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Contributions of *in situ* hybridization of sRNA to the study on spatio–temporal gene expression in *Hevea brasiliensis*

Alemanno L., Guilhaumon C., Marteaux B., Conejero G., Verdeil J.L., Dessailly F., Montoro P.
UMR BEPC, CIRAD, TA 80 / 03, Avenue Agropolis, 34398 Montpellier Cedex 5, France.

Abstract

Since the dawn of the genomics era, much research has focused on functional studies of genes of interest. *In situ* hybridization is a method that can be used to precisely localize the expression of a gene in tissues and cells. This article describes how the method has been adapted to the analysis of *Hevea* tissues. Initially, the conventional method of digoxigenin detection with NBT/BCIP revealed the expression of strongly expressed genes in tissues of different differentiation intensity. A new digoxigenin detection method using Alexa488 fluorochrome–labelled antibodies has been used to detect the expression of more weakly expressed genes. This method, combined with observation under a confocal microscope, has enabled very precise localization of expression. Some examples of *in situ* hybridization use are described for *Hevea* gene expression in somatic plantlets and shoot bark: the *uidA* gene in callus and transgenic somatic plantlets, the *HEV2.1* gene encoding hevein and the *ACO-H5* gene involved in ethylene metabolism. Cell imaging methods therefore open up fundamental prospects for studying the different molecular mechanisms involved in some agronomic traits of *Hevea*.

1. Introduction

The availability of latex, which consists of the cytoplasm of laticifer cells (metabolites, proteins and mRNA), has enabled in-depth work on latificer metabolism with regard to natural rubber production. Of course, such production also depends on functions outside the latificer system. An overall understanding of "production mechanisms" in the bark encouraged our team to adapt new cell imaging tools to gain a clearer picture of where the expression of each gene is localized and thereby propose interaction and functioning models for the tissues of *Hevea* bark.

When using conventional molecular methods to analyse gene expression, such as Western blot analysis, molecular hybridization of the Northern blot type or semi-quantitative RT-PCR, tissue fragments have to be ground up. This means a mixture of proteins and mRNA from the different cells, hence different tissues tangled up with each other. On the other hand, the development of cell imaging techniques combining histological analyses and molecular biology techniques, makes it possible to precisely localize a protein or mRNA at cell level.

Immunolocalization makes it possible to localize proteins arising from the expression of a gene, using specific antibodies. For this technique, proteins have to be purified and monoclonal antibodies have to be produced.

ISH enables spatio-temporal localization of the transcipts of a target gene through hybridization with a specific labelled RNA probe transcribed *in vitro* from cDNA. It is thus possible to determine in which tissues and cells, and at which moment, a gene has been expressed. This information is important for gene characterization.

2. Adaptation of *in situ* hybridization techniques to the rubber tree

In situ hybridization, which was first tested in animal biology towards the end of the 1970s, came about through a meeting of histology with molecular biology. It was first applied to plant biology at the end of the 1980s on *Arabidopsis thaliana* (Meyerowitz, 1987); (Smith et al., 1987). The first results revealed how difficult it was to implement this technique on plants, notably because of secondary metabolites and the existence of cell walls. In *Hevea*, the existence of latex in all the tissues of the plant, and the substantial differentiation of bark tissues, complicated its development (Vidal, 2003). However, some worthwhile results have been obtained in recent years.

In situ hybridization stages

Sample preparation. The samples for analysis are first fixed, dehydrated and embedded in paraffin. 10µm slivers are then cut with a microtome and deposited on glass slides. All these operations are carried out under RNAse-free conditions.
Probe synthesis. Specific probes are synthesized: they are complementary with the RNA sequence transcribed by the target gene and labelled with digoxigenin. Two types of probe are used for each experiment: antisense probes, which recognize the mRNAs to be detected, and sense probes that act as a negative control. Synthesis is carried out in two stages: the matrix fragments are amplified by an initial PCR with specific primers including, either at 5' or at 3', an extension corresponding to the T7 promoter sequence. A second PCR makes it possible to amplify only the population of fragments containing the complete T7 using a specific primer of the T7 end. Using the same cDNA, it is possible in this way to obtain sense and antisense probes separately. Then the amplified DNAs are transcribed *in vitro* into RNA by an enzyme: RNA polymerase. During transcription, digoxigenin-labelled UTP nucleotides are incorporated into the probes. This is known as labelling.
Hybridization. During hybridization on the explant sections, the antisense probes fix themselves to the RNA strands transcribed from the target gene.
Detection. During detection, the RNA-RNA complex formed by the antisense probe and the RNA transcribed by the target gene is brought into contact with an antibody, anti-digoxigenin combined with an enzyme, alkaline phosphatase. The antibody recognizes the "UTP-digoxigenin" antigen, to which it is specific, and thus forms an immune complex. The enzymatic activity of alkaline phosphatase is visualized by adding enzyme substrate: NBT/BCIP. When the substrate recognizes the enzyme, an "enzyme-substrate" complex is formed and its reaction gives a stained product.

Protocol improvements

One of the problems often encountered with this detection method using NBT/BCIP is background noise. Indeed, staining of secondary metabolites often occurs on explant sections, along with non-specific labelling due to probe adsorption on cell wall compounds. This is known as background noise and is quite different from true labelling, which indicates gene activity on a cytoplasmic level. Background noise may also be found on control slides hybridized with antisense probes, on which no staining should be visible.

Several changes have been made to the basic protocol in order to reduce background noise. Firstly, it was found that adding NBT/BCIP (substrate of alkaline phosphatase) to explant sections of bark from non-hybridized shoots induced significant staining. Endogenous phosphatases therefore exist in shoot bark, especially in the laticifer cell zone. By increasing

tissue pH from 9.5 to 10.5 before adding substrate they were effectively inhibited. Then, several parameters of the *in situ* hybridization protocol were modified: a pre-hybridization stage was added before the hybridization stage, denaturation of tRNAs, used to block non-specific transcripts, before they were added to the hybridization medium (de Almeida Engler et al., 2001), detection at constant temperature reduced to 30°C and precise monitoring of the detection time. The cumulation of all these factors led to a significant reduction in background noise on bark.

Different detection systems

Despite significant improvements to the *in situ* hybridization protocol, detection by alkaline phosphatase and NBT/BCIP remained approximative. Indeed, whilst it is possible to determine the overall expression of a gene in an explant, it is difficult to descend precisely to cell level. Other more sensitive detection methods have been tested to improve the detection of gene expression.

Detection with Vector Blue™. This substance is another substrate of alkaline phosphatase which, in some cases, gives finer detection and localization of gene expression than with NBT/BCIP. Formation of the "alkaline phosphatase –Vector Blue™" complex leads to blue staining. Using this substrate for the rubber tree did not improve sensitivity.

Probe labelling with Psoralen-Biotin. In this case, rather than being labelled with digoxigenin during transcription, the probes were labelled under UV light with psoralen-biotin, itself coupled to alkaline phosphatase. Detection was then carried out by adding NBT/BCIP. When applied to the rubber tree, this method did not improve detection sensitivity.

Detection with Alexa488 fluorochrome. With this technique, digoxigenin-labelled probes were detected by a fluorochrome: Alexa488. After hybridization of the probes, mouse anti–digoxigenin monoclonal antibodies were deposited on the slides then detected using two antibodies coupled to Alexa488 fluorochromes. One was a rabbit anti-mouse IgG (ImmunoGlobulin), the other was a goat anti-rabbit IgG. This assembly of antibodies coupled to fluorochromes amplified the signal. Use of this technique improved the detection of gene expression in *Hevea.*

Different samples

Rubber tree *in situ* hybridization studies have been carried out on several planting materials displaying different degrees of differentiation. The studies were conducted on embryogenic calli produced from the inner integument of seeds, then on stems, roots and leaves of somatic plantlets, and lastly on the barks of shoots grown in the greenhouse. The ultimate objective was to develop this method on the bark of tapped mature trees.

The *in situ* hybridization conditions defined for one type of explant are not necessarily appropriate for all the others. Thus, it is necessary to define optimum hybridization conditions for each gene and each explant studied.

3. Examples of expression localization for a few genes in rubber tree calli, somatic plantules and shoot bark

The *uidA* gene controlled by the CaMV 35S RNA promoter

Expression of this gene was studied in rubber tree calli transformed by the *uidA* gene controlled by the CaMV 35S RNA promoter using the method described in Blanc *et al.* (Blanc et al., 2005). Expression of this gene, detected by hybridization of digoxigenin–labelled probes and detected with NBT/BCIP, was uniform in all the cells of the callus. Expression of this gene was high. Labelling was specific as it was clearly visible in cell cytoplasm. The negative controls made up of antisense probes hybridized on sections of non-transformed callus and sense probes hybridized on transformed callus did not display any specific staining.

These calli regenerated somatic embryos, subsequently converted into plantlets. Expression of this gene was studied inside the transformed plantlets. All the organs of the transformed plantlets hybridized with the antisense probe revealed staining on all the cells, except those of the xylem and the sclerenchyma. Labelling was localized in the cytoplasm and nuclei. Leaves appeared to be labelled more than roots and stems for the same detection time. The same hybridization on non-transformed plantlets did not display any specific labelling.

The *HEV2.1* gene encoding hevein

Expression of this gene, which is highly expressed in laticifers, was studied in somatic plantlets and shoot bark. Hybridization was carried out with the HEV2.1 probe labelled with digoxigenin and detected by NBT/BCIP. On roots and stems of plantlets, and on shoot bark hybridized with the antisense probe, slight specific labelling was seen for laticifers. The messengers of *uidA*, a gene alien to *Hevea*, were more effectively detected by *in situ* hybridization in transformed plantlets than the native *HEV2.1* gene in wild plants.

The *ACO* gene encoding ACC oxidase involved in ethylene biosynthesis

In rubber, several members of the *ACO* multigenic family have been isolated (Kuswanhadi et al., 2004). Expression of the *ACO-H5* gene was studied in shoot bark and somatic plantlets. This gene was weakly expressed and required a longer detection time than for *HEV2.1*, for example.

Detection of digoxigenin-labelled probes with NBT/BCIP revealed overall expression in shoot bark. In the case of plantlets, all the organs displayed labelling of varying intensity throughout the tissues. After detection for 8h, leaf tissues displayed non-specific labelling in the chloroplasts, making it difficult to observe labelling. For roots and stems, the optimum detection time was over 24 h. However, for such a long detection time, substrate precipitation was also seen which was responsible for substantial background noise. It thus became difficult to distinguish between labelling and background noise.

Probe detection with Alexa488 fluorochrome made it possible to fine-tune localization of *ACO-H5* gene expression, both in bark and in the stems and roots of somatic plantlets. Observations were carried out under a confocal microscope. The bark materials hybridized with the antisense probe displayed cytoplasm labelling in the cortical parenchyma and the phloem: this labelling was even visible in the laticifers. For somatic plantlets, stems displayed cytoplasm labelling in the cortex, pith and mostly in the phloem in the laticifer zone. Roots displayed very weak cytoplasmic labelling of a few cortex and pith cells.

4. Conclusions and prospects

We now have several *in situ* hybridization methods for studying the expression of genes that are expressed to varying degrees in more or less differentiated rubber tree tissues.

Thus, the NBT/BCIP detection method is perfectly suitable for all types of tissues and for strongly expressed genes. On the other hand, when tissues are highly differentiated, or when the gene studied is weakly expressed, detection with Alexa488 fluorochrome makes it possible to localize expression much more finely and precisely.

However, the methods developed do not yet make it possible to distinguish between the expression of several members of the same multigenic family. For that, consideration needs to be given to using short probes specific to the little-conserved regions of genes of the same family.

If that is not possible, and in reference to results obtained with the hevein gene, consideration could be given to functional characterization of the promoters of the genes studied in transgenic plants in fusion with the *iudA* gene. That approach has been used to specifically localize *HEV2.1* in laticifers (Montoro et al., 2005) or *ACS* in rice (Zhou et al., 2002). This method can therefore be applied to weakly expressed genes but it is necessary to clone the promoter regions of the genes studied.

For very weakly expressed genes, the development of *in situ* PCR is another alternative. With that method, the transcripts sought are amplified from specific primers in the explant sections before hybridization with the corresponding probes.

The examples described in this paper clearly demonstrate the feasibility and merits of *in situ* hybridization for fine characterization of the expression of key genes in *Hevea* metabolism.

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