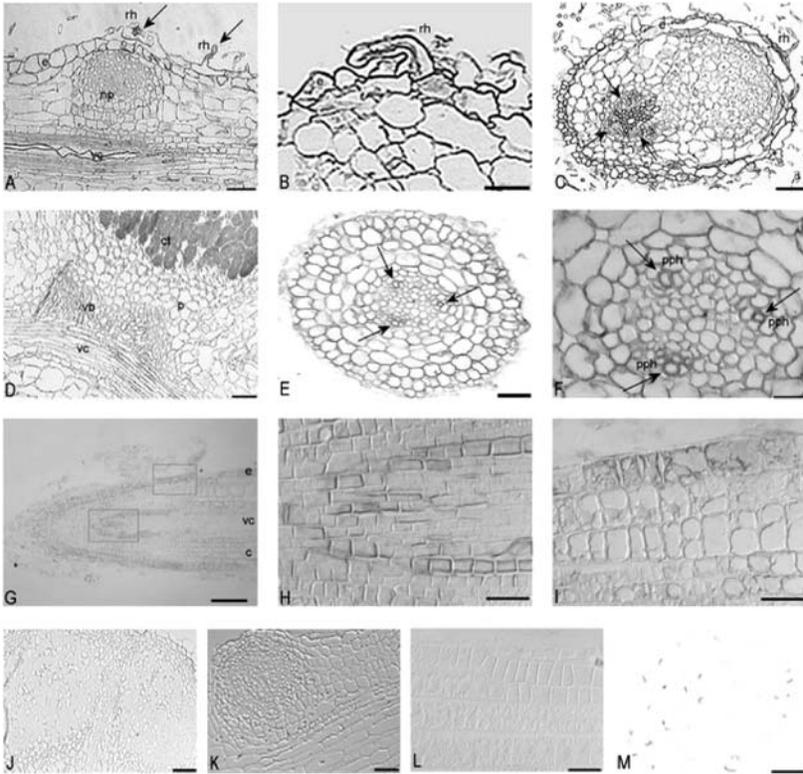
RESULTS

Figure 1. Localisation of LjSBP1 in root tips and roots. Immunolocalisation of the selenium binding protein (SBP) of Lotus japonicus *on roots, nodules and root tips. Detection was performed with alkaline phosphatase conjugated secondary antibodies. The embedding of the root tissue in BMM resulted in sections of high cellular resolution, enabling the detection of SBP in cellular structures that are otherwise difficult to preserve in detail, like root hairs (A), infection threads (B), and the phloem of the vascular cylinder (C). Furthermore, signal was detected in the vascular bundles and the infected cells of the central tissue in mature nodules (D). H and I are differential interference contrast microscopy pictures of the marked regions in picture G. Signal was observed in the cells of the epidermis and the outer cortex of the root tip (I) as well as on the vasculature (H). Transverse sections of that area allowed the identification of the protophloem cells of the stele as the cells with high expression levels of the protein (E and F). J-M sections incubated with pre-immune serum. A to D, G, J, K, bars=50µm. E, M, bars=25µm.*

F, H, I, L, bars=15µm. c, cortex; e, epidermis; np, nodule primordium; p, parenchyma; pph, protophloem; rh, root hair; vb, vascular bundle; vc, vascular cylinder.
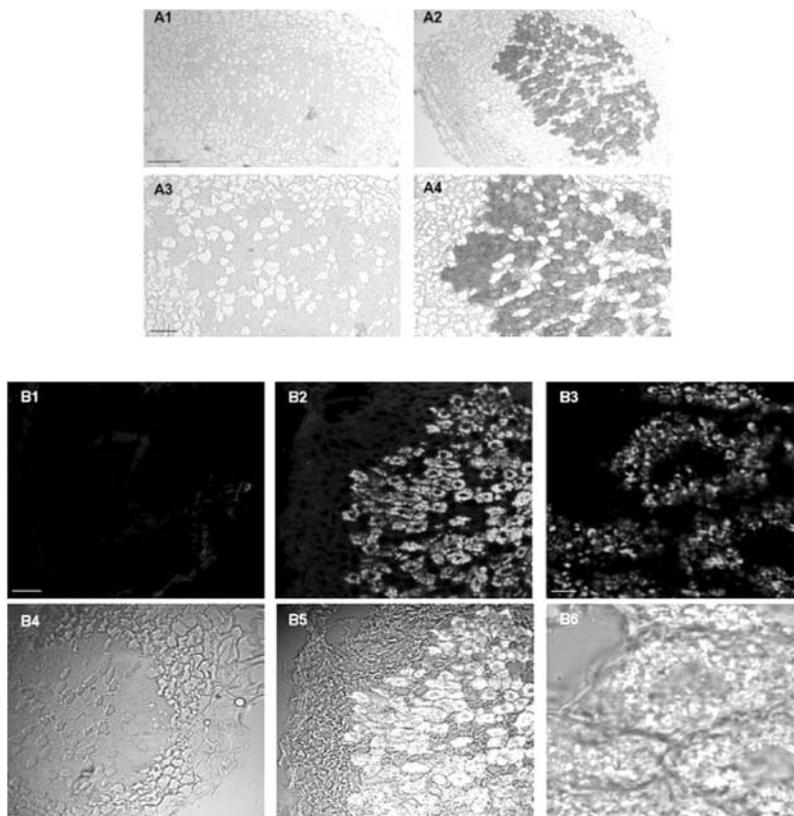

Figure 2. Comparison of results obtained with the same primary antibody (MnSOD) and either AP-detection (A) or fluorescent detection (B). Immunolocalisation of MnSOD in Lotus japonicus nodules. Images represent longitudinal sections of 13 days post inoculation (dpi) nodules. Alkaline phosphatase-conjugated (A) and alexa488-conjugated (B) anti-rabbit immunoglobulin Gs were used as secondary antibodies. A3 and A4 are magnifications of A1 and A2, respectively. B1, B2, and B3, are the confocal microscope images showing the signal in the infected cells of the central tissue (background subtracted). B4, B5, and B6, are overlays of B1, B2, and B3, and the corresponding transmitted light images. B3 and B6 are a high magnification of the central tissue. No signal was observed in the controls (A1, A3, B1, B4). Bars = 50 µm (A1 and A2), 30 µm (B1, B2, B4, and B5), 20 µm (A3 and A4), and 3 µm (B3 and B6). See colour plate 3 (G) for a colour version of B1-B6.
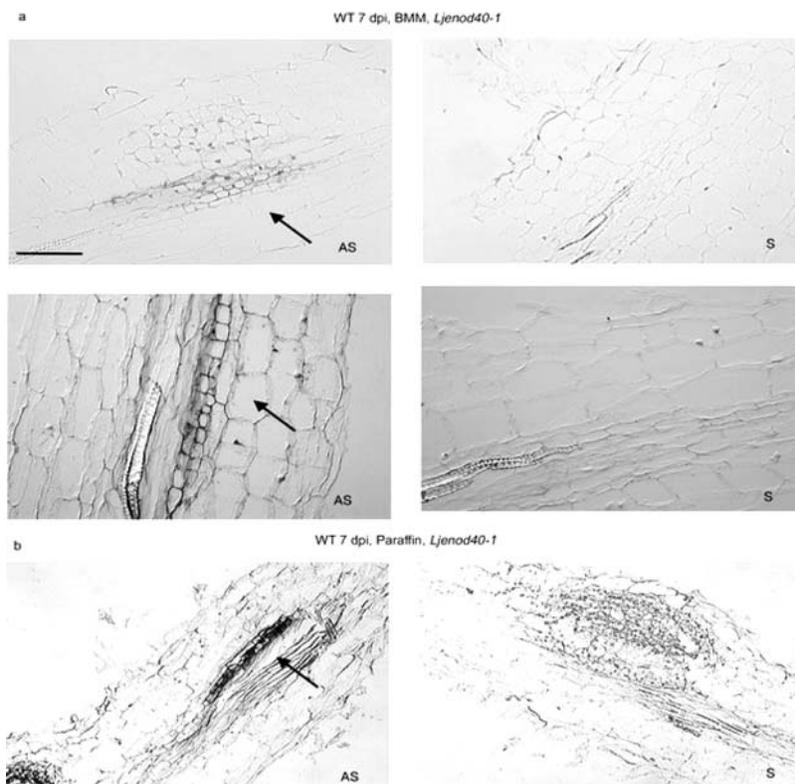
Figure 3. The weak signals obtained for RNA localisation of Ljenod40-1 transcripts are compared to signals obtained on paraffin sections. Ljenod40-1 localises in the pericycle cells at the base of dividing cortical cells. The signal obtained on BMM embedded tissue (a) is much lower, but the cellular resolution/tissue preservation better compared to the paraffin embedded tissue (b). AS, sections probed with antisense Ljenod40-1 transcript. S, sections probed with sense Ljenod40-1 transcript, as negative control. Arrows indicate signal. Detection was performed with alkaline phosphatase-conjugated anti-DIG primary antibody. Bar = 100 μm.

CONCLUDING REMARKS

As seen in Figures 1 and 2, immunolocalisation works well on plastic embedded *L. japonicus* root and nodule tissue, with only minor adjustments of the original protocol. Adjustments mainly concern the speed of polymerisation for different tissue types, as for example the need for slow polymerisation of nodules, to allow also the central tissue to polymerise. Furthermore, one should determine for each protein/antibody pair whether a denaturation step has to be included in the immunolocalisation protocol.

From Figure 3 it is evident that more parameters should still be tested in order to enhance the signals obtained on plastic embedded tissue used for *in situ* RNA:RNA

hybridisation studies. However, even though we do not achieve signal strengths as high as those obtainable with paraffin sections, we believe it worth working to optimise the protocol, considering the much higher cellular resolution in sections of plastic embedded root tissue, in particular.

ACKNOWLEDGEMENTS

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