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In situ immunofluorescent localization of ribulose-1,5-bisphosphate carboxylase/oxygenase in mesophyll of C_4 dicotyledonous plants

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Abstract

The location of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) in the leaf mesophyll of some dicotyledonous C_4 plants was confirmed by immunofluorescent labelling. The anti-RuBPCO immune serum was obtained by inoculating a rabbit with commercially obtained RuBPCO. Specificity of these antibodies was tested by immunodiffussion, immunoelectrophoresis, and Western blotting. Fresh hand-cuts of leaves from dicotyledonous C_4 plants, *Amaranthus caudatus*, *A. dubius*, *Gomphrena globosa*, and *Portulaca oleracea*, were incubated with the conjugated anti-RuBPCO immune serum and then with a commercial FITC-anti-rabbit IgG conjugate. *Nerium oleander* was used a control C_3 plant pattern and *Zea mays* as a C_4 plant pattern. The immunofluorescent label was distributed in both mesophyll and bundle sheath in all the C_4 plants tested. It is an unequivocal proof that in the C_4 dicotyledonous plants the RuBPCO is not only located in the chloroplasts of the bundle sheath cells but also in the chloroplasts of the mesophyll cells. In these plants therefore, the C_4 pathway cannot exclusively be viewed as an intercellular level concentration mechanism. In the mesophyll cytoplasm, phosphoenolpyruvate carboxylase traps CO_2, while in the mesophyll chloroplasts, RuBPCO operates with atmospheric CO_2 and CO_2 from the C_4 decarboxylation step at an intracellular level, which could mean a significant energetic economy. The CO_2 from photorespiration could be saved and reincorporated. Location of RuBPCO in the mesophyll and/or bundle sheath chloroplasts is a matter of inter- and intra-cellular compartmentation which makes another variation of C_4 photosynthetic pathway possible.

Additional key words: *Amaranthus*; *Gomphrena*; *Portulaca*; *Nerium*; *Zea*.

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Introduction

Plants with C_4 photosynthetic pathway have a specialized leaf anatomy which is complementary to the CO_2 metabolism, eliminating photorespiration and increasing the CO_2 assimilation capacity (Black 1973, Hatch 1987, Brown and Hattersley 1990). The C_4 pathway is a metabolic cooperation between mesophyll (M) and bundle sheath (BS) cells and has evolved in both monocotyledonous and dicotyledonous families (Moore 1982). The *in situ* intercellular localization of C_4 photosynthetic enzymes has been reported (Hattersley *et al.* 1977, Matsumoto *et al.* 1977, Perrot-Rechenmann *et al.* 1982, Bauwe 1984, Reed and Cholett 1985). Recently, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39, RuBPCO) activity was detected in mesophyll and bundle sheath cells of six C_4 dicotyledonous species: *Gomphrena globosa*, *Portulaca oleracea*, *Amaranthus caudatus*, *A. retroflexus*, *Atriplex canescens* and *A. halimus* (Castrillo and Whatley 1994). The fairly high RuBPCO activities found in the mesophyll cell fraction could not be explained in terms of bundle sheath contamination. In the present work we report the succesful *in situ* immunofluorescent labelling of RuBPCO in hand-cut leaf blade sections of four C_4 dicotyledonous plants, *G. globosa*, *P. oleracea*, *A. caudatus* and *A. dubius* in a C_4 monocotyledonous plant *Zea mays* (the classical C_4 plant), and in a C_3 dicotyledonous plant *Nerium oleander*.

Materials and methods

Plants: *P. oleracea* and *A. dubius* were collected near the beach in the xerophic coast at the west of La Guaira port, near Las Salinas village, Distrito Federal (10°31'N, 67° 15'W). *G. globosa* and *A.caudatus* were bought in an ornamental plantshop. A botanical sample of the plants was taken to the herbarium for proper identification of the species. The seeds of *Zea mays* L. (C_4) were obtained from Fondo Nacional de Investigaciones Agropecuarias (FONAIAP), germinated in plastic trays and transplanted to plastic pots (4000 cm^3). Leaf samples of *N. oleander* (C_3) were taken from the University gardens. Fully expanded young leaves were used in all cases. For crude bean leaf extract preparation, *Phaseolus vulgaris* L. cv. Tacarigua was used.

Preparation of antibodies: To obtain the anti-RuBPCO antibodies, a 2.5 kg young adult male rabbit (New Zealand stock) was injected twice in 30-d intervals with 5 mg of D-ribulose-1,5-bisphosphate carboxylase from *Sigma*, using a complete Freund's adjuvant for the first inoculation and an incomplete adjuvant for the second one. After 15 d from the second injection, the rabbit was bled from the marginal ear vein. The immune sera obtained were kept at -20 °C until used.

Anti-RuBPCO specificity test: The specificity of the rabbit immune serum was tested by three different immunological methods. The Ouchterlony (1958) double diffusion assay in 1 % agarose gel in 0.02 M buffer phosphate saline, pH 8.0 (PBS) containing 0.15 M NaCl was used. Different double serial dilutions of the commercial RuBPCO (*Sigma*) in PBS were tested against several dilutions of the normal and

immune rabbit sera. Additionally immunoelectrophoresis was done in 1 % agarose gel in 0.1 M Tris-0.05 M acetic acid buffer, pH 8.0. Frozen (-20 °C in a freezer) and fresh prepared RuBPCO (both dissolved in PBS, pH 8.0) as well as a crude bean leaf extract (kept frozen at -70 °C in a freezer) (extraction medium: 0.2 M Tricine-NaOH, pH 8.1 containing 17.1 mM NaCl, 0.2 mM EDTA sodium salt, 10 mM $NaHCO_3$, 10 mM $MgCl_2$; 1 g bean leaf fresh mass per 5 cm^3 extraction medium) were separated by electrophoresis and incubated for 24 h with several dilutions of normal and immune rabbit sera. The immune blot technique was performed by a SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After the electrophoretic run, the polypeptides were electrotransferred to a nitrocellulose paper (230-250 mA, 45 min at 4 °C). The nitrocellulose paper was incubated with 1:100 dilution of anti-RuBPCO rabbit serum and after washing with 1:7500 diluted commercial anti-rabbit IgG-peroxidase conjugate.

***In situ* immunofluorescent labelling of RuBPCO:** The method reported by Hattersley *et al.* (1977) was employed. Fresh leaf segments (2 × 5 mm) including minor veins were taken and cut by hand with new razor blades. The cuts were immersed in 70 % ethanol for 2 h, then washed briefly with PBS (0.1 M K_2HPO_4 in 0.2 M NaCl), incubated for 1.5 h in anti-RuBPCO serum diluted 1:2 in PBS, and placed inside wet chambers. The cuts were rinsed for 20 min in three changes of 2 cm^3 PBS with constant and gentle agitation. Immediately afterwards the cuts were incubated in conjugated goat anti-rabbit IgG coupled with fluorescein isothiocyanate (FITC) diluted 1:10 in PBS. The sections were incubated in darkness inside a wet chamber for 1.5 h and then rinsed again for a period of 20 min with three changes of PBS. The sections were mounted on slides in aqueous solution of 50 % glycerol containing 1 % thymol, and kept in darkness at 4 °C. The procedure was performed at room temperature. A preimmune control was also set, following the same procedure but using normal rabbit serum. The second control used was an autofluorescent one, fixed with ethanol and directly mounted without any sera. The mounted sections were observed using a *Dialux Leitz-Wetzlar* microscope with an *Orthomat* photographic camera 3.2:1, set up for epifluorescence (*Ploemopak* 2.3), blue excitation with lamp *200Z* and a two position filter system (excitor filter, dichromatic mirror, and barrier filter). The ocular lens was 12.5×. *Kodak* colour film (*Gold-400*) 400 ASA (rapid exposure) was used, utilizing automatic exposure facilities incorporated in the microscope. Sections were observed and photographed within 24 h.

Results and discussion

The tests for the specificity of the rabbit immune serum are shown in Figs. 1 to 3. Fig. 1 shows the results of immunodiffusion assays. The central well of set *A* contained fresh commercial RuBPCO preparation (0.5 kg m^{-3}). In the central well of set *B*, frozen RuBPCO preparations were used, and in set C the central well was filled with crude bean leaf extract. In the external wells (numbered *1*, *2* and *3*), the anti-RuBPCO sera (well *1*) and two sera dilutions 1:2 and 1:4 (wells *2* and *3*

respectively) were present. Wells *4, 5* and *6* contained another batch of the same immune serum. The immune serum at different dilutions recognized the commercial RuBPCO (set *A*). Also the antibodies were able to react against the enzyme from the crude bean leaf extract (set *C*). The antibodies reacted weakly with the frozen enzyme. Hence during freezing the enzyme probably underwent a change in its topology in a way that the main antigenic epitopes were not well recognized. When normal rabbit serum was used under the same conditions, no reactions were observed in the Ouchterlony assay.

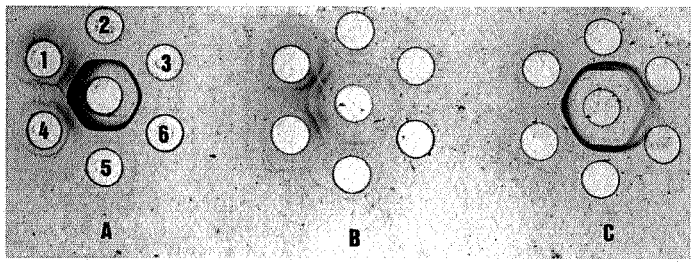

Fig. 1. Immunodiffusion. The method of Ouchterlony (1958) double diffusion. *A*: The central well contained recently suspended 0.5 kg m^{-3} fresh preparation of the enzyme obtained from *Sigma*. *B*: The well contained the same concentration of enzyme preparation but previously frozen. *C*: The well contained a crude leaf extract from bean. The external wells *1*, *2* and *3* contained the anti-rabbit sera nondiluted (*1*) and/or diluted 1:2 (2) or 1:4 (*3*). Wells *4*, *5*, and *6* contained other batches of the same serum.

Fig. 2 shows the immunoelectrophoresis of the rabbit anti-RuBPCO immune serum against fresh and recently prepared RuBPCO: 0.5 kg m^{-3} (*A*) and 1 kg m^{-3} (*B*); frozen RuBPCO: 0.5 kg m^{-3} (*C*) and 1 kg m^{-3} (*D*); and crude bean leaf extract (*E*). Only one precipitating arc was observed in the anionic zone with commercial fresh RuBPCO (*A* and *B*), indicating that only electronegative antigen was recognized by the rabbit antibodies. The frozen enzyme (*C* and *D*) was not recognized by the immune serum. Two bands were recognized in the crude bean leaf extract, one of them in the same position as in the commercial RuBPCO.

Fig. 3 shows the SDS-PAGE and immunobloting with the anti-RuBPCO serum. A very strong polypeptide band of 55 kDa and another weaker of 14 kDa are recognized by the antibodies either in frozen RuBPCO (line *2*), fresh enzyme preparation (line *3*), and crude bean leaf extract (line *4*). These results are consistent with the fact that the immune serum obtained is able to react with the two polypeptides which are part of the RuBPCO molecule (large and small subunits). Additionally, the obtained anti-RuBPCO immune serum was able to recognize denaturalized antigens, which might be present in the frozen enzyme preparation (line *2*). More important is the fact that only two bands were recognized in the crude bean leaf extract indicating that this antiserum reacted mostly with the two RuBPCO subunits. These results demonstrated the specificity of the *Sigma*'s partially purified RuBPCO with its prepared anti-RuBPCO serum and that the antibodies were able to

recognize the enzyme in conditions similar to those found under native conditions (crude leaf extract).

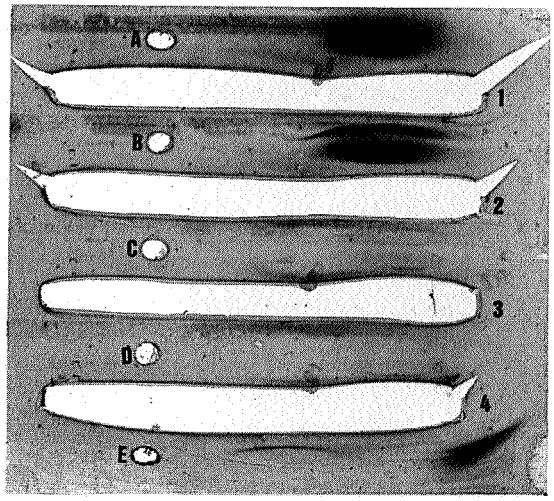

Fig. 2. Immunoelectrophoresis. The rabbit anti-RuBPCO immune serum against: (*A*) 0.5 kg m^{-3} RuBPCO fresh and recently prepared, (*B*) 1 kg m^{-3} RuBPCO fresh and recently prepared, (*C*) 0.5 kg m^{-3} frozen RuBPCO, (*D*) 1.0 kg m^{-3} frozen RuBPCO, (*E*) crude bean leaf extract (diluted 1:2).

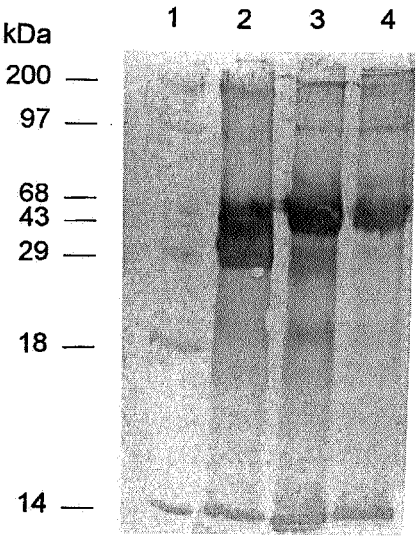

Fig. 3. Western blot. SDS-PAGE and immunobloting with the anti-RuBPCO serum. A very strong polypeptide band of 55 kDa and other weaker of 14 kDa are recognized by the antibodies against RuBPCO: line *1* - markers; line *2* - frozen RuBPCO; line *3* - fresh enzyme prepared; line *4* - crude bean leaf extract.

In the leaf sections of *N. oleander* (C_3) a specific fluorescence corresponding to RuBPCO was located in palisade and spongy parenchyma, just above a stomatal cavity (Fig. 4*A*) and an unspecific fluorescence was observed mainly from an

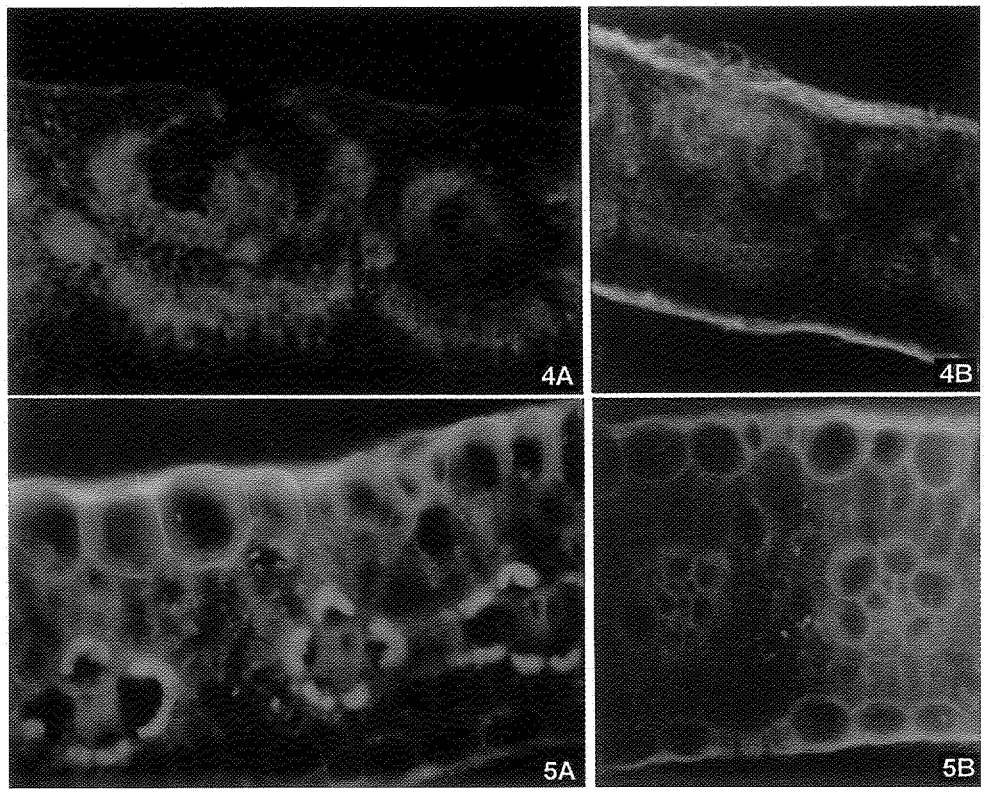

Fig. 4 (*top*). Leaf sections of *Nerium oleander* (C_3). *A*: A specific fluorescence corresponding to RuBPCO was located in palisade and spongy parenchymas, just above the stomatal cavity. *B*: in the normal rabbit serum treated section leaf, an unspecific fluorescence was observed mainly at the epidermis level.

Fig. 5 (*bottom*). *Zea mays* (C_4) leaf sections. *A*: A specific fluorescence corresponding to RuBPCO was located in the bundle sheath chloroplasts in centrifugal position. *B*: In the normal rabbit serum treated section leaf, an unspecific fluorescence was observed mainly from epidermis cells and from the walls of vascular bundles.

epidermal level (Fig. 4*B*). In *Z. mays* (C_4), a specific fluorescence corresponding to RuBPCO was located in the bundle sheath chloroplasts in a centrifugal position (Fig. 5*A*) and an unspecific fluorescence was found mainly in epidermis cells and in the walls of vascular bundle (Fig. 5*B*). In the *G. globosa* leaf section, a specific fluorescence corresponding to RuBPCO was observed in mesophyll and bundle sheath tissues (Fig. 6*A*) and an unspecific fluorescence was mainly observed in epidermis and vascular bundles (Fig. 6*B*). In *A. dubius*, a distinctive specific fluorescence corresponding to RuBPCO was located in mesophyll and bundle sheath tissues (Fig. 7*A*) and an unspecific fluorescence was spread throughout the section (Fig. 7*B*). In leaf section of *A. caudatus*, both the specific (Fig. 8*A*) and unspecific (Fig. 8*B*) fluorescences corresponding to RuBPCO were spread throughout the leaf section. The "Kranz anatomy pattern was not observed. In *P. oleracea*, the specific flurescence was observed throughout the leaf section (Fig. 9*A*) and only a weak unspecific fluorescence was observed (Fig. 9*B*). The "Kranz" anatomy pattern was not observed.

The present results confirm those reported before about localization of RuBPCO activity in the mesophyll cells in some plant species (*G. globosa*, *A. caudatus* and *P. oleracea*) (Castrillo and Whatley 1994). These results confirm that RuBPCO localization in the mesophyll and bundle sheath of C_4 plants is a fact even in plants having high PEPC activity and with or without the "Kranz" anatomy, and that this makes sense if it is considered that the C_4 photosynthetic complementary pathway represents a real way to reduce the photorespiration without the energetic cost of metabolite transport and compartmentation.

There have been many evidences on differences in the localization of enzyme activities, immunolocalization, anatomy and photorespiration with respect to the classical and typically established C_4 structure/function scheme. Hattersley *et al.* (1977) reported some specific fluorescence, arising from labelled RuBPCO in mesophyll chloroplasts of some monocots and dicots (*Gomphrena celosioides* and *Salsola kali*); for *G. celosioides*, this specific fluorescence persisted upon using different fixation times, leaves of different ages and of different antisera, but it did not persist only in a control with FITC-labelled sheep anti-rabbit serum. Even if these observations may reflect the presence of some RuBPCO in mesophyll cell chloroplasts of certain C_4 species, the authors conclude that their results for species with "classical" C_4 leaf anatomy demonstrate that RuBPCO is located almost exclusively in the bundle sheath cells. According to Nelson and Langdale (1992), in spite of the consistent correlation of the "Kranz" feature with the C_4 metabolism, there is not an absolute relationship between structure and function. Several combinations have already been described, for example, C_3 plants with the "Kranz" anatomy (Hedge and Patil 1981, Edwards *et al.* 1990) and C_4 plants showing variations of the normal "Kranz" scheme (Shomer-Ilan *et al.* 1985, Dengler and Dengler 1990, Dengler *et al.* 1990).

Flaveria brownii was initially classified as a typical C_4 plant (Bassüner *et al.* 1984) but its leaves lack the strict intercellular compartmentation of several photosynthetic enzymes, including RuBPCO and phosphoenolpyruvate carboxylase

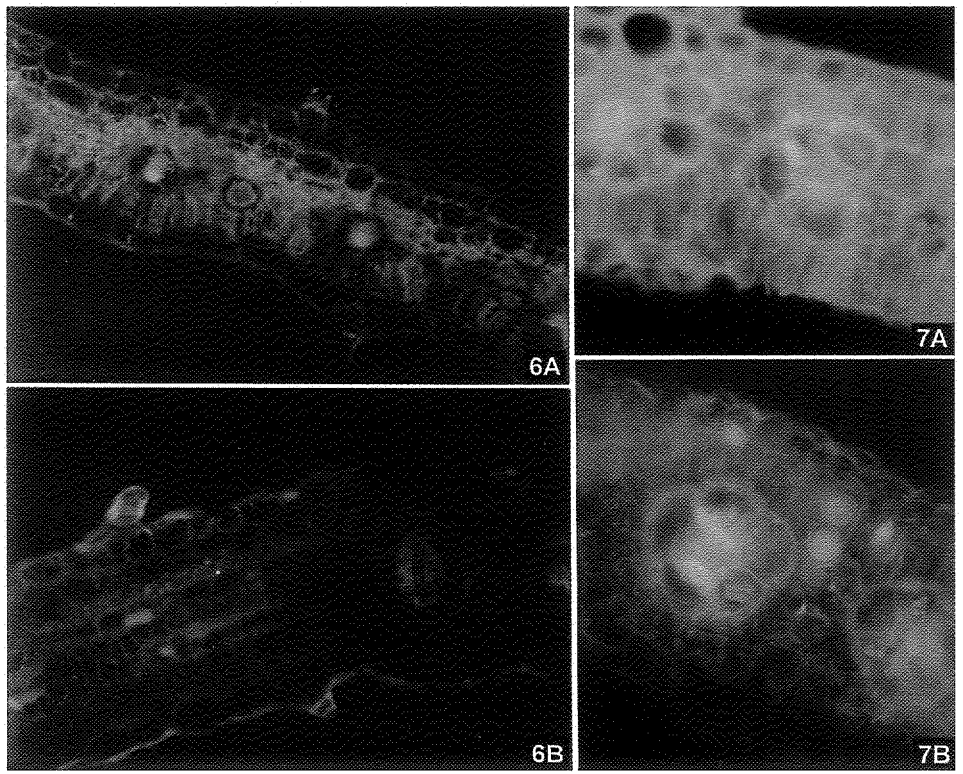

Fig. 6 (*left*). *Gomphrena globosa* leaf sections. *A*: A specific fluorescence corresponding to RuBPCO was observed in mesophyll and bundle sheath tissues throughout the leaf section. *B*: In the normal rabbit serum treated leaf section, an unspecific fluorescence was mainly observed from epidermis and vascular bundles.

Fig. 7 (*right*). Leaf sections of *Amaranthus dubius*. *A*: A distinctive specific fluorescence corresponding to RuBPCO was observed in mesophyll and bundle sheath tissues throughout the leaf section. *B*: In the normal rabbit serum treated leaf section, an unspecific fluorescence was spread throughout the section.

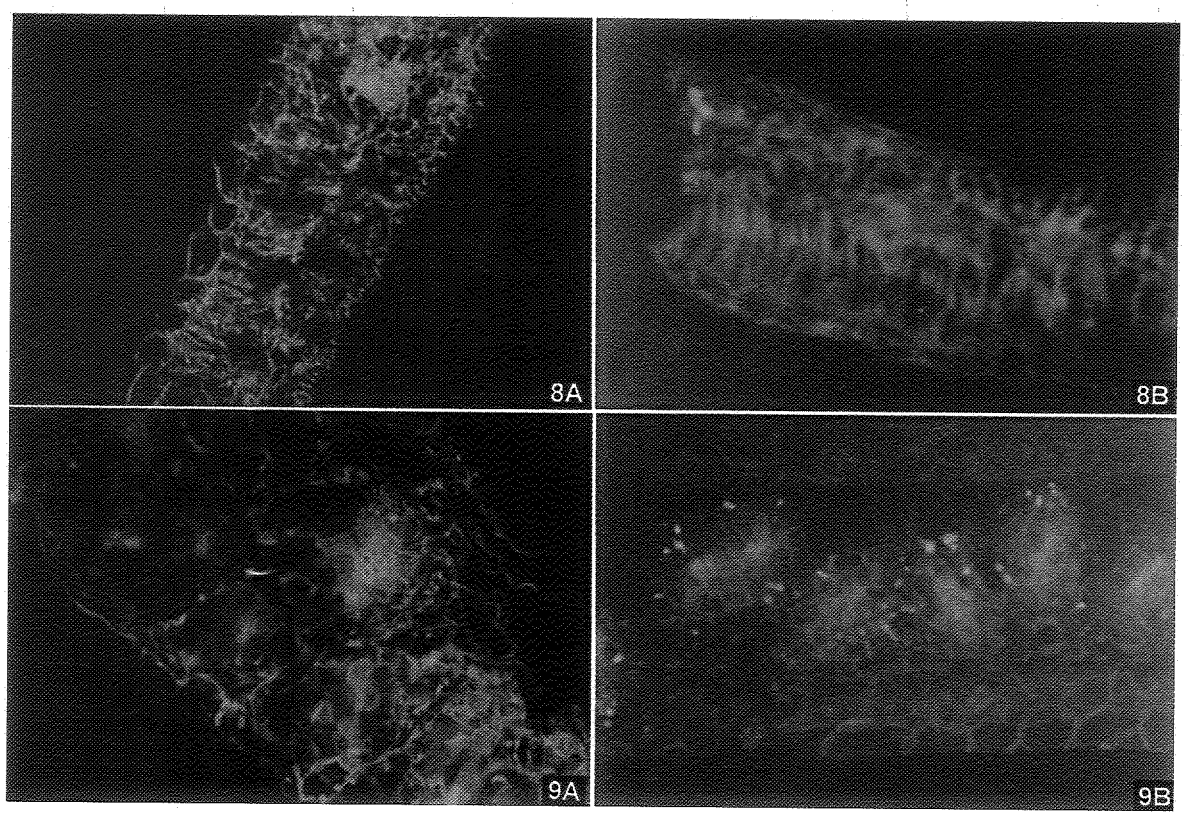

Fig. 8 (*top*). Leaf sections of *Amaranthus caudatus*. *A*: A specific fluorescence corresponding to RuBPCO was observed throughout the leaf section. *B*: In the normal rabbit serum treated leaf section, an unspecific fluorescence was spread throughout the leaf section. The "Kranz" anatomy pattern was not observed.

Fig. 9 (*bottom*): Leaf sections of *Portulaca oleracea*. *A*: A specific fluorescence corresponding to RuBPCO was observed throughout the leaf section. *B*: In the normal rabbit serum treated leaf section, a weak unspecific fluorescence was observed. The "Kranz" anatomy pattern was not observed.

(PEPC) (Bauwe 1984, Reed and Chollet 1985, Cheng *et al*. 1988.). Cheng *et al.* . (1988). conclude that *F. brownii* has some capacity for C_3 photosynthesis in the mesophyll cells and should be considered a C_4-like species; they also state that there is a gradient of PEPC activity decreasing and a gradient of RuBPCO activity increasing towards the nearest vein. In *Hydrilla,* RuBPCO is present in the chloroplasts and PEPC in the cytosol of all leaf chlorenchyma cells suggesting that an intracellular C_4 cycle may function there (Reiskind *et al.* 1989). On the other hand, there have been reports about the photosynthetic plasticity of several C_4 species related to leaf ontogeny, light growth conditions and cell position with respect to veins (Hatch *et al.* 1969, Sheen and Bogorad 1985, Cheng *et al.* 1989). Callus cultures derived from *P. oleracea* exhibit reduced photorespiration without vascularization and the "Kranz" anatomy differentiation, leading to the conclusion that the lack of detectable photorespiration is not related to the anatomy (Kennedy 1976). Our results on *P. oleracea* confirm the results of Kennedy. Reiskind *et al.* (1989) conclude that the reduced apparent photorespiration in *Moricandia* is not achieved through separation of CO_2 fixation events into different cells but rather through the efficient refixation of photorespiratory CO_2 by RuBPCO. Brown (1980) suggests that the intermediacy of many C_3-C_4 species is primarily due to a refixation of photorespired CO_2, rather than to a CO_2 concentrating mechanism. Recently, Reyes, Fernandes and Baker (1995) reported immunogold localization of RuBPCO and glycine decarboxylase (enzyme of the photorespiratory pathway): in the leaf mesophyll of C_3 plants, in the bundle sheath of the "classical" C_4 standard and in the C_3-C_4 intermediate plants, RuBPCO was located in the mesophyll and bundle sheath, while glycine decarboxylase was exclusively found in the bundle sheath.

According to our results, it seems that the function of the C_4 photosynthetic pathway is to reduce photorespiration through the CO_2 concentrating mechanism around RuBPCO; this fact appears to be independent of the location of RuBPCO. The CO_2 from photorespiration could be saved and reincorporated again. The RuBPCO could be located in the mesophyll and/or bundle sheath chloroplasts, it is a matter of inter- or intracellular compartmentation, and this is an evidence of another variation of C_4 photosynthetic pathway. The C_3-C_4 intermadiate species could be then considered as a stabilized photosynthetic alternative that will not evolve to the level of the "classical" C_4 (Monson *et al.* 1984).

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