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A γ-TIP cross-reacting protein is abundant in the cortex of soybean N_2-fixing nodules

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Abstract. The distribution and abundance of tonoplast intrinsic protein (γ-TIP), a putative aquaporin which is abundant in the tonoplast of osmoregulated pulvinus motor cells, were determined in nodules of *Glycine max* (L.) Merr. using chemical fixation and immunolocalization. This protein was highly expressed in the tonoplast of the inner cortical cells of the nodules but poorly expressed in the vascular transfer cells and in infected cells. It is concluded that the differentiation of the inner cortical cells of the nodules like that of pulvinus motor cells, is accompanied by an increased expression of γ-TIP. This result is consistent with our previous hypothesis that a reversible exchange of intercellular water by the inner cortical cells plays a role in the regulation of nodule conductance to O_2 diffusion, and hence in subsequent N_2-fixing activity.

Key words: Aquaporin – Glycine – Nodule conductance – Oxygen – Symbiosis

The existence of a physical limitation to O_2 diffusion in legume root nodules was postulated more than 30 years ago, and suggested, by theoretical modeling and measurements with O_2-microelectrodes, to be located in cellular layers of the nodule cortex that contain water-filled intercellular spaces (reviewed by Minchin 1997). Moreover, the conductance of root nodules to O_2 diffusion has been shown to vary with manipulations of the plant or its environment (reviewed by Hunt and Layzell 1993). Thus, elucidating how this conductance is modulated in the rhizobium-legume symbiosis may contribute to the understanding of the regulation of nodule nitrogenase activity, and the agronomic improvement of symbiotic N_2 fixation.

It was hypothesized that the variations in nodule conductance to O_2 diffusion are determined by contraction/expansion of nodule inner-cortex cells (IC-cells), i.e. two to four parenchyma layers adjacent to the central infected zone (IZ; Fig. 1A; Drevon et al. 1995). This hypothesis is substantiated by the similar physiological characteristics of the decrease in nodule conductance to O_2 diffusion (Roy 1993) and the contraction of pulvinus tissues (Satter and Galston 1981), and by the common ultrastructural features of nodule IC-cells and pulvinus osmocontractile motor cells (Serraj et al. 1995). Both nodule IC-cells and pulvinus motor cells are specialized and able to change their shape and volume rapidly, presumably using water exchange (Drevon et al. 1995; Fleurat-Lessard et al. 1997).

Recently, it has become clear that water fluxes across biological membranes occur not only through the lipid bilayer but also across specific aquaporins in the membranes (reviewed by Maurel 1997; Schäffner 1998). Moreover, a high abundance of a tonoplast intrinsic protein (γ-TIP) was detected in the tonoplast of motor cells of *Mimosa pudica* pulvini (Fleurat-Lessard et al. 1997) and was found to be associated with the ability of motor cells to exhibit large and rapid turgor variations in response to external stimuli. Therefore, the purpose of the present work was to measure and compare the relative abundances of this γ-TIP in the tonoplast of nodule IC-cells, in cells of the infected zone at the center of the nodule and in the pericycle of the vascular bundles at the edge inner cortex/middle cortex (Fig. 1A).

Soybean [*Glycine max* (L.) Merr. cv. Kingsoy] seeds were surface-sterilized and inoculated with *Bradyrhizobium japonicum* PJ17, then grown in a liquid and aerated nutrient solution (Serraj et al. 1995).

Fresh nodules were cut into 1- or 2-mm-thick pieces and fixed for 15–30 min in a mixture of 2% (w/v) paraformaldehyde, 0.5% glutaraldehyde in 0.1 mM phosphate buffer, pH 7.2. Abundant washing in this buffer (+7.5% sucrose) was followed by a 4-min postfixation in 1% (v/v) OsO_4, rapid dehydration in an ethanol series and embedding in LR White resin (TAAB

Abbreviations: γ-TIP = tonoplast intrinsic protein; IC-cells = inner-cortex cells

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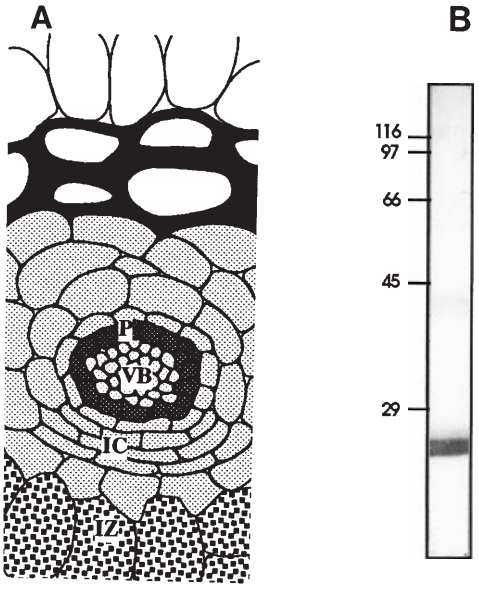

Fig. 1A. Diagram to show the arrangement of tissues in a soybean nodule: inner-cortex (*IC*) cells are adjacent to the infected zone (*IZ*) and to the vascular bundle (*VB*). *P*, pericycle transfer cells. **B** Microsomal fractions isolated from *Glycine max* were processed for immunoblotting with 1/750-dilluted anti-radish VM23. A band with an apparent molecular weight of 23 kDa corresponds to the aquaporin. The VM23 protein was detected in 40 μg total proteins of the microsomal fraction

Laboratories, J Delville, St. Germain en Laye. France). Polymerization was allowed to occur in gelatin capsules at 60 °C for 24 h. Thin sections were collected on parlodion-coated gold grids. The immunogold reaction was performed as previously described (Fleurat-Lessard et al. 1997). Observations were made at 80 kV with a 100C Jeol microscope (Tokyo, Japan). Samples for quantification of gold particles included tissues from three independent chemical fixations. For each procedure, at least four immunoreactions were performed. Membrane isolation, SDS-PAGE and Western blot analyses were performed as described previously (Fleurat-Lessard et al. 1997).

Polypeptides in the microsomes of soybean specifically cross-reacted with the polyclonal antibody directed against the purified γ-TIP VM23 of radish vacuolar membrane (Maeshima 1992; Fig. 1B). We therefore proceeded to immunolocalize and quantify this protein at the subcellular level.

The γ-TIP was immunodetected in IC-cells (Fig. 2A) in chemically fixed nodules and found to be abundant in the vacuolar membrane (Fig. 2C). The evaluation of gold particle distribution (Table 1) gave a density of 21.2 ± 1.8 in IC-cells and 2.8 ± 0.3 in pericycle transfer cells (Fig. 2D,E). These transfer cells were characterized by finger-like wall ingrowths (Fig. 2B,C). The γ-TIP was poorly expressed in the infected cells (1.1 ± 0.3; Fig. 2F). Controls in which the antibody was saturated with the purified protein showed a density of 0.3 ± 0.1 (Fig. 2G).

Though the γ-TIP-antibody we used may not react with all tonoplast aquaporins, its higher concentration in IC-cells suggests intense water exchanges in these cells. Indeed, aquaporins may play a pivotal role in water transport from one compartment to another. Because of its large volume, the vacuole is the cell's water reservoir. Changes in the water status of a cell and concomitant water fluxes across the plasma membrane may induce fluxes across the vacuolar membrane. Although it is not known whether membrane intrinsic proteins MIPs are expressed in the plasma membrane of IC-cells, our result is consistent with the possibility that water fluxes across the vacuole membrane play a role in the changes in IC-cell volume and shape postulated to be involved in the regulation of nodule O_2 diffusion (Drevon et al. 1995).

Table 1. Comparative distribution of the γ-TIP VM23 in IC-cells, vascular transfer cells and infected cells of soybean root nodules. Counts were made on six nodules, 30 cells of each type were analyzed, and the total membrane length was at least 500 μm for each cell type. Mean ± SE is given for 10 μm of membrane

Cell type	IC-cells	Vascular transfer cells	Infected cells
Gold particle number (γ-TIP antibody)	21.2 ± 1.8	2.8 ± 0.4	1.1 ± 0.3
Control (saturation of the γ-TIP antibody by the purified protein)	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1

Nodule IC-cells have structural features similar to those of pulvinus motor cells (Serraj et al. 1995). The rapid changes in shape of motor cells (Fleurat-Lessard et al. 1993) depend upon cell turgor variations associated with changes in the intracellular compartmentation of ions (Satter and Galston 1981). A large increase in K^+ concentration occurs in the apoplast during the bending of pulvini (Kumon and Tsurumi 1984). The IC-cell contraction in nodules would also require fast ionic efflux, through outward rectifying channels, followed by water fluxes from the symplast to the apoplast. Though there is no conclusive evidence for changes in ionic fluxes in the nodule cortex, Roy (1993) and Denison and Kinraide (1995) have observed IC-cell membrane depolarization associated with the O_2-induced decrease in nodule conductance to O_2 diffusion. These changes in the electrical potential of the cells might be linked to K^+ export, the associated water efflux resulting in the observed O_2-induced IC-cell contraction (Serraj et al. 1995). Also, the high levels of intrinsic capacitance and elasticity are consistent with modulation of nodule permeability by the release and uptake of intercellular water in the nodule cortex (Purcell and Sinclair 1993).

The pericycle transfer cells with a less developed vacuolar compartment and a 7-fold lower distribution of aquaporin sites than the IC-cell, might be involved in the loading of N_2-fixation products into the xylem vessels for their distribution to the rest of the plant. The export of nitrogenous compounds would occur via a symplastic route from the bacterial tissues to the bundle apoplast (Walsh 1995) and their concentration has been found to be more than 6-fold greater in the sap from bleeding nodules than in other nodule compartments (Pate et al.

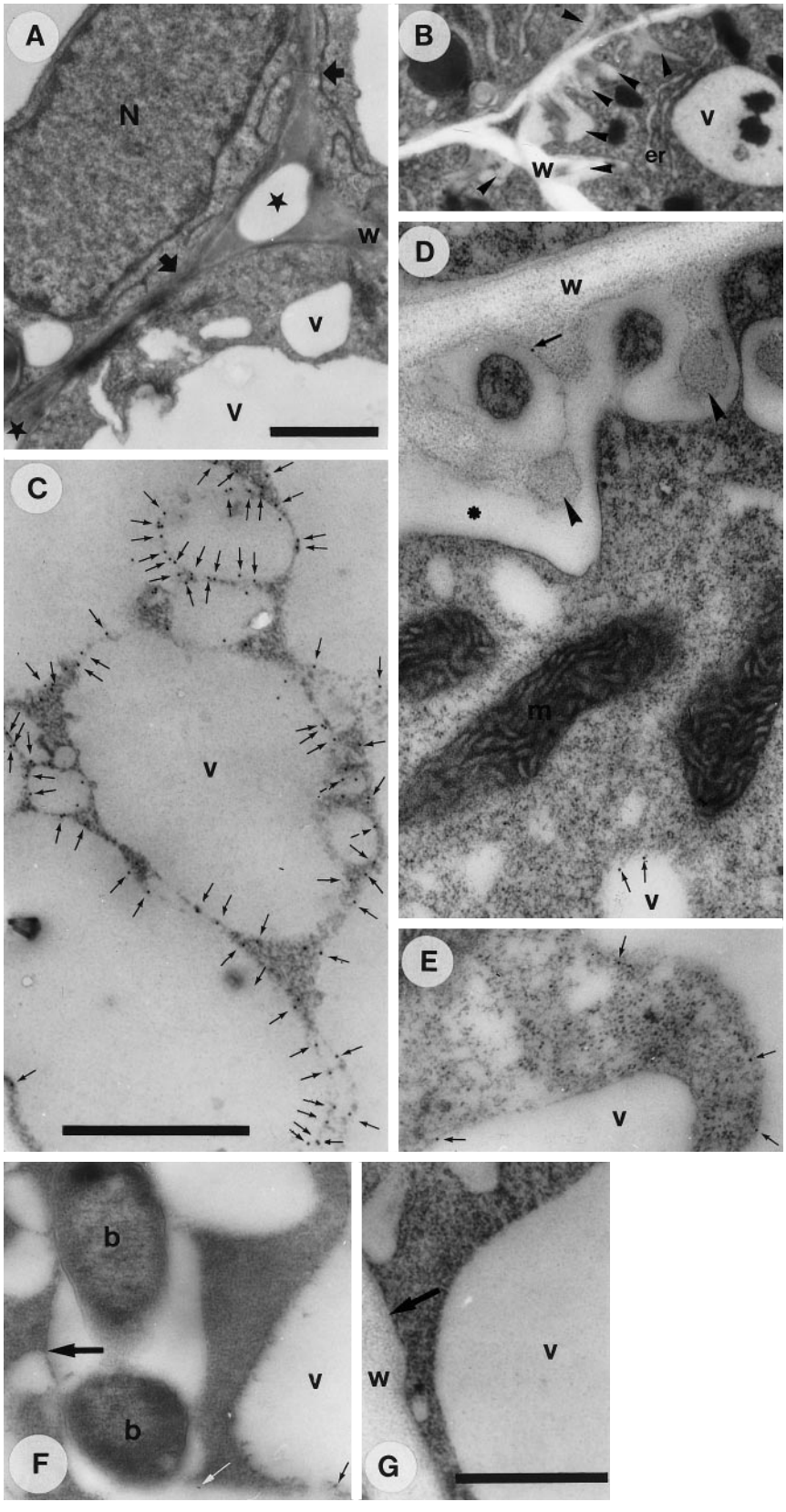

Fig. 2A–G. Comparative distribution of the vacuolar membrane in IC-cells, vascular transfer cells (VT) and the infected zone (IZ) of *Glycine max* nodules. **A** Part of an IC-cell showing the thin layer of cytoplasm, large nucleus (*N*), several vacuole profiles (*v*), and numerous plasmodesmata groups (*thick arrows*). *w*, wall, asterisk, intercellular space. Bar = 2 μm. **B** Part of a pericycle transfer cell showing the finger-like wall ingrowths (arrowheads), abundant cytoplasm, and dilated profiles of ER (*er*). Scale as in **A**. **C** Sections incubated with 1/50-diluted anti-VM23 antibody followed by goat antirabbit IgG-15 nm gold, and showing high immunolabelling (*thin black arrows*) in IC-cells. Bar = 1 μm. **D, E** Low labelling in vascular transfer cells whose large periplasmic space (*asterisk*) underlies wall ingrowths (*arrowheads*). Numerous mitochondria (*m*) with a dense matrix and dilated cristae are present. *White arrow*, non-specific labelling; *thin black arrows* show immunolabelling of γ-TIP Vh 23. Scale as in **C**. **F** Poor labelling in the vacuolar membrane of infected cells, and some non-specific labelling on the plasma membrane (*thick arrow*) surrounding bacteroids (*b*). **G** No labelling occurred in the inner cortical cells when the antibody saturated by the purified VM23 protein was used. Bar = 1 μm; same scale for **F**

1969). On the other hand, wall ingrowths enlarge the volume of the apoplast (Fig. 2B) but also the absorption surface of the plasma membrane.

To conclude, the findings in this study indicate that the differentiation of nodule cortical cells is accompanied by an increased expression of γ-TIP aquaporin. Moreover,

the vacuole may be responsible for ionic and water fluxes associated with the mechanisms of nodule conductance changes by causing the reversible contraction of IC-cells. Further investigations on γ-TIP aquaporin density and ion concentrations in the IC-cells of nodules differing in their conductance to O_2 diffusion are now required.

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