Plant Physiol. (1982) 69, 273–277
0032-0889/82/69/0273/05/$00.50/00

Localization of Ribulose Bisphosphate Carboxylase in the Guard Cells by an Indirect, Immunofluorescence Technique[1]

Received for publication March 9, 1981 and in revised form September 2, 1981

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ABSTRACT

Ribulose bisphosphate carboxylase, a key enzyme in the photosynthetic carboxylation process, has been localized through an indirect immunofluorescent technique in the guard cells of some of the 41 species of plants examined. This sample includes 17 families of both dicotyledons and monocotyledons, one gymnosperm, and one pteridophyte. Plants were selected to represent all of the three major photosynthetic categories, namely C_3, C_4, and Crassulacean acid metabolism. Antibodies raised against tobacco (*Nicotiana tabacum* L.) ribulose bisphosphate carboxylase were used for this immunofluorescent study. A good degree of fluorescence was observed in the guard cells of seven out of 21 species exhibiting Crassulacean acid metabolism. C_3 plants exhibited a very low degree (almost negligible) of fluorescence, while the C_4 species did not exhibit any fluorescence.

RuBP[2] carboxylase has long been known as an essential enzyme for carbon assimilation in C_3, C_4, and CAM plants. A variety of experiments has been designed to detect the amount and activity of this enzyme in various parts of plant tissues. One of the most widely used and recently developed methods to determine the amount of this enzyme is the immunoquantitation method.

Guard cells of leaf epidermal tissues in many species are known to possess high levels of PEP carboxylase (29, 30). The major pathway of carbon dioxide fixation in guard cells of *Commelina communis, C. benghalensis, C. cyanea, Tulipa gesneriana, Tridax procumbens,* and *Allium cepa* has been found to be through this enzyme (5, 10, 19–21, 24, 26, 28, 29). Fixation of CO_2 via PEP carboxylation leads to the formation of malate and aspartate in the isolated, detached epidermis of the species which have been investigated (26). The production of malate has also been linked to the control of stomatal aperture by CO_2 concentration, and the concentration of malate has been shown to increase during stomatal opening (2, 12, 14, 15, 25). It is suggested that this malate accumulates in the vacuoles of guard cells and provides one of the balancing anions for the influx of cations (probably potassium) during stomatal movement. There is a strong indication that when stomates close, the malate is removed from the vacuole and is converted to starch or other polysaccharides, possibly by gluconeogenesis (6, 27), or is transferred from the guard cells to adjacent epidermal cells and, possibly, even to the mesophyll (18, 22).

It has also been suggested recently that, although the leaf epidermal tissues contain high levels, PEP carboxylase does not make a large contribution to the metabolism of the epidermal tissues (27, 31). Raschke and Dittrich (16) suggested that the Calvin cycle is absent from the epidermal tissues of plants that they investigated, but there has been some speculation regarding Calvin cycle activity in the epidermis of *Vicia faba* (1, 10). This may be due to contamination of the epidermal tissue with mesophyll fragments, as it was more difficult to free the epidermis from mesophyll in this tissue than it was in the other species studied.

Outlaw *et al.* (13) have suggested that RuBP carboxylase activity is absent from the guard cells of *V. faba*. Almost all of the studies concerned with guard cell enzymes have been carried out with plants possessing C_3 photosynthetic metabolism. Although it is difficult to get a good supply of detached epidermis from most of the C_4 plants, CAM plants are usually not chosen for epidermal studies for fear of contamination by mesophyll tissue.

In the present study, an attempt has been made to localize RuBP carboxylase in the guard cells following an indirect, *in situ* immunofluorescent method of Hattersley *et al.* (8). Forty-one species belonging to different photosynthetic categories have been surveyed for the presence of this enzyme in their guard cells.

MATERIALS AND METHODS

Crystalline RuBP carboxylase was prepared from fully expanded leaves of tobacco (*Nicotiana tabacum* L.) following the techniques described by Chan *et al* (3). Antisera for immunofluorescence was directed against 4-times recrystallized tobacco RuBP carboxylase. Four albino rabbits were given subcutaneous injections of a total of 10 mg crystalline RuBP carboxylase resuspended in Freund's complete adjuvant. Rabbits were given an intravenous booster containing 1 mg/ml RuBP carboxylase 1 week prior to collection of serum. Antiserum collected from all of the rabbits showed an optimal proportion of <0.5 titer (23). Serum specificity was determined qualitatively by Ouchterlony double diffusion (11). Normal serum was obtained from untreated rabbits.

For the immunofluorescent labeling of RuBP carboxylase in the transections of leaves, the indirect labeling approach described by Hattersley *et al.* (8) was used. The same method was altered slightly for the epidermal strips.

Epidermal strips were obtained from young and fully expanded leaves (in some CAM plants, the modified stems) of plants listed in Table I and were carefully screened for freedom from mesophyll contamination. In some cases, the lower and upper epidermal strips were obtained. The adhering mesophyll cells on the epidermal strips from some of the CAM plants were totally removed by rubbing the strips on their mesophyll side with a dissecting needle in distilled H_2O.

Epidermal strips were then immersed for 1 h in 70% ethanol. This alcohol treatment seems essential for the penetration of chloroplasts by antibodies (8). Strips were then rinsed in buffered saline (0.1 M K-phosphate in 0.2 M NaCl, pH 7.5) for about 30 s and transferred to a small watchglass containing 10 μl of RuBP

[1] Supported in part by grants from the Associated Students of Brigham Young University and from the Brigham Young University Research Division.

[2] Abbreviations: RuBP, ribulose biphosphate; PEP, phosphoenolpyruvate.

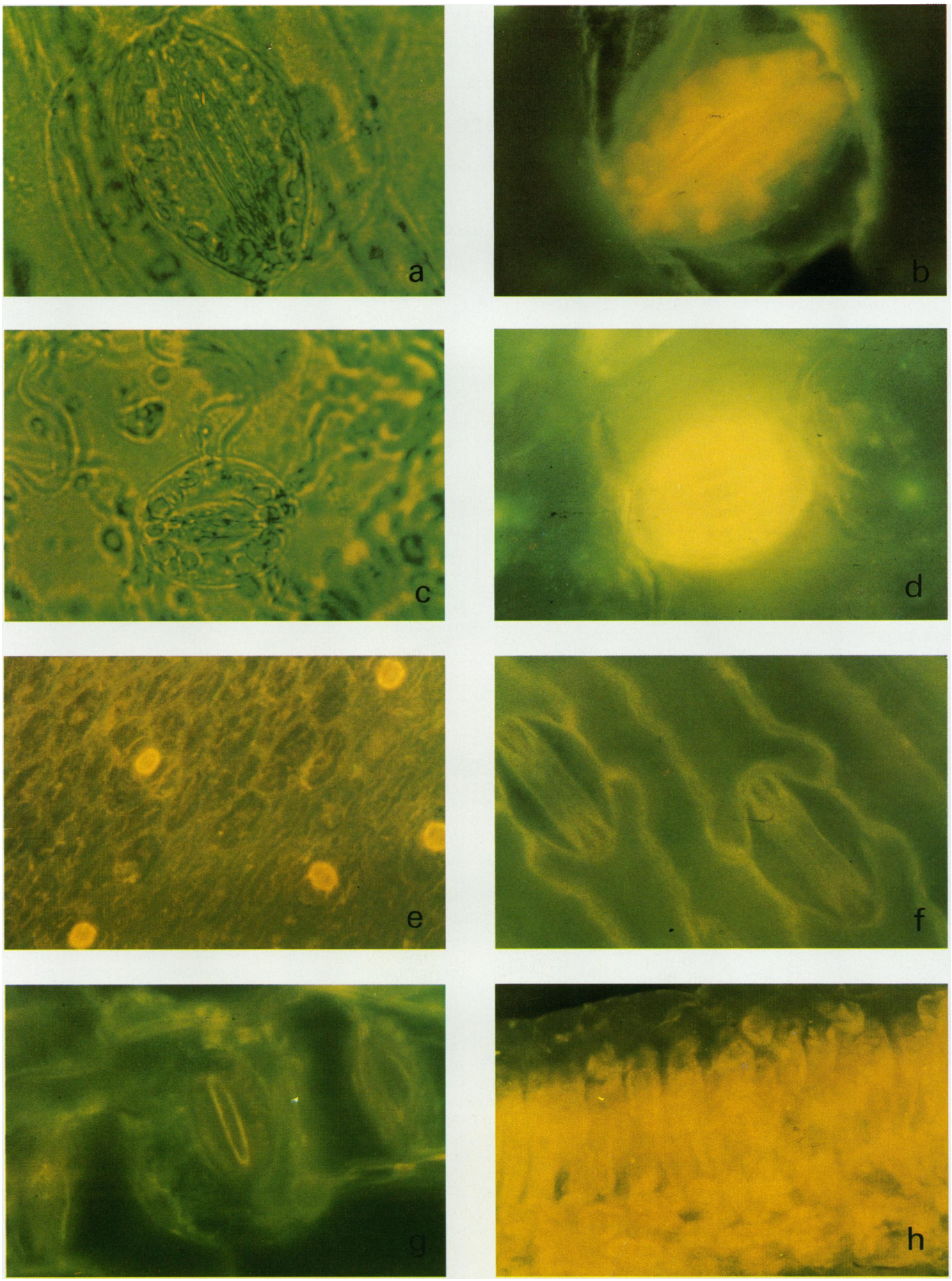
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Table I. *Species Examined for Labeling of RuBP Carboxylase in Their Guard Cells Using an in Situ Immunofluorescent Technique*

Species	Photosynthetic Pathway Type	Degree of Fluorescence[a] Observed
Aizoaceae		
Fenestraria aurantiaca N. E. Brown	CAM[b]	+
Gibbaeum velutinum Haw.	CAM[b]	–
Mesembryanthemum crystallinum L.	CAM[c]	+
Rhombophyllum nelii Schwantes.	CAM[b]	+
Araceae		
Syngonium podophyllum Schott.	C_3	–
Asclepiadaceae		
Edithcolia rubra N. E. Br.	CAM[b]	+
Stapelia variegata L.	CAM[c]	++
Asteraceae		
Chrysanthemum anethifolium Brouss.	C_3	–
Senecio herreianus DTR.	CAM[c]	++++
Cactaceae		
Opuntia basilaris Engelm and Bigel.	CAM[c]	–
Zygocactus truncatus Haworth	CAM[c]	++
Commelinaceae		
Hadrodemas warszewiczianum (Kunth & Bouche) H. E. Moore	CAM[b]	+++
Setcreasea purpurea Schum and Sydow	C_3	–
Tradescantia bracteata Small.	C_3	–
Zebrina pendula Schnizl.	C_3	–
Crassulaceae		
Cotyledon peacockii Baker.	CAM[c]	+
Crassula arborescens (Mill) Willd.	CAM[c]	++
Echeveria gibbiflora D. C.	CAM[c]	+
Kalanchoe daigremontiana HAM. and PERR.	CAM[c]	+
Sedum divergens S. Wats.	CAM[d]	+++
S. pachyphyllum Rose	CAM[c]	++
Cucurbitaceae		
Cucurbita foetidissima HBK	C_3	+
Cycadaceae		
Zamia tenuis Willd.	C_3	–
Euphorbiaceae		
Euphorbia flanaganii N. E. Br.	CAM[b]	+
E. tirucalli L.	CAM[c]	+
Fabaceae		
Phaseolus vulgaris L.	C_3	+
Geraniaceae		
Geranium pratense L.	CAM[c]	+
Gramineae		
Bambusa fastuosa Mak.	C_3	–
Saccharum officinarum L.	C_4	–
Sorghum bicolor (L.) Moench.	C_4	–
Zea mays L.	C_4	–
Labiatae		
Plectranthus australis L. Her	C_3	+
Liliaceae		
Haworthia cuspidata Haw.	CAM[c]	+
Malvaceae		
Hibiscus rose—sinensis L.	C_3	+
Oleandraceae		
Nephrolepis exaltata (L.) Schott.	C_3	–
Onagraceae		
Fuchsia hybrida Hort.	C_3	–
Orchidaceae		
Epidendrum alatum Bateman	CAM[c]	–
Solanaceae		
Capsicum annuum L.	C_3	+
Datura stramonium L.	C_3	+
Lycopersicon esculentum Mill.	C_3	+

[a] The degree of fluorescence observed has been scored in a scale of 1 to 4: +, minimum fluorescence; ++++, maximum fluorescence.
[b] S. Madhavan and B. Smith, unpublished.
[c] See Ref. 19.
[d] See Ref. 17.

carboxylase antiserum (neat). After 2 h of incubation, the epidermal peels were rinsed for about 30 min in three changes of buffered saline (10 ml per wash) with thorough but gentle agitation. The strips were then transferred to another watchglass containing fluorescent isothiocyanate, labeled swine anti-rabbit immunoglogulin (Bio Rad Laboratories, Richmond, CA; 4.63 mg antibody protein/ml, with a fluorescence/phosphorescence ratio of 2.76).

After 90 min of incubation in the dark, the strips were rinsed for another 30 min in buffered saline (three changes with 10 ml per wash). The epidermal peels were then mounted in 50% glycerol (aqueous) containing 1% (w/v) thymol. Slides were kept in the

FIG. 1. Immunofluorescent labeling of ribulose-1,5-bisphosphate carboxylase in guard cells (lower epidermis) and in a handsectioned leaf blade. a) *Hadrodemas warszewiczianum* (CAM: Commelinaceae), lower epidermis; normal serum control (× 1,600). b) *H. warszewiczianum* (CAM: Commelinaceae), lower epidermis; anti-tobacco RuBP carboxylase serum test showing specific fluorescence (× 1,600). c) *Senecio herreianus* (CAM: Asteraceae), lower epidermis; normal serum control (× 1,600). d) *S. herreianus* (CAM: Asteraceae), lower epidermis; anti-tobacco RuBP carboxylase serum test showing specific fluorescence (× 1,600). e) *S. herreianus* (CAM: Asteraceae), lower epidermis; anti-tobacco RuBP carboxylase serum test showing specific fluorescence in all the guard cells throughout the epidermis (× 250). f) *Saccharum officinarum* (C_4: Gramineae), lower epidermis; anti-tobacco RuBP carboxylase serum test showing no specific fluorescence (× 1,600). g) *Phaseolus vulgaris* (C_3: Fabaceae), lower epidermis; anti-tobacco RuBP carboxylase serum test showing very little specific fluorescence (× 1,600). h) *P. vulgaris* (C_3: Fabaceae), handsection of the leaf blade; anti-tobacco RuBP carboxylase serum test showing specific fluorescence associated with chloroplasts of all chlorenchymatous cells (× 640).

dark until observation. An autofluorescence control and a normal serum control test for the antiserum-labeling test were also run, following the method of Hattersley *et al.* (8). All procedures were carried out at room temperature. Fine sable-hair brushes were used for the transfer of strips between watch glasses, with one brush per serum treatment. A Carl Zeiss Photomicroscope II was used to observe the mounted sections and peels.

Preparations were photographed within 12 h of preparation. As the unsuitability of black and white film for recording results had been recognized in earlier studies (it often fails to distinguish clearly between specific fluorescence and autofluorescence of chloroplasts), Kodak high-speed Ektachrome reversal film (EHB 135; tungsten light; ASA 160; stored in the refrigerator until 1 day before use) was used. This is, perhaps, the best way to satisfactorily keep the visual records as color transparencies. The automatic exposure facilities of the Zeiss Photomicroscope camera (using integrated measurement) were used for photographing all frames.

RESULTS AND DISCUSSION

Of the 41 species examined for the immunofluorescent localization of RuBP carboxylase, many of the CAM species, alone, showed a positive response in their guard cells (Table I; Fig. 1, B, D, and E). Species following the C_3 pathway exhibited fluorescence, though at a very low degree (Fig. 1G). Plants following the C_4 pathway did not exhibit fluorescence at all (Fig. 1F). Some of the CAM species also did not clearly exhibit fluorescence, and the degree of fluorescence observed in these species is also of a varied nature. This may perhaps be due to an inefficient penetration of the antiserum of RuBP carboxylase in the epidermal cells, as these species often possess a very thick cuticle. In order to enhance the effective penetration of the antiserum into the epidermal cells, the epidermal peels of all plants examined were rubbed on their mesophyll side with a dissecting needle. This procedure did not improve the results obtained from unrubbed epidermal peels. To serve as a reference to this study, leaves and/or modified stems of all plants examined for guard cell immunofluorescence were also tested for their *in situ* immunofluorescent labeling of RuBP carboxylase in their mesophyll tissues. The observations on these leaves are consistent with the observations of Hattersley *et al.* (8) (Fig. 1H).

The assumption that the RuBP carboxylase antiserum, which was raised against tobacco RuBP carboxylase, may not be specific to the RuBP carboxylase found in the guard cells of C_3, C_4 and CAM plants cannot be held valid for the simple reason that the mesophyll of these tissues exhibited a very high degree of fluorescence with the same antiserum. It is also highly unlikely that the RuBP carboxylase of the mesophyll tissue would be different from that of the guard cells in the same plant.

There was very little autofluorescence observed in the guard cell chloroplasts, and, hence, whatever fluorescence was observed during the experiment must have been specific fluorescence. It has been demonstrated (8, 9) that detection of specific fluorescence was not hindered by chloroplast autofluorescence, inasmuch as this is rarely at or near the wavelengths of fluorescent isothiocyanate fluorescence. There was also no fluorescence observed in the normal serum control test (Fig. 1, A and C), and, hence, the fluorescence observed in the anti-RuBP carboxylase serum test can be regarded as specific fluorescence.

There has been increasing doubt concerning the degree of C_3 activity in the epidermal cells of plants examined so far. The presence of RuBP carboxylase has been detected but with a low activity (29). The activity of RuBP carboxylase, on both a Chl basis and a protein basis, has been found to be higher in leaf tissue minus epidermal tissue in the species so far investigated (7, 29). The ratio of RuBP carboxylase to PEP carboxylase was also found to be considerably lower in the epidermal extract of *C. communis* (7).

In the absence of the occurrence of the C_3 cycle in the epidermal tissue (13, 16), it is currently believed that carbon must flow from the mesophyll to the epidermal tissue and, specifically, to the guard cells to maintain a carbon balance (27). In their study on the carbon isotope discrimination of epidermal tissue and mesophyll tissues from the leaves of various plants to resolve the question of whether PEP carboxylase makes a significant contribution to net carbon gain in the epidermal tissue (and guard cells) independent of the underlying mesophyll, Willmer and Firth (28) observed that the values for the epidermal tissues were marginally, but consistently, less negative than were values for the mesophyll tissues. All values, however, were typical of C_3 plants. This has been suggested to be indicative of a metabolite transport from mesophyll to epidermis, and the PEP carboxylase does not make a large contribution to net carbon gain of epidermal tissue (31).

The detection of RuBP carboxylase in amounts necessary to display a relatively good fluorescence in at least some of the CAM species is intriguing. Experiments have not yet been carried out to determine the degree of activity of this enzyme relative to PEP carboxylase in these tissues. The role of PEP carboxylase has already been speculated, however, in the few species hitherto investigated. Localization of PEP carboxylase by the same immunoflourescent technique is also in progress in this laboratory.

Stomatal opening at night and closure during the day are essential characteristics of CAM. In general, stomates close in response to high, and open in response to low, CO_2 concentration. It has also been reported that there may be a possibility for causal relationship between internal CO_2 concentrations and stomatal resistance in CAM plants (4). The internal CO_2 concentration in CAM tissues has been found to be greater than that of ambient air in the light (4). Although it is premature to attach any regulatory role for the RuBP carboxylase in the guard cells of CAM plants in the absence of suitable enzyme studies, it may be possible to assume some role for this enzyme in the CO_2 fixation process.

Acknowledgments—Grateful thanks are due to Dr. Ferron L. Andersen for permitting us to use his Carl Zeiss Photomicroscope and to Dr. William R. Andersen for providing us with the antiserum of RuBP carboxylase.

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