Leaf inner structure and immunogold localization of some key enzymes involved in carbon metabolism in CAM plants

Ayumu Kondo1,2, Akihiro Nose1 and Osamu Ueno2,3

1 Faculty of Agriculture, Saga University, Honjo, Saga 840–8502, Japan
2 Department of Plant Physiology, National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305–8602, Japan

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Abstract

There are relatively few reports on the leaf structure and in situ immunolocalization of carbon metabolism enzymes in crassulacean acid metabolism (CAM) plants, compared with reports on C4 plants. The leaf inner structure and the subcellular location of some key CAM enzymes for a phosphoenolpyruvate carboxykinase (PCK) CAM species, Ananas comosus, and three malic enzyme (ME) CAM species, Mesembryanthemum crystallinum, Kalanchoë daigremontiana, and K. pinnata, was investigated by immunogold labelling and electron microscopy in this study. The leaves of these species had few intercellular air spaces in the mesophyll. A large vacuole occupied the mesophyll cells, and many vesicles of various sizes occurred in the cytosol. Immunocytochemical study revealed that labelling was present for phosphoenolpyruvate carboxylase in the cytosol and for ribulose-1,5-bisphosphate carboxylase/oxygenase in the chloroplasts of the mesophyll cells in all species. No specific labelling for pyruvate orthophosphate dikinase (PPDK) was observed in the PCK-CAM species. In the ME-CAM species, the patterns of labelling for PPDK differed. In M. crystallinum labelling for PPDK was present only in the chloroplasts, whereas in the two Kalanchoë species it occurred in the cytosol as well as in the chloroplasts. These results suggest that the subcellular localization of PPDK varies with ME-CAM species, in contrast to the conventional belief that it is localized in the chloroplasts.

Key words: Crassulacean acid metabolism, immunolocalization, leaf inner structure, phosphoenolpyruvate carboxylase, pyruvate orthophosphate dikinase.

Introduction

Crassulacean acid metabolism (CAM) represents one of three major photosynthetic modes, together with the C3 and C4 pathways. In CAM plants, malic acid is accumulated in the vacuoles of mesophyll cells at night as a result of fixation of CO2 by phosphoenolpyruvate carboxylase (PEPC). During the day, malic acid is decarboxylated, and the released CO2 is refixed in the C3 cycle (Osmond, 1978; Cushman and Bohnert, 1997). CAM plants can be divided into two groups, phosphoenolpyruvate carboxykinase (PCK) CAM and malic enzyme (ME) CAM plants, based on the decarboxylation process. In ME-CAM plants, malic acid is decarboxylated by NAD(P)-ME and generates pyruvate with CO2. Pyruvate is phosphorylated to PEP by catalysis of pyruvate orthophosphate dikinase (PPDK), and then it is conserved in gluconeogenesis. In PCK-CAM plants, oxaloacetic acid produced from malic acid is decarboxylated by PCK, and generates PEP with CO2 without the catalysis of PPDK (Dittrich et al., 1973; Dittrich, 1976; Osmond, 1978; Holtum and Osmond, 1981; Winter and Smith, 1996). These biochemical processes operate within a single cell, but are separated between day and night (Osmond, 1978). Compared with leaves of C4 plants, leaves of CAM plants have a simple inner structure (Kluge and Ting, 1978). There are relatively few reports on leaf anatomy of CAM plants.
To elucidate a metabolic pathway, it is important to determine the subcellular localization of the enzymes involved. This is fairly clear for CAM enzymes. However, this knowledge is based on the biochemical analysis of cell fractions, and some conflicting results, such as in the subcellular distribution of PEPC, have been reported (Brandon, 1967; Spalding et al., 1979; Schnarrenberger et al., 1980; Perrot-Rechenmann et al., 1982). Recently, immunocytochemical techniques have been used. Immuno-electron microscopy shows the subcellular localization of enzymes in plant cells at high resolution (Herman, 1988). To our knowledge, no report describes the cellular localization of CAM enzymes with immunogold labelling and electron microscopy, although some immunofluorescent studies have been reported (Perrot-Rechenmann et al., 1982; Taborsky et al., 1983).

The intracellular localization of PEPC, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and PPDK in leaves of some CAM species with different decarboxylation types was investigated here by immunogold electron microscopy. The inner structure of the leaves in relation to the mechanism of CAM was also examined.

Materials and methods

Plant materials

Four species of CAM plants were examined; Ananas comosus (L.) Merr. (cv. smooth cayenne N67–10), Mesembryanthemum crystallinum (L.), Kalanchoë daigremontiana Hamet et Perr., and K. pinnata (Lam.) Pers. A. comosus is a PCK-CAM plant, and other species are ME-CAM plants (Dittrich et al., 1973; Dittrich, 1976; Holtum and Osmond, 1981; Winter et al., 1982). A. comosus was planted in 10 dm³ pots filled with sand. The Kalanchoë species were planted in 1 dm³ pots filled with vermiculite. Plants were grown in a naturally illuminated greenhouse (20–30 °C) for 3 months and watered every third day. Compound fertilizer (Otsuka I, Otsuka Chemicals Co., Osaka, Japan) diluted to 0.1% was supplied every 2 weeks. Plants were subsequently transferred to a growth chamber and grown for 2 weeks. The temperature in the growth chamber was maintained at 25 °C during the light period (14 h) and at 20 °C during the dark period (10 h). Photon irradiance was provided by metal halide lamps at about 300 μmol m⁻² s⁻¹. Fully expanded leaves of A. comosus and the fully expanded fifth or sixth leaves (numbered from the apex) of Kalanchoë were used for the experiments.

Seeds of M. crystallinum were germinated in 1 dm³ pots filled with a mixture of soil and vermiculite (1:1, v/v) in the greenhouse. During the 7 weeks after germination, a fifth-strength of Hoagland’s solution that contained 1 mM NaCl was supplied every day. Subsequently, a fifth-strength of Hoagland’s solution that contained 350 mM NaCl was supplied every other day to induce CAM. After 6 weeks, the fully expanded second or third leaves (numbered from the apex) were used for the experiments. These leaves were confirmed to have high activities of PEPC (above 1000 μmol mg⁻¹ chlorophyll h⁻¹), indicating full expression of CAM.

Light and electron microscopy of leaf structure

Leaf samples were collected at 09.00 h. Small segments of leaves were fixed immediately in 3% glutaraldehyde in 50 mM sodium phosphate (pH 6.8) for 1.5 h. They were then washed in phosphate buffer and post-fixed in 2% OsO₄ in buffer, as described by Ueno (1992). They were then dehydrated through an acetone series and embedded in Spurr’s resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Semithin sections were stained with 1% toluidine blue O.

Antisera

Antisera raised against PEPC and PPDK from maize leaves were generously provided by Dr M Matsuoka (Nagoya University, Nagoya, Japan). Antiserum raised against the large subunit of Rubisco from pea leaves was a kind gift of Dr S Muto (Nagoya University).

Analysis by SDS-PAGE and Western blotting

Leaf samples (0.5 g fresh weight) were ground with 0.5 g of sea sand and 25 mg of insoluble polyvinylpyrrolidone in 1 ml of 50 mM HEPES-KOH (pH 8.2) that contained 5 mM dithiothreitol, 0.2 mM disodium EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% (v/v) Triton X-100, and 0.1% (w/v) leupeptin. In the case of A. comosus, 1 mM p-chloromercuric benzoic acid (PCMB) was added to the buffer. Each homogenate was centrifuged at 10000 g for 10 min at 4 °C. Each supernatant was subjected to SDS-PAGE and Western blotting as described by Ueno (1992).

Immunoelectron microscopy

Preparations and immunostaining of samples for immuno-electron microscopy followed the method reported by Ueno (1992). Leaf samples were collected at 09.00 h. Small segments of leaves were fixed immediately in 3% glutaraldehyde in 50 mM sodium phosphate (pH 6.8) for 4 h on ice, and were then washed in phosphate buffer. They were dehydrated through an ethanol series at −20 °C and embedded in Lowicryl K4M resin (Chemische Werke Lowi, Wald Kraiburg, Germany) at −20 °C. Ultrathin sections were collected on 100 mesh nickel grids coated with Formvar. Sections on grids were incubated for 20 min in 0.5% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) that contained 10 mM sodium phosphate (pH 7.2), 150 mM NaCl, and 0.1% (v/v) Tween 20, and then for 3 h in antiserum that had been diluted with 0.5% BSA in PBS. For controls, antiserum was replaced by non-immune serum. The grids were washed four times with PBS and incubated in a 40-fold-diluted suspension of 15 nm protein A-colloidal gold particles (EY Laboratory Inc., San Mateo, California, USA) for 30 min. After several washes with PBS and distilled water, the sections were stained with uranyl acetate and lead citrate.

Quantitative analysis of immunogold particles

The density of PPDK labelling was determined by counting the gold particles on electron micrographs at 13000 × magnification and calculating the number per unit area (μm²). For profiles of chloroplasts, the areas occupied by starch grains were excluded from estimation. Ten to twelve individual cells were examined in several immunolabelled sections from each species.
Results

Structural features of leaves

Leaves of *A. comosus* possessed thick-walled epidermis, and two or three layers of thick-walled hypodermal cells were present just inside both the adaxial and abaxial epidermis (Fig. 1A). Stomata were present only on the abaxial epidermis. Water storage tissue, which is composed of colourless cells, existed inside the adaxial epidermis, and occupied a quarter to a third of the leaf thickness. Spherical, swelling mesophyll cells were densely packed and had few intercellular air spaces. Aerating canals were present. Fibre strands were scattered in the mesophyll (Fig. 1A). In *M. crystallinum*, *K. daigremontiana*, and *K. pinnata*, stomata were present on both adaxial and abaxial epidermis (not shown). The mesophyll exhibited almost no differentiation into palisade or spongy tissue, and large swelling mesophyll cells were densely packed (Fig. 1B, C, D). In both species of *Kalanchoë*, cells that contained dense material were arranged near the abaxial epidermis (Fig. 1C, D). The epidermis of *M. crystallinum* was covered with many transparent bladder cells (not shown).

A large vacuole occupied the mesophyll cells of the four CAM species. The organelles thus appeared pressed against the cell periphery (Fig. 1). Most chloroplasts were distributed at the sides facing the intercellular spaces. The chloroplasts were granal and included starch grains and plastoglobules (Fig. 2). No peripheral reticulum was observed in the chloroplasts. Many vesicles of various sizes occurred in the cytosol of mesophyll cells. The development of vesicles was prominent in *M. crystallinum* and the *Kalanchoë* species, and the vesicles frequently clustered around chloroplasts (Fig. 2B, C, D). In all four species, the bundle sheath cells showed structural features similar to those in adjacent mesophyll cells.

Immunogold localization of photosynthetic enzymes

Western blotting was used to examine the cross-reactivities of proteins extracted with antisera against PEPC, PPDK, and the large subunit of Rubisco (Fig. 3). The antisera against PEPC and the large subunit of Rubisco gave strong single bands on the blots (Fig. 3), which indicates that they were a reliable tool for immunocytochemical localization of these enzymes in sections of leaves. The PCK-CAM species *A. comosus* showed no band for PPDK. The ME-CAM species *M. crystallinum* showed a single band for PPDK and the two *Kalanchoë* species showed strong bands, which consisted of two or more sub-bands (Fig. 3). The largest sub-band suggested a larger molecular size than PEPC.

When leaf sections were incubated in non-immune serum, only non-specific and negligible labelling with colloidal gold was observed (Fig. 4A). Immunogold labelling for the large subunit of Rubisco was present only in the chloroplasts (Fig. 4B, C). Labelling for PEPC was present only in the cytosol, including the fraction that included vesicles (Figs 4D, E, F, 5A). No significant labelling for PEPC was observed in the epidermal cells, the vascular bundles, or the water storage tissues (not shown).

Leaves of *A. comosus* showed no significant labelling for PPDK (Fig. 5B), which was consistent with the result of Western blotting. In *M. crystallinum*, labelling for PPDK was present only in the chloroplasts (Fig. 5C); in *Kalanchoë* it occurred in the cytosol as well (Fig. 5D, E). In the *Kalanchoë* species, labelling for PPDK was also found in the fraction of cytosol that included vesicles, showing similar density of labelling to the other fraction of cytosol (Fig. 5E). In the epidermal cells and vascular bundles, no labelling for PPDK was observed (not shown).

The density of labelling of PPDK was calculated for the mesophyll cells of the three ME-CAM species (Table 1). In *M. crystallinum*, the density of labelling was high in the chloroplasts, but negligible in the cytosol and other organelles. In *Kalanchoë*, the density of labelling in the cytosol was approximately twice that in the chloroplasts, indicating that the amount of accumulation of PPDK differs between two subcellular sites.

Discussion

Reports on the subcellular localization of PEPC in CAM plants conflict. Brandon (1967) found PEPC in the mitochondrial fraction in leaves of *Bryophyllum calycinum* (= *Kalanchoë pinnata*). By contrast, Schnarrenberger et al. (1980) reported that PEPC is present in the chloroplasts in *K. pinnata* and *Crassula lycopodioides*. An immunofluorescent study detected PEPC mainly in the cytosol but also slightly in the chloroplasts in *K. blossfeldiana* (Perrot-Rechenmann et al., 1982). In general, however, PEPC is considered to be present in the cytosol in CAM plants (Spalding et al., 1979; Winter et al., 1982; Taborsky et al., 1983). This study confirms that PEPC is localized unambiguously in the cytosol of the mesophyll cells in all four species examined.

No labelling for PPDK was found in leaves of *A. comosus*, as PCK-CAM plants lack PPDK activities ( Holtum and Osmond, 1981; Black et al., 1996). Previous studies indicated that PPDK is distributed in the chloroplasts in ME-CAM plants (Spalding et al., 1979; Winter et al., 1982). This study confirmed such localization of PPDK in *M. crystallinum*. However, it also revealed that PPDK is found not only in the chloroplasts but also in the cytosol of the *Kalanchoë* species. Compared with studies of C4 plants, there are relatively few reports on PPDK in CAM plants ( Kluge and Osmond, 1971; Sugiyama and Laetsch, 1975). Recently, a chloroplastic
gene for PPDK, which includes a domain representing a transit peptide, has been isolated from *M. crystallinum* (Fisslthaler et al., 1995). In this plant, PPDK is encoded probably by a single gene (Fisslthaler et al., 1995). This agrees with the results of this study. The *Kalanchoë* species accumulated more PPDK in the cytosol than in the chloroplasts. Further research is necessary to determine whether the difference in the blotting band patterns of PPDK in the ME-CAM species reflects a difference in the subcellular patterns of PPDK. In the amphibious
Fig. 2. Ultrastructure of mesophyll cells of CAM species. (A) *A. comosus*. (B) *M. crystallinum*. (C) *K. daigremontiana*. (D) *K. pinnata*. The cytosol contains vesicles of various sizes. Bars = 0.5 µm. C, chloroplast; Cw, cell wall; mt, mitochondrion; P, plastoglobule; S, starch grain; Vc, vesicle.
forms (Agarie et al., 1996) and the cytosolic PPDK have been isolated, and the starch content and granal development (Gibeaut and ME-CAM plants, associated with chloroplasts are often observed in the conditions. Therefore, some environmental factors may distinct cellular di

Peperomia protein is an active form so as to elucidate the metabolic ATPase activity, as do the tonoplasts of the central

involved in the regeneration of PEP and is localized in were also observed in the cytosol of mesophyll cells and way to synthesize sugars and glucan. Instead, PEP for structure may extend the surface area of the tonoplast,

Table 1. Immunogold labelling of PPDK in mesophyll cells of ME-CAM species

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of gold particles (μm^-2)</th>
<th>Cytosol</th>
<th>Chloroplasts</th>
<th>Other organelles</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. crystallinum</td>
<td>1.0 ± 0.1</td>
<td>13.8 ± 1.4</td>
<td>0.3 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>K. daigremontiana</td>
<td>26.6 ± 2.6</td>
<td>13.2 ± 1.2</td>
<td>0.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>K. pinnata</td>
<td>18.3 ± 1.5</td>
<td>8.0 ± 1.3</td>
<td>0.4 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

sedge Eleocharis vivipara, which expresses the C₄-like mode under terrestrial conditions and C₃-like mode under submerged conditions. PPDK is also accumulated in both the chloroplasts and the cytosol of the photosynthetic cells (Ueno, 1996). The genes encoding the chloroplastic and the cytosolic PPDK have been isolated, and the expression patterns of the genes differ between the growth forms (Agarie et al., 1997). In CAM plants also, the degree of CAM expression is controlled by growth con-

differences would be required to understand the taxonomic variation in the subcellular localization of PPDK in ME-CAM plants. CAM plants are divided into four groups based on carbohydrate partitioning strategies (Christopher and Holtum, 1996). According to this classi-fi-

starch formers. Thus no relationship could be found between the localization patterns of PPDK and the types of carbohydrate partitioning strategies.

The CAM species examined had succulent leaves, in which highly vacuolate mesophyll cells were densely packed and had few intercellular air spaces. These structural features have also been observed in leaves of other CAM plants (Kluge and Ting, 1978). Recently, a very low internal conductance to CO₂ diffusion has been reported for leaves of K. daigremontiana (Maxwell et al., 1997). The mesophyll exhibited almost no differentiation into palisade and spongy tissues. In leaves of CAM species in Peperomia, three types of parenchyma tissue differentiate. These tissues differ not only in the levels of starch content and granal development (Gibeaut and Thomson, 1989) but also in the pattern of accumulation of CAM enzymes (Nishio and Ting, 1987; Ting et al., 1994). In leaves of the CAM species examined here, such distinct cellular differentiation was not observed. Balsamo and Uribe (1988a, b) reported that cytosolic vesicles associated with chloroplasts are observed in the mesophyll cells of K. daigremontiana, and they also exhibit ATPase activity, as do the tonoplasts of the central vacuoles. In this study, many vesicles of various sizes were also observed in the cytosol of mesophyll cells and tended to cluster around the chloroplasts. The fraction of cytosol including these vesicles also accumulated PEPC and PPDK, as did the other fraction of the cytosol. The structure may extend the surface area of the tonoplast,
Fig. 4. Immunogold labelling of mesophyll cells of CAM species. (A) Labelling of *K. daigremontiana* with non-immune serum. Only non-specific labelling of gold particles (arrows) is present. (B) and (C) Labelling of *A. comosus* (B) and *K. pinnata* (C) with antiserum to the large subunit of Rubisco. Note dense labelling in the chloroplasts. (D–F) Labelling of *A. comosus* (D), *M. crystallinum* (E), and *K. daigremontiana* (F) with antiserum to PEPC. Note dense labelling in the cytosol. Bars = 0.5 μm. Abbreviations as in Fig. 2.
Fig. 5. Immunogold labelling of mesophyll cells of CAM species. (A) Labelling of \textit{K. pinnata} with antiserum to PEPC. (B–E) Labelling of \textit{A. comosus} (B), \textit{M. crystallinum} (C), \textit{K. daigremontiana} (D), and \textit{K. pinnata} (E) with antiserum to PPDK. \textit{A. comosus} shows no significant labelling. Note labelling in the chloroplasts in \textit{M. crystallinum} and labelling in both the chloroplasts and cytosol of both \textit{Kalanchoe} species. Bars = 0.5 μm. Abbreviations as in Fig. 2.
which may result in efficient transport of metabolites between the cytosol and the vacuole.

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