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# In situ hybridization of a radioactive RNA probe on resin-embedded legume root-nodule sections: a tool for observing gene expression in the rhizosphere?

Olivier SCHUMPP<sup>a</sup>, Hassen GHERBI<sup>a</sup>, Jacques ESCOUTE<sup>b</sup>, Hélène PAYRE<sup>a</sup>, Jean-Jacques DREVO<sup>a\*</sup>

<sup>a</sup> UMR Rhizosphère et Symbiose, INRA, place Pierre Viala, 34060 Montpellier Cedex 1, France

<sup>b</sup> CIRAD/BIOTROP UMR BDPPC, avenue d'Agropolis, 34060 Montpellier Cedex, France

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**Abstract** – In this work we performed in situ hybridization of a carbonic anhydrase RNA <sup>35</sup>S-labeled probe on nodule sections previously embedded in a methacrylate resin. The results were more precise and reproducible than those obtained on paraffin-embedded nodules. Thanks to the small thickness of the sections and the good preservation of tissue during sectioning, the specific localization of the carbonic anhydrase gene expression in the inner cortex of the nodule could be described quite precisely. It is argued that these results are consistent with the hypothesis of osmoregulation of the symbiotic nitrogen fixation. Moreover, the quality of these results with a radioactive probe makes it possible to consider using ISH for such rhizospheric applications as localizing and quantifying a microbial invasion of the endorhizosphere or the rhizoplan by using bacterial- or fungal-specific RNA probes, and counting the number of radioactive dots per cell. However, major artifacts observed with a digoxigenin RNA cold probe makes it necessary to recommend the use of a radioactive RNA probe for these prospects.

common bean / microscopy / rhizobia / rhizosphere / symbiotic nitrogen fixation

**Résumé** – Hybridation in situ d'une sonde ARN radioactive sur des coupes de nodosités de légumineuses incluses dans une résine méthacrylique : un outil pour observer l'expression génique dans la rhizosphère ? Dans ce travail, nous avons réalisé une hybridation in situ d'une sonde ARN d'anhydrase carbonique marquée au <sup>35</sup>S, sur des coupes de nodosités préalablement incluses dans une résine méthacrylique. Grâce à la faible épaisseur des coupes et la bonne conservation des tissus durant la coupe, la localisation spécifique de l'expression du gène d'anhydrase carbonique dans le cortex interne des nodosités a pu être décrite très précisément. Il est argumenté que cette localisation est en accord avec l'hypothèse d'osmorégulation de la fixation symbiotique d'azote. De plus, la qualité de ces résultats avec une sonde radioactive suggère qu'il serait possible d'utiliser l'hybridation in situ pour des applications telles que la localisation et la quantification d'une invasion microbienne de l'endorhizosphère ou du rhizoplan en utilisant des sondes ARN spécifiques de champignons ou de bactéries, et en comptant le nombre de signaux radioactifs par cellule. Il est recommandé d'utiliser un marquage radioactif des sondes, en raison des artefacts observés dans ce travail avec une sonde ARN marquée à la digoxygénine.

fixation symbiotique d'azote / haricot / microscopie / rhizobia / rhizosphère

## 1. INTRODUCTION

The embedding of plant tissues for in situ hybridization is generally performed within paraffin. However, the inclusion in paraffin requires 2–3 days at 60 °C, which alters the cellular structures and the antigenic sites of proteins [1]. Moreover, the cut has to be thick in order to avoid cell distortions [18]. By contrast, methacrylic resins allow inclusions at low temperatures with a better preservation of antigenic sites. In addition,

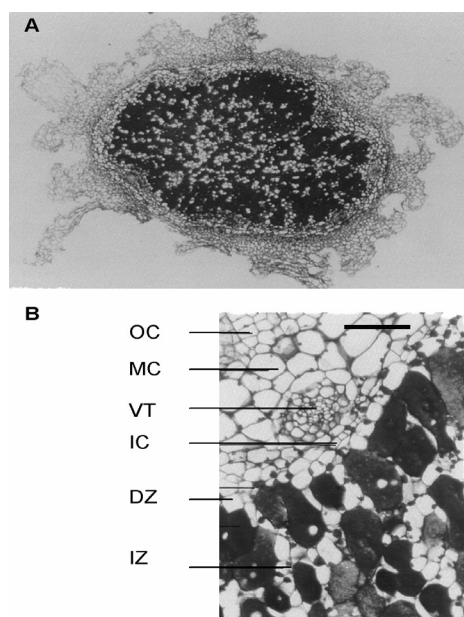
their toughness allows cutting without distortions [1] for a better analysis of morphological differences between cells, as previously shown with soybean nodules [20]. Since such resins can be deresined, they may be quite interesting for in situ hybridization.

In our group we are researching genes whose overexpression in the cortical parenchyma of legume root-nodules may relate to the function of this tissue to osmoregulate the oxygenation of the nodule [5, 6]. The nodular cortex, as illustrated in

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\* Correspondence and reprints  
drevonjj@ensam.inra.fr



**Figure 1.** Nodule morphology. (A) transverse section stained with periodic acid-schiff and naphtol blue black; (B) cortex is subdivided into: outer cortex (OC), middle cortex (MC), i.e. the cell-layers localized between the vascular traces and the outer cortex [10], inner cortex (IC), i.e. the cell-layers located between the vascular traces and the most internal non-infected cells surrounding the infected zone [20], and the distributing zone (DZ), that separates the IC from the infected zone (IZ) where atmospheric  $N_2$  is fixed by bacteroids, the organite form of the symbiotic rhizobia [14]. The vascular traces (VT) are distributed in-between MC and IC. Dark bar corresponds to 50  $\mu$ m.

Figure 1 for *Phaseolus vulgaris*, can be divided into external (EC), middle (MC), i.e. the cell-layers localized between the vascular traces and the outer cortex [10], and internal (IC), i.e. the cell-layers located between the vascular traces and the most internal non-infected cells surrounding the infected zone [20]. Vascular traces run in-between the IC and MC. A cell-layer named the distributing zone (DZ) would separate the IC from the infected zone (IZ) where atmospheric  $N_2$  is fixed by bacteroids, the organite form of the symbiotic rhizobia [14]. A major function of the specialization of the legume root-nodule is to preserve a microaerobic environment for the assimilatory reduction of  $N_2$  into  $NH_3$  by nitrogenase. Indeed, this enzyme is inactivated by  $O_2$  traces, and its gene-transcription requires a partial  $O_2$  pressure lower than 0.2% [3]. Moreover, the ATP requirement for  $N_2$  reduction is so high that the concentration of  $O_2$  seems to be regulated at a sublimiting level for nitrogenase in the infected-cells [7, 9].

To test the technical reliability of in situ hybridization on resin-embedded nodules, we used a carbonic anhydrase (CA) gene that is specifically expressed in the nodule inner-cortex [4]. Based on reproducible results with  $^{35}S$  radiolabeling shown in this work, we discuss the potential of using similar methodology with bacterial- or fungal-specific RNA probes to localize and quantify a microbial invasion of the endorhizosphere or the rhizoplan.

## 2. MATERIALS AND METHODS

### 2.1. Culture of plants and bacteria and preparation of nodule sections

Seeds of common bean (*Phaseolus vulgaris*) line BAT477 were surface-sterilized and inoculated with *Rhizobium tropici* CIAT 899, then grown in a liquid and aerated nutrient solution [21]. Nodules of 3–4 mm diameter were harvested 5 weeks after inoculation, cut into 2 hemispheres in a transverse plan [18] and immediately transferred into the following fixative at room temperature: 0.9% (w/v) paraformaldehyde with 100 mM cyclohexylamine, 10 mM EGTA (ethyleneglycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetracetic acid), 10 mM  $MgCl_2$  and 1 mM DTT (dithiotreitol) in 100 mM 1,4-piperazinediethane sulfonic acid (Pipes) buffer, pH 7.5 [12]. Vacuum was applied during the 6 h of incubation, with several interruptions until the nodule halves were all sunk into the fixative. Thereafter, the nodule halves were washed 3 times for 15 min with 10 mM EGTA, 10 mM  $MgCl_2$ , 1 mM DTT and 100 mM glycine in 10 mM Pipes buffer at pH 7.5, and incubated overnight in the same buffer without glycine.

The embedding in resin was performed with the procedure of Baskin et al. [1] with the following modifications: nodule halves were passed through ethanol-methacrylate series, 3:1 (v/v) for  $3 \times 30$  min, 1:1 (v/v) for  $2 \times 1$  h, 1:3 (v/v) for 30 min, then incubated for  $2 \times 1$  h in methacrylate, and overnight in methacrylate with added 0.5% (w/v) benzoin ethyl ether (Flucka). 10 mM DTT was added to all resin mixtures and they were previously bubbled for 15 min with gaseous nitrogen before each incubation to displace dissolved oxygen. Nodule halves in liquid resin were transferred into plastic moulds (Leica), and covered with agarose film (Melinex film, Agarscientific) and an excess of resin for polymerization at 4 °C at 30 cm above a UV source of 365 nm for 20 h to solidify the resin. The embedding in paraffin was performed at 60 °C in series of toluidin, toluidin:paraffin (1:1, v/v) and paraffin.

Nodule sections of 7  $\mu$ m in paraffin and 4  $\mu$ m in resin were performed with a microtome. They were floated on a drop of water on slides previously treated with Vectabon (Vector), heated at 45 °C for 4 h and incubated twice for 10 min in acetone to remove the resin. Tissues were then rehydrated, treated with proteinase K and incubated in triethanolamine-HCl (pH 8) with 0.5% acetic anhydride according to Kronenberger et al. [11]. All solutions were made in RNase-free glassware with DEPC-treated water.

### 2.2. Preparation of RNA probe

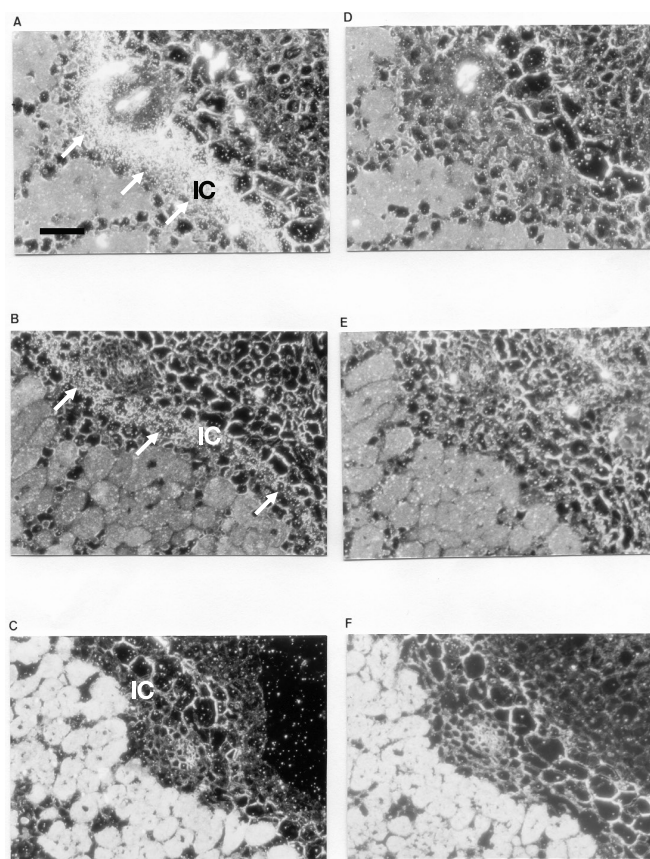
The cloning of a PvCA cDNA fragment for subsequent probe synthesis was performed from total RNA prepared from a *P. vulgaris* root-nodule cortex with a RNeasy Plant Mini Kit (Qiagen, GMBH, Germany). This RNA was treated with RNase-free DNase (Promega, USA) in order to avoid genomic DNA contamination. Two  $\mu$ g of RNA were reverse transcribed with MMLV reverse transcriptase (Promega) in the presence of an oligo-d(T) primer and dNTPs in a final volume of 50  $\mu$ l. Two CA primers, forward CTKYTSAGR-GAGAAGRMKGA, and reverse CCACAGCTTGAAT-TCTCC, were designed from conserved regions of several

CA coding sequences for amplifying a 700-bp fragment [13]. The PCR reaction was carried out in a total volume of 25  $\mu$ l, including 20  $\mu$ M of each dNTPs, 200  $\mu$ M of each CA primer, 0.5 unit of Taq DNA polymerase (Promega) and 1 $\times$  Taq Promega buffer. The thermocycling profile was 35 cycles at 94  $^{\circ}$ C for 1 min, 55  $^{\circ}$ C for 1 min 30 s and 72  $^{\circ}$ C for 1 min. A control reaction where RNA was treated as above but without reverse transcriptase gave no amplification. The amplified fragment was cloned into a pGEM-T vector (Promega). It was used for sequencing with the automatic sequencing system from Applied Biosystems 373A (Foster City, CA, USA) and for synthesizing the  $^{35}$ S-labeled RNA probes. The sequence was given the accession No. PVU547634 standard. It shares 86 and 90% of identity with the *Glycine max* and *Medicago sativa* CA, respectively. To generate the sense or antisense probes, the plasmid was linearized with *Apa*I or *Pst*I and transcribed with RNA polymerase SP6 or T7, respectively. Radioactive labeling was performed as previously described [8].

### 2.3. In situ hybridization

The in situ hybridization (ISH) with a radioactive probe was carried out according to Cox et al. [2] with the following modifications: radioactive RNA probes were heated at 80  $^{\circ}$ C for 5 min in tRNA 0.15  $\text{g}\cdot\text{l}^{-1}$ , Poly A 0.5  $\text{g}\cdot\text{l}^{-1}$  and DTT 300 mM, quickly cooled on ice and the following were added: formamide 50%, NaCl 300 mM, TE buffer pH 7.5 (Tris-HCl 10 mM, EDTA 1 mM), Dextran sulfate 10%, 1 $\times$  Denhardt's reagent (QuantumBioprobe) and DTT 60 mM. The mixture (5  $\mu\text{l}\cdot\text{cm}^{-2}$ , 4000 cpm $\cdot\mu\text{l}^{-1}$ ) was applied onto slides and covered with coverslips to hybridize overnight at 42  $^{\circ}$ C in a humid box containing Whatmann paper saturated with 2 $\times$  SSC (1 $\times$  SSC = 0.15 M NaCl and 0.015 M Na-citrate, pH 7), and protected from light. The coverslips were carefully removed and the slides were washed for 4 $\times$  10 min in 4 $\times$  SSC with 5 mM DTT, then incubated for 5 min in RNase buffer (Tris-HCl 10 mM pH 7.5, EDTA 1 mM and NaCl 0.5 M). The slides were then treated for 30 min with RNase A (50  $\mu\text{g}\cdot\text{ml}^{-1}$ ) at 37  $^{\circ}$ C, and washed for 4 $\times$  15 min with RNase buffer with 5 mM DTT. They were incubated for 30 min in 2 $\times$  SSC with 5 mM DTT, and for 1 h in 0.2 $\times$  SSC with 5 mM DTT, both at room temperature. The slides were coated with autoradiographic emulsion (LM1, Amersham) diluted in water (2/1), processed as recommended by the Amersham Company and stored for 1 month at 4  $^{\circ}$ C. Development was performed for 1 min with a D-19 developer (Kodak), and colored with 0.02% toluidine blue. Observations were made with a Leica microscope DMRXA. Numeric pictures from ISH with resin or paraffin embedding were computerized using Photoshop software (Adobe).

ISH with a cold probe was performed for 1 h in a Tris-HCl buffer at pH 7.4 containing the antibody anti-Dig coupled to an alkaline phosphatase (Boehringer) at 1/250 and a blocking agent at 1%, and for 3 h in a similar renewed mixture. The Dig-RNA labeling mix was performed with a kit (Boehringer) including 10 mM A/C/GTP and 3.5 mM Dig-UTP. The slides were washed afterwards in the similar Tris-HCl buffer, and a buffer containing 100 mM NaCl, 50 mM  $\text{MgCl}_2$  and 100 mM Tris-HCl pH 9.5 with added NBT-BCIP and 1 mM levamisole, an inhibitor of endogenous alkaline phosphatases. The



**Figure 2.** Variations in ISH signal with radioactive antisense (A, B and C) and sense- (D, E and F) *PvCA* probes on 2 consecutive sections from 3 resin-embedded nodules. White arrows show ISH signals. Dark bar corresponds to 50  $\mu$ m.

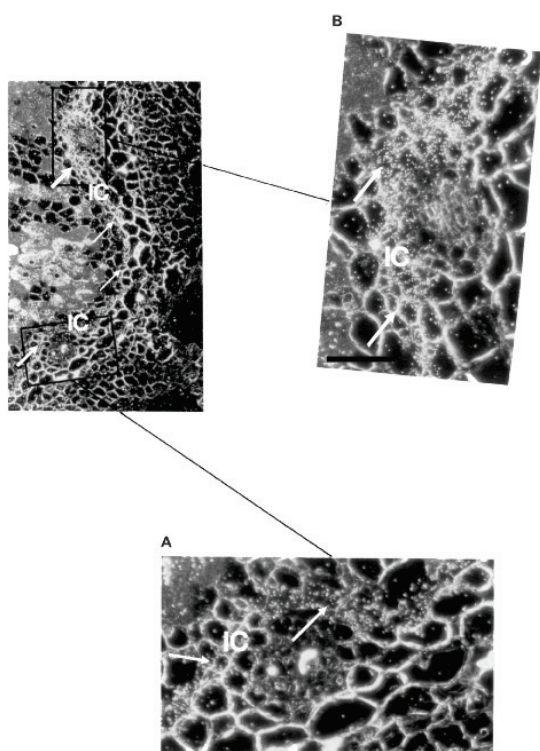
coloration was regularly checked. Generally a hybridization signal could be seen after 2–3 days.

## 3. RESULTS

### 3.1. Hybridization with methacrylate embedding and a radioactive probe

The radioactive signal observed in Figures 2A, 2B and 2C, shows that it is actually possible to obtain an ISH positive result with an antisense radioactive RNA probe on nodule sections that were initially embedded in a methacrylate resin. As shown in Figures 2D, 2E and 2F, there was no radioactive signal on consecutive sections treated with a similar ISH procedure but with the sense *PvCA* probe. Since the latter could not hybridize with transcripts because it was homologous and not complementary to the CA mRNA, this result confirms that the radioactive signal observed in Figures 2A, 2B and 2C was not artefactual.

The precision of the signal localization in methacrylate-embedded nodules made it possible to investigate the topographic distribution of the *PvCA* transcripts within nodule



**Figure 3.** Differences in ISH signal with radioactive antisense-*PvCA* probe in the nodule inner cortex (IC) in the vicinity (A and B) and between the vascular traces (VT). White arrows show ISH signals. Dark bar corresponds to 50  $\mu\text{m}$ .

tissues. Whatever the intensity of the signal in Figure 2, the *PvCA* gene was expressed exclusively in the inner-cortex.

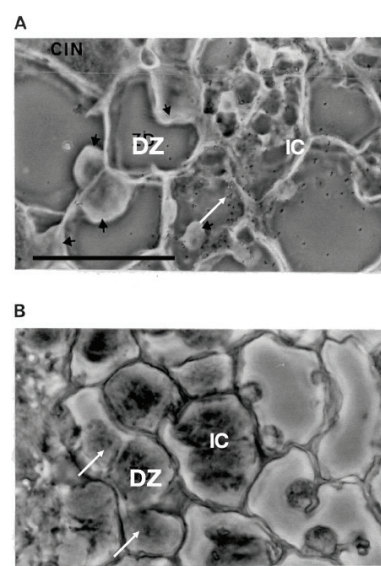
### 3.2. Variations in nodular expression of *Pvca*

Furthermore, the intensity of the ISH signal varied dramatically between nodules as shown by comparing A, B and C in Figure 2. Such variation was associated with nodules from different plants, though some variations were also found between nodules from one plant (data not shown). In addition, some variation in this signal could be found within one nodule. Thus, Figure 3 shows a lower ISH signal close to the vascular trace magnified in Figure 3A than that in Figure 3B, although both vascular traces were within one nodule. Moreover, the *PvCA* expression was more intense in the vicinity than in-between the vascular traces, as illustrated by the thickness of the white arrows in Figure 3.

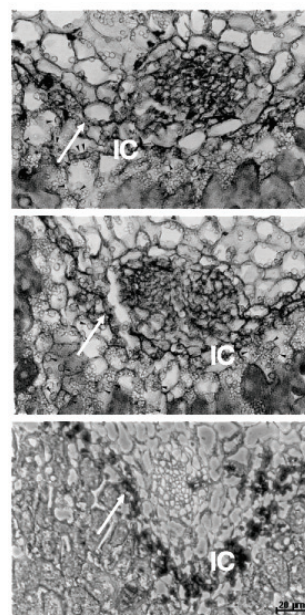
These variations were not artefactual since they were distinct from the background ISH signal. The latter could be recognized by its random distribution in other nodule tissues and outside the section (see Figs. 2A and 2C, for example), or in the nodule cuts exposed to the sense probe (compare Figs. 2B and 2E, for example).

### 3.3. Hybridization with a digoxigenin cold probe

The use of a radioactive probe is limited by the requirement of a suitably-equipped laboratory and by the 1-month duration



**Figure 4.** Differences in ISH signals with radioactive (A) or digoxigenine (B) *PvCA* probes in nodule inner cortex. White arrows show ISH signals. Digoxigenine signal appears as a purple stain. Black arrows show amyloplasts. Dark bar corresponds to 50  $\mu\text{m}$ .



**Figure 5.** ISH signals with sense (A) and antisense (B) digoxigenine *PvCA* probes, and with (C) digoxigenine GUS probe in nodule inner cortex. White arrows show digoxigenine signals, as a brownish-purple stain. Black arrows show amyloplasts. Dark bar corresponds to 50  $\mu\text{m}$ .

of the procedure. However, sections of resin-embedded nodules were hybridized with digoxigenin-labeled *PvCA* probes. By contrast with the above results with a radioactive probe, major artefacts were found with the digoxigenin probe: (i) in the distributing zone (ZD), no signal was observed with anti-sense radioactive-*PvCA* (Fig. 4A) although a signal was

observed with antisense digoxigenin-*PvCA* (Fig. 4B), and (ii) in the inner-cortex (IC), a similar signal was observed with the sense (Fig. 5A) and the antisense (Fig. 5B) digoxigenin-*PvCA* probe.

In order to test whether the above signals with digoxigenin-*PvCA* were artefactual, hybridization was performed with a digoxigenin-GUS probe that could not hybridize with any *P. vulgaris* mRNA. The aspecificity of the digoxigenin signal is demonstrated in Figure 5C, where the signal of the digoxigenin GUS probe is localized similarly to that of the digoxigenin-*PvCA* in Figure 5B, i.e. close to amyloplasts.

#### 4. DISCUSSION

In this work we show in situ hybridization results with a mRNA radioactive probe on sections of legume root-nodules previously embedded in a methacrylate resin. Thus methacrylate embedding is a reliable methodology for radioactive ISH with plant tissue, as previously reported with a cold probe [11] or with animal tissue [22].

Moreover, the precision of the results shown here with the radioactive antisense *PvCA* probe makes it possible to consider using the ISH not only qualitatively, by localizing precisely the gene expression at the cellular level, but also quantitatively by counting the number of radioactive dots per cell, with image analysis software if needed.

In this work, we could localize the *PvCA* expression precisely within the IC-cells of the common-bean nodule cortex. These IC-cells were previously characterized as exhibiting reversible contraction associated with a decrease in nodule permeability [20], and high content in tonoplast aquaporins [19]. Further work is required to verify whether the intensity of *PvCA* and aquaporin expressions in IC-cells increases under deficiencies of phosphorus or oxygen, which increase nodule permeability [16, 17].

In addition, the high resolution of the ISH signals shown in this work with a radioactive RNA probe on methacrylate-embedded biological material may lead to such rhizospheric applications as localizing and quantifying a microbial invasion of the endorhizosphere or the rhizoplane by using bacterial- or fungal-specific RNA probes.

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