

New data on the *Azolla-Anabaena* symbiosis

II. Cytochemical and immunocytochemical aspects

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Abstract

Cytochemical and immunocytochemical studies were made on the *Azolla-Anabaena* symbiosis to try to understand how metabolites are interchanged between the prokaryotic colony and the fern. The detection of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) by immuno-gold staining was considered to be important in order to understand the behaviour of *Anabaena* cells in the association and the role of the plastids present in *Azolla* transfer hairs and mesophyll cells. We have detected the presence of catalase and glycollate oxidase activity in peroxisomes of *Azolla*. The relationship between these two sets of data is discussed.

Introduction

The physiology and biochemistry of nitrogen fixation in different plants has been studied intensively during the last few years, particularly in the *Rhizobium*-legume symbiosis (Reynolds *et al.*, 1982; Sprent, 1984). These studies have been extended to other plant associations, *e.g.* the *Azolla-Anabaena* symbiosis (Lumpkin and Plucnett, 1980; Peters, 1975; Ray *et al.*, 1978). The most important characteristic of this symbiotic association is that a cyanobacterium (blue-green alga) *Anabaena azollae*, lives in a cavity of the dorsal lobe of the fern leaf, where the ecological conditions stimulate high heterocyst frequency in the cyanobacterium.

The exchange of metabolites between the two organisms is assumed to be an active process of photosynthate translocation from the host to the algae and translocation of fixed nitrogen compounds from the algae to the host.

In this work, cytochemical and immunocytochemical research has been developed, with special attention to the relation between the catalase and glycollate oxidase activities and the presence of RuBisCO in the *Azolla* cells. The detection of this

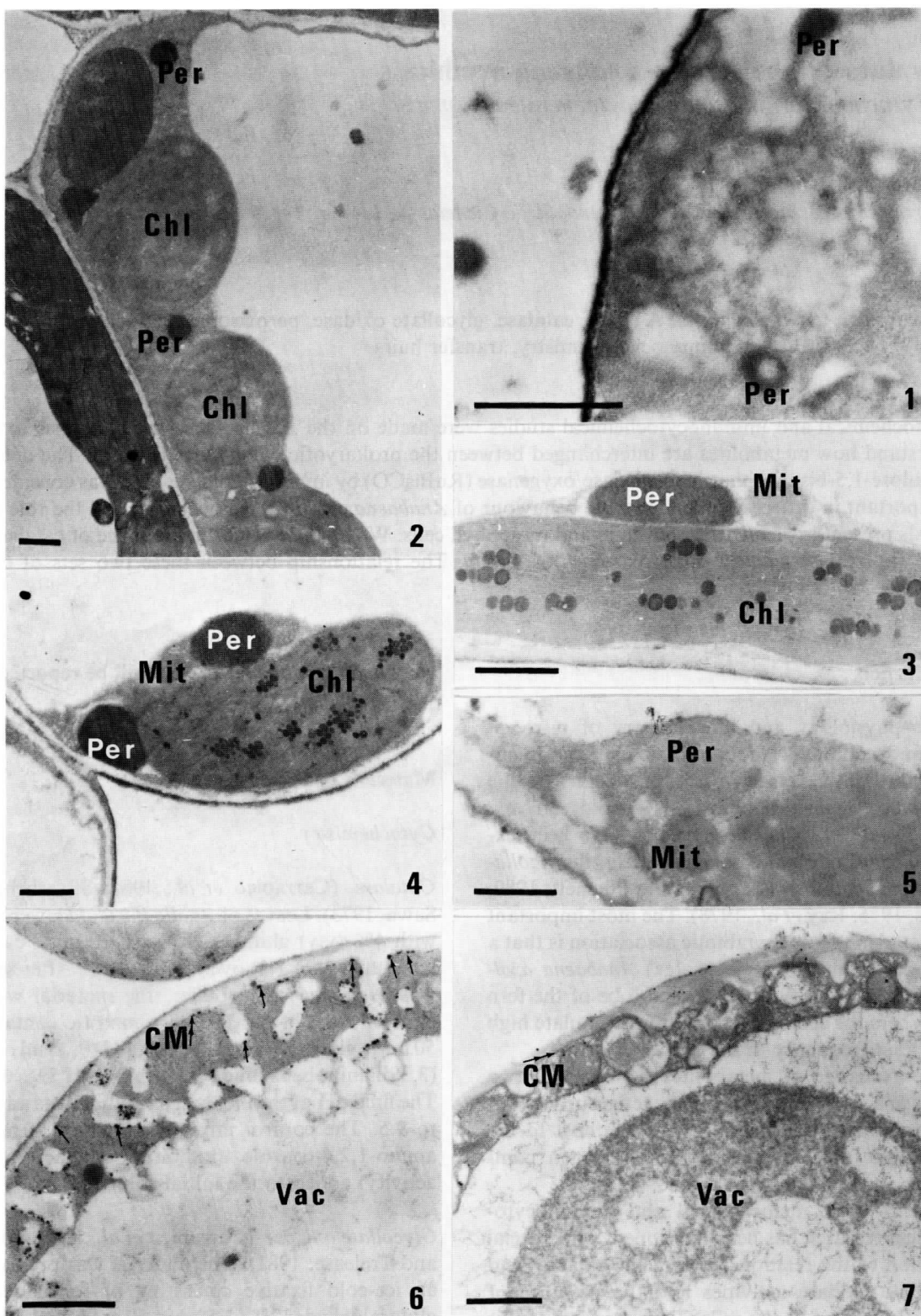
enzyme in the *Anabaena* cells will be reported elsewhere.

Materials and methods

Cytochemistry

Catalase. (Carrapiço *et al.*, 1985; Silverberg and Sawa, 1973). Leaves of *Azolla filiculoides* were fixed with 4% (v/v) glutaraldehyde in 100 mM cacodylate buffer (pH 7.4) overnight at 4°C. After several washes in the same buffer, the material was incubated for 1 h, at 37°C, in a mixture containing: 50 mM glycine/NaOH buffer pH10, 5 ml; DAB (3,3'-diaminobenzidine), 10 mg; 3% H₂O₂, 0.1 ml. The final pH of the incubated solution was adjusted to 9.5. The control mixture contained 20 mM 3-amino-1,2,4-triazole (inhibitor of the catalase activity) added to the initial solution.

Glycollate oxidase. (Carrapiço *et al.*, 1985; Thomas and Trelease, 1981). The material was pre-fixed in an ice-cold fixative consisting of formaldehyde-glutaraldehyde (2%–2.5%) in 50 mM PIPES buffer



(pH 7.4) for 15 min at 4°C. The formaldehyde was freshly prepared from paraformaldehyde immediately before use.

After three washes (10 min each) in the same fixative buffer, the material was pre-incubated overnight, at 4°C, in 100 mM Tris-maleate buffer (pH 7.8), containing 3 mM CeCl_3 and 50 mM 3-amino-1,2,4-triazole. After this step, the samples were incubated at 37°C, for 3 h, in an aerated mixture, containing 100 mM Tris-maleate buffer (pH 7.8), 50 mM 3-amino-1,2,4-triazole, 20 mM cerium chloride (CeCl_3) and 60 mM sodium glycollate. The final pH of the mixture was adjusted to 7.8. The control of the reaction was made with the same medium, but without sodium glycollate.

ATPase. (Hulstaert *et al.*, 1983). *Azolla* leaves fixed in the same way as for glycollate oxidase detection, were pre-incubated overnight at 4°C in a solution consisting 100 mM Tris-maleate buffer (pH 7.2) and 1 mM CeCl_3 . After this step the material was incubated for 45 min at 37°C in a solution consisting 100 mM Tris-maleate buffer (pH 7.2), 1 mM CeCl_3 , 2.5 mM ATP and 4 mM MgSO_4 . The final pH of the mixture was adjusted to 7.2. The control was done without substrate (ATP). All the solutions containing CeCl_3 were made with boiled double-distilled water to prevent carbonate precipitation.

After incubation, the specimens were washed several times in 50 mM cacodylate buffer (pH 6.0) to remove non-specific cerium precipitates, and fixed overnight at 4°C in the same pre-fixative mixture. The samples were washed in 50 mM PIPES buffer (pH 7.4) (10 min each) and post-fixed in 1%

OsO_4 in 50 mM cacodylate buffer (pH 6.8) for 2 h at room temperature. All the samples were dehydrated in an acetone series and embedded in Epon-Araldite (Mollenhauer, 1964).

Immunocytochemistry

RuBisCO (*Ribulose-1,5-biphosphate carboxylase/oxygenase*). (Vaughn, 1987). *Azolla* leaves were fixed in an ice-cold fixative consisting of formaldehyde-glutaraldehyde (2%–2.5%) in 50 mM PIPES buffer (pH 7.4) for 3 h at 4°C and washed in three changes of the same buffer (10 min each). The samples were dehydrated through an ethanol series and embedded in LR White's resin. Thin sections (60–80 nm) of the specimens were cut with a glass knife and Porter-Blum Mt-2 ultramicrotome and mounted on nickel grids.

After this step the grids were floated on 1% (w/s) bovine serum albumin (BSA) in 50 mM sodium phosphate (pH 7.4) with 0.85% sodium chloride (= BSA-PBS) for 30 min to block non-specific antibody sticking. The grids were then transferred to a drop of anti-RuBisCO (antitobacco) diluted 1:20 (v/v) in BSA-PBS for 3 h. After several washes in the same buffer (BSA-PBS) the grids were floated on a 1:30 (v/v) dilution of protein A-colloidal gold (15 nm gold, EY Laboratories, San Mateo, CA) in BSA-PBS for 60 min. After several washes in phosphate-buffered saline (PBS) and water the grids were post-stained in 2% (w/v) uranyl acetate and Reynold's lead citrate. The controls were made with pre-immune sera. The observations were made in a Hitachi-12 electron microscope at 75 Kv.

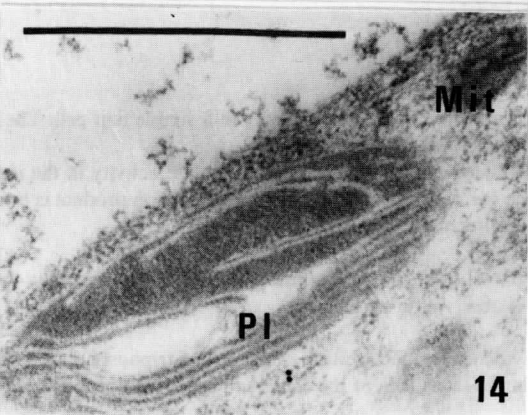
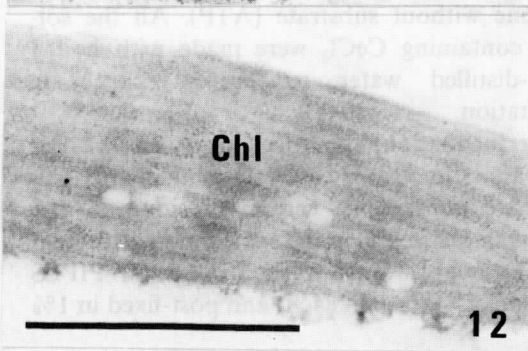
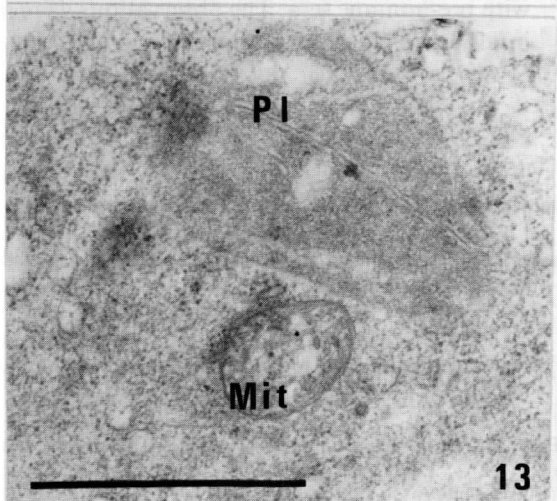
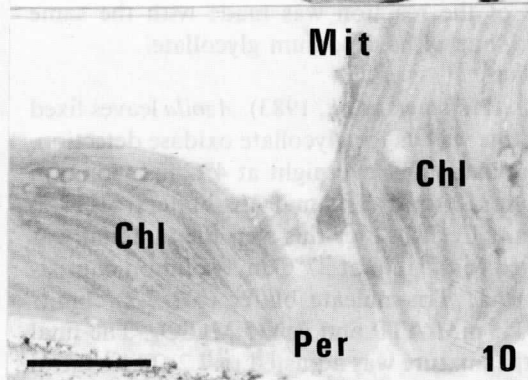
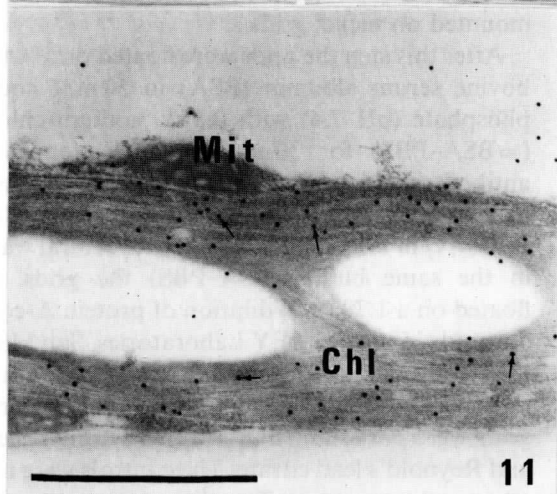
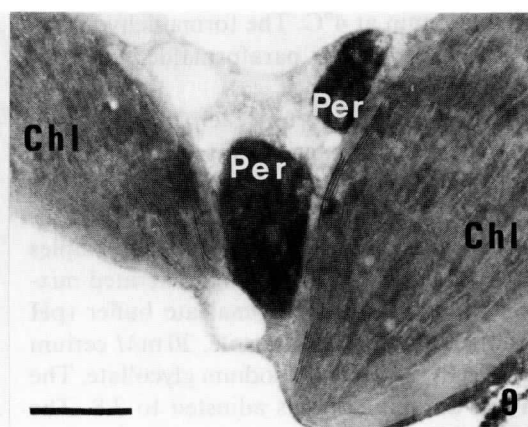
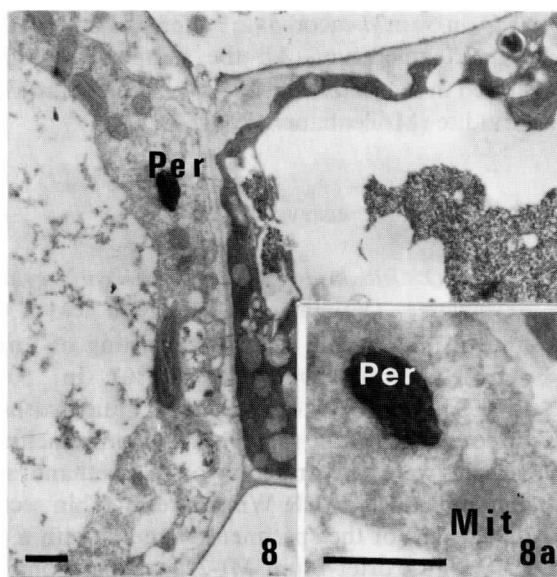
Fig. 1. Localization of catalase activity in a simple hair cell. The stained organelles inside the cell are peroxisomes (Per).

Figs. 2–4. Cytochemical localization of catalase activity in the mesophyll cells of the dorsal leaf lobe (fig. 2 and 3) and in epidermal cells which surround the cavity (fig. 4). The reaction product is only observed in the peroxisomes (Per). No stain is seen in chloroplasts (Chl) and mitochondria (Mit).

Fig. 5. Control of the DAB catalase cytochemical test made by the addition of aminotriazole to the incubation medium. No reaction product is observed in peroxisomes.

Fig. 6. Cytochemical localization of adenosine triphosphatase (ATPase) activity in simple hair cells. The reactivity (arrows) is mainly located in the inner face of the cell membrane (M) (double arrow).

Fig. 7. Control of ATPase by omission of substrate (ATP) from the incubation medium. No reaction product is observed in the cell membrane (CM) (double arrow).



Results and discussion

After incubation of the *Azolla* leaves in a DAB medium to detect catalase activity and in a CeCl_3 glycollate solution to detect glycollate oxidase activity, stained peroxisomes were observed both in mesophyll and cavity epidermal cells of the fern in close association with chloroplasts and mitochondria (Figs. 2, 3, 4, 9). These two organelles did not show any reaction to these tests (Figs. 3, 8, 8a). Stained peroxisomes were also present in the cells of simple hairs (Figs. 1, 8, 8a). The controls of these two tests gave no reaction in peroxisomes (Figs. 5, 10).

The material submitted to the ATPase test revealed the presence of electron-dense deposits of cerium phosphate in the inner face of the cell membrane in simple hair cells (Fig. 6). The control gave a negative reaction in the plasmalemma (Fig. 7).

The immuno-gold staining of RuBisCO was apparent only in the stroma of chloroplasts present in the mesophyll cells (Fig. 11) and no colloidal gold particles were seen in plastids of simple hairs (Fig. 13). The control gave no labelling in these organelles (Figs. 12, 14).

The presence of reactive peroxisomes to catalase and glycollate oxidase tests showed that these organelles contained these two enzymes and are associated with an active metabolic pathway, especially for degradation of glycollate. This product (glycollate), formed in the chloroplasts by the action of RuBisCO's oxygenase activity, is transferred to the peroxisomes, where by the action of glycollate oxidase, it is converted into glyoxylate and initiates the photorespiration process (Tolbert, 1982). The glycollate oxidase activity generates H_2O_2 which is broken down by the action of

catalase, also present in the peroxisomes. In these conditions, the results obtained by the glycollate oxidase and catalase cytochemistry tests in *Azolla* mesophyll cells suggest that these organelles are involved in glycollate degradation and are probably involved in the photorespiration process (Peters *et al.*, 1982).

RuBisCO is an important enzyme in the photosynthetic carbon reduction pathway (Grey and Kekwick, 1974; Vaughn, 1987) and its immunodetection in the chloroplasts of the *Azolla* mesophyll cells confirms the capacity of these organelles to fix CO_2 . This result is normal, but the absence of this enzyme of the plastids in the transfer hair cells was not expected. These results suggest that in simple hair cells, plastids are not involved in CO_2 fixation and in glycollate synthesis. The presence of a few starch granules in these organelles could be explained by a flow of sugars through these hairs, where an excess of monosaccharides produced in the mesophyll cells could participate in starch formation. The detection of ATPase activity in the cell membrane of the transfer hairs confirms this idea and probably suggests the existence of a proton co-transport of sugars from the mesophyll cells into the mature leaf cavity. This enzyme may be involved in ion movements and could account for the stimulation of sugar loading (Malek and Baker, 1977; Peters and Calvert, 1983).

If the presence of the glycollate oxidase activity in the mesophyll peroxisomes is normal, the existence of this enzyme in the peroxisomes of hair cells is unexpected. Since there is no RuBisCO in the plastids of these cells, no glycollate can be formed there. In these conditions, the presence of glycollate oxidase in hair cell peroxisomes could

Figs. 8–9. Cytochemical localization of glycollate oxidase activity. Electron dense deposits of cerium perhydroxide are only observed in peroxisomes (Per) from simple hair cells (figs. 8, 8a) or mesophyll cells (fig. 9). No reaction product is observed in mitochondria (Mit).

Fig. 10. Control of glycollate oxidase test by omission of glycollate from the incubation medium. No reaction product is seen in peroxisomes (Per).

Fig. 11. Immuno-gold staining of RuBisCO in mesophyll cells. Colloidal gold particles are mainly restricted to the chloroplasts (Chl). No particles are observed in mitochondria (Mit).

Fig. 13. Immuno-gold staining of RuBisCO in simple hair cells. No colloidal gold particles are seen in the plastids (Pl) of these cells.

Figs. 12 and 14. Control of immunocytochemical localization of RuBisCO, by treatment with the pre-immuno serum. No colloidal gold particles are observed in the chloroplasts of mesophyll cells (fig. 12) or in the plastids of simple hair cells (fig. 14).

Bar in figures = 1 μm

indicate that a flow of glycollate occurs from the mesophyll cells or from another source to the simple hairs. Another possible explanation of these results may be found in the affinity of this enzyme to other substrates in addition to the glycollate, such as L-lactate and other hydroxy acids (Thomas and Trelease, 1981).

In conclusion, we suggest that the simple hair cells are involved in a process of carbohydrate translocation to the cavity and that sugars are formed in the chloroplasts of the mesophyll cells. The plastids of the transfer hairs are probably involved in the formation of lipophilic compounds found in the vacuoles of these cells.

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