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Localization of legume nodule components by immunocytochemistry techniques

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The use of immunocytochemistry techniques to localize nodule components in legume is very much helping to the understanding of the symbiotic nitrogen fixation process, including the development and metabolism of *Rhizobium*-nodule symbiosis.

In this review it will be mentioned some immunocytochemical works that have been carried out to localize nodule components with the aim to understand the relationships between structure and function in the complex N_2-fixation process.

The association between *Rhizobium* bacteria and host legumes results in the development of specialized organs called root nodules. The development and the proper function of nodules involve the specific expression of several host-encoded proteins, nodulins. Nodule-specific host proteins have been described in various legume species. Some functions have been assigned to several nodulins, among which are some enzymes and proteins as uricase, glutamine synthetase, leghemoglobin etc. Cellular and subcellular localization of both nodulins and its gene products are essential for the understanding of the molecular basis of the plant-*Rhizobium* symbiosis.

A few "in situ" immunological studies have been carried out on the tissue specificity of some of the nodulins including uricase (VandenBosch and Newcomb, 1986), leghemoglobin (Robertson et al., 1984; VandenBosch and Newcomb, 1988; Vivo et al., 1989), and glutamine synthetase (Hara et al., 1988; Brauman et al., 1989). Besides nodulins, another nodule component, a glycoprotein related with the O_2-diffusion to bacteroids has been localized by immunogold techniques (VandenBosch et al., 1989; de Lorenzo et al., 1991). Recently, the membrane antigenic determinants of different *Rhizobium* strains have been also localized by the same techniques (Lucas et al., 1992).

Uricase

The pathway of ureide synthesis involves the "de novo" synthesis of purines from aminoacids and the oxidation of purines to form ureides. The enzyme uricase (EC 1.7.3.3) is near the end of this pathway and is responsible for the breakdown of uric acid, the product of purine catabolism, to allantoin. In 1983 Bergman et al. identified a nodule-specific protein from soybean, nodulin-35, as a 33-kDa subunit of uricase, one of the most abundant proteins in mature soybean nodules and it is believed to be responsible for the large increase in uricase activity which occur following the onset of nitrogenase activity. Uricase is sometimes found along with catalase in peroxisomes in plants and animal tissues (Vaughn and Stegink, 1987). The presence of numerous enlarged peroxisomes in uninfected cells of the central infected zone of soybean nodules led Newcomb and Tandon (1981) to predict that the last steps of ureide synthesis would be found to take place in these cells. They tried to seperate infected and uninfected cells, measuring uricase activity in the different fractions, but it was unclear to predict that only in the uninfected cells of soybean occur ureide formation. Furthermore, Newcomb et al. (1985) have reported that infected cells contain small peroxisome-like bodies that could conceivably function in the production of ureides. Shelp et al. (1983) using similar techniques on nodules of cowpea, found several enzymes of the ureide pathway distributed through both infected and uninfected cells and also the cortex, indicating considerable contamination between fractions since homogenization procedures can also change cellular

components, releasing soluble proteins. At this respect Hanks et al. (1981) found only 15% of the total uricase activity in the peroxisomal fractions, following a differential centrifugation, probably because of the fragile nature of these organellas.

So that, a method capable of demonstrating the presence of uricase and allantoinase "in situ" was needed to demostrate the presence of these enzymes in the microbodies of both types of nodule cells. Immunocytochemistry is specially well suited for this type of investigation. No disruption of tissue is required, since the procedures are carried out on fixed, embedded and thin sectioned material. Furthermore, the technique combines the specificity of an immunological reaction with the high resolution afforded by the electron microscopy. VandenBosch and Newcomb (1986) used the immunogold technique to follow the time of appearance of a nodule specific uricase in peroxisomes of differentiated nodules. This study was one of the first to correlate the induction of a nodulin with morphological events. They were able to determine whether uricase is located in both types of peroxisomes (P) corresponding to uninfected (UI) and infected (IC) cells. Heavy labelling occurs over the large peroxisomes in uninfected cells (Fig. 1). At the contrary, the small peroxisome-like bodies seen occasionally in the periphery of infected cells appear without labelling, allowing to the authors to conclude that uricase has not been expressed in the peroxisomes of the infected cells. In 1989 Newcomb et al. observed localization of uricase in the inner three cortical layers of nodule cortex by immunogold labelling enhanced with silver, for visualization in the light microscope, suggesting that with respect to ureide production the cells of the inner layers of the cortex are functionally similar to the interstitial uninfected cells of the infected region. So that, by means of immunocytochemistry, it is now recognized that the uninfected cells of the central region collaborate with the inner cortex cells in the production of the principal export products, allantoin and allantoic acid. These cells could carry out the terminal steps in ureide production.

Leghemoglobin

One of the most important components of legume nodules is the leghemoglobin (LHb), a monomeric protein that function by providing an optimum concentration of O_2 to bacteroids in order to maintain nitrogenase and respiratory activities.

Leghemoglobin localization has been a controversial subject. There have been used conventional histochemical techniques, as the one with 3,3'-diaminobenzidine and X-Ray microanalisis (Bergensen and Goldchild, 1973) for its localization. Most of these preliminary studies agreed in the finding of the red pigment in the bacteroidal membranes and in the peribacteroidal spaces. In 1978 Robertson et al. isolated the peribacteroidal membranes from nodules and came to the conclusion that the LHb was located only in the host cell not in the microsymbiont components. However in 1981 Bergensen and Appleby isolated the intact membranes of the bacteroids of soybean nodules and found that they contained LHb. These controversial results indicate that the fractionation and centrifugation processes gave a lot of contamination among the different fractions with little reliable results with respect to specific localization.

The controversy was over, when Robertson et al. (1984), applying immunogold techniques, localized LHb in the cytoplasm of pea nodule, and at the contrary to the preceding authors, they did not find LHb in the peribacteroidal space, neither in the bacteroidal membrane.

Therefore confirmation of the sites of location of LHb seemed important with regard to the other legumes. Vivo et al. (1989) confirmed the localization of Robertson et al. in the cytosol (cy) of *Lupinus* nodules (Fig. 2) using immunogold techniques. These authors also found LHb localization in the peribacteroidal membrane (pbm) and in the nuclei.

In 1989 VandenBosch and Newcomb observed for first time LHb in the uninfected

cells of soybean, although it remains not clear if this is the functional protein or it is only the apoprotein. The presence of LHb in the uninfected nodule cells corroborates O_2 consumption by bacteroids in infected cells. So that, the LHb could play an important role for the mitochondria of infected and uninfected cells of legume nodules.

Glutamine synthetase

One of the key enzymes involved in nitrogen processing is glutamine synthetase (GS) (EC 6.3.1.2) whose function, regulation and localization have been the object of numerous studies, in particular its role in the incorporation of ammonia either via nitrogen fixation or nitrate reduction.

GS in higher plants has been shown to occur as a number of distinct isoenzymes in different organs and subcellular compartments.

In legumes, the primary assimilation of fixed nitrogen is catalized by the GS in the so called GS-GOGAT pathway. GS is composed of distinct polypeptides in the nodule of legumes where this enzyme has been studied. Nonetheless, most of the observation concerning the enzyme regulation were made using averaging biochemical methods and extraction of whole organs. A few exceptions have dealt with the immunohistochemical GS detection of nodule cells (Verma et al., 1986). However information concerning compartmental subcellular or preferential intracellular localization was lacking or controversial.

GS is composed of distinct polypeptides in the nodule of legumes where this enzyme has been studied. The differential expression of the GS monomers in bean nodules sets up the question of their functional significance in the assimilation of the ammonia derived from the bacteroids. Lara et al. (1986) carried out the subcellular localization of GS searching for the role that the different GS polypeptides play in the ammonia assimilation during symbiosis. Before applying the "in situ" localization of the enzyme, they obtained the western blotted analysis of nodule crude extract, soluble proteins and peribacteroidal fraction, peribacteroidal membrane (PBM) plus peribacteroidal space (PBS), observing that GS is only in the peribacteroidal fraction. These results were confirmed by immunocytochemistry also by Brangeon et al. (1989) as shown in the Fig. 3, in which GS is detected in the PBM surrounding the bacteroids. These results indicate that the ammonia assimilation could take place in this compartment and high lights the metabolic significance of the peribacteroidal compartment in the symbiosis.

Nodule glycoprotein

Recent investigations of physiological factors regulating the rate of N_2 fixation in legumes have placed emphasis on the importance of the regulation of the oxygen concentration within nodules (Layzell and Hunt, 1990). There is evidence that a barrier to O_2 diffusion exits in the inner cortex of legume nodules. The resistance of this barrier to O_2 diffusion is variable and changes under various environmental and physiological conditions of the plant. The rate of nitrogenase activity may be limited by limiting the availability of O_2 for respiration by the bacteroids in the nodule.

In legumes treated with nitrate, resistance to O_2 diffusion in nodules increases and nitrogenase activity decreases (Fernández-Pascual et al., 1991). Therefore, to follow the morphology and composition of the oxygen-diffusion barrier under normal and stress conditions appeared to be necessary.

According to different works carried out to study where this barrier is located, it seems clear that it is situated in the inner cortex but varies with the plant (James et al., 1991). Another important feature of investigation is the finding of a glycoprotein occluding the intercellular spaces of the cells forming the O_2 diffusion barrier, that can be responsible of the limitation of O_2 to the bacteroids. The occurrence of glycoprotein in the inner cortex was firstly found by VandenBosch et al. (1989) in pea plants and recently by de Lorenzo et al. (1991)

in lupin plants (Fig. 4).

Competitiveness studies of *Rhizobium* strains

The recognition of different *Rhizobium* strains in the same nodule is another example of the potentiality of immunogold techniques at electron microscope level. The investigation has been carried out in our laboratory (Lucas et al., 1992) with the aim to show that the membrane immunological constituents of two *Bradyrhizobium* sp. (*lupini*) strains growing together in both a free nutritive medium and in root nodules of *Lupinus* plants keep the immunological properties which allow to distinguish them by ELISA and immunocytochemical reaction. (Fig. 5).

Perspectives

In the last few years the application of immunogold techniques to the *in situ* localization of nodule components has received a great impulse. However the localization of enzymes and proteins related with the interchange of metabolites between the cytosol and bacteroids needs major attention. In the same manner that immunocytochemical studies of the enzymes related with nitrogen assimilation have been carried out, it would be of great interest the localization of enzymes and metabolites related with carbon metabolism. Besides, it would be important to follow the immunological characteristics and localization of the nodule components when varying the environmental conditions of nodule development.

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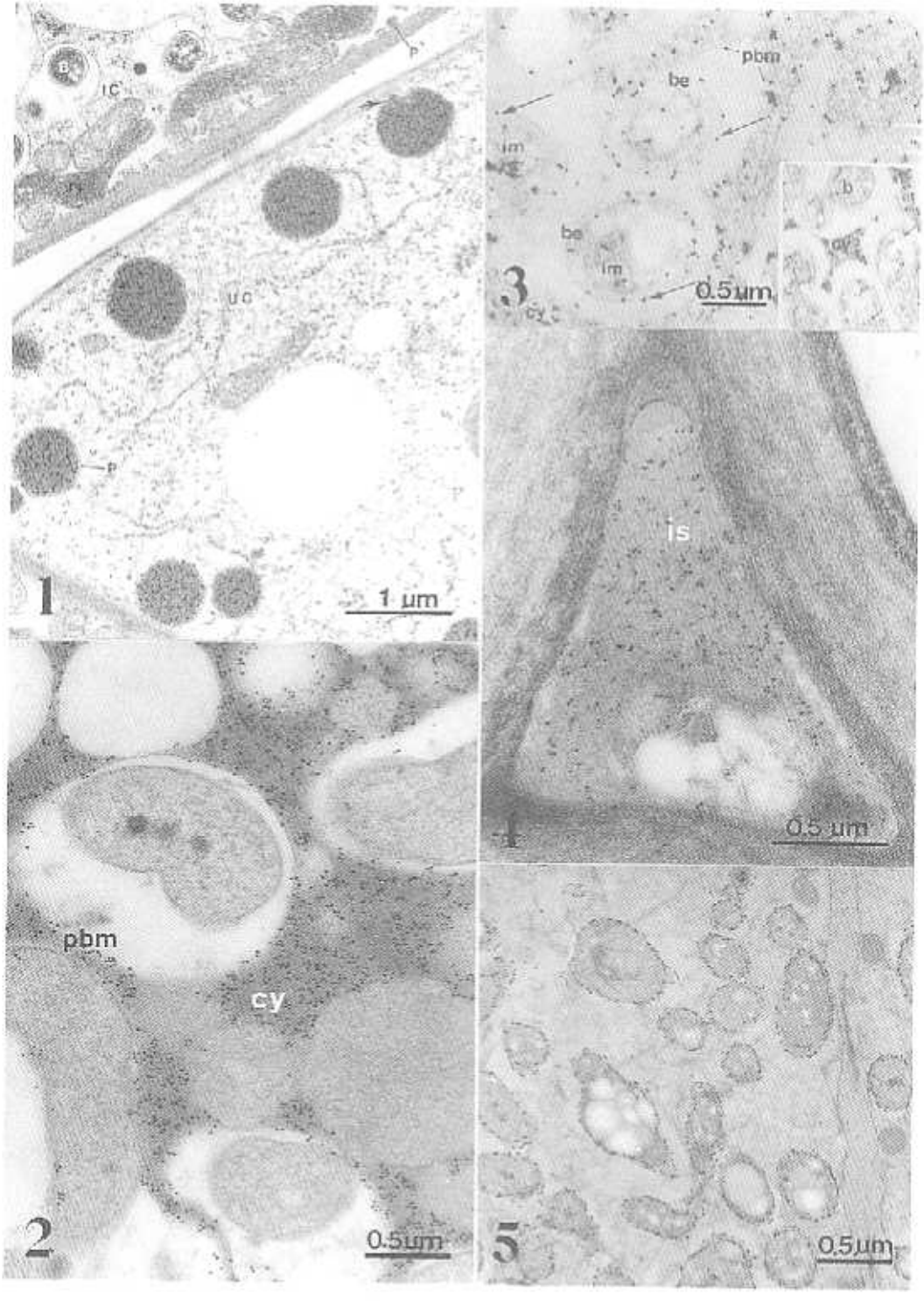
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