

Changes in Activities and Levels of Pyruvate, Orthophosphate Dikinase with Induction of Crassulacean Acid Metabolism in *Mesembryanthemum crystallinum* L.*

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Abstract : In *Mesembryanthemum crystallinum* L. leaves exhibiting C_3 photosynthesis, Crassulacean acid metabolism (CAM), as characterized by CO_2 uptake during the dark period, was induced by exposing the plant roots to high NaCl concentration over a week. The increase in malate content in the leaves at the end of the dark period correlated with the increases in the activities of phosphoenolpyruvate carboxylase (PEPCase), NADP malic enzyme (NADP-ME) and pyruvate, orthophosphate dikinase (PPDK) which exhibited a maximum about a week after NaCl treatment. It was found that PPDK existed in C_3 -*M. crystallinum* and the increases in the activities of PEPCase and PPDK were accompanied by the *de novo* synthesis of these proteins.

Key words : Crassulacean acid metabolism induction, *Mesembryanthemum crystallinum*, Phosphoenolpyruvate carboxylase, Pyruvate, Orthophosphate dikinase.

Mesembryanthemum crystallinum* L. の CAM 型光合成の誘導にともなうピルビン酸正リン酸ジキナーゼの活性及びレベルの変化 :** 斎藤和幸・中村保典**・川満芳信・松岡 信**・鮫島宗明**・縣 和一 (九州大学農学部・**農業生物資源研究所)

要 旨 : C_3 型光合成を行っている *Mesembryanthemum crystallinum* L. の葉身は植物体の根を NaCl 処理することによって処理後 1 週間で暗期に CO_2 を吸収するようになり、光合成型が C_3 型から Crassulacean acid metabolism (CAM) 型へシフトすることが認められた。葉身の暗期の終わりのリンゴ酸含量の増加の推移はホスホエノールピルビン酸カルボキシラーゼ (PEPCase)、NADP-リンゴ酸酵素 (NADP-ME) 及びピルビン酸正リン酸ジキナーゼ (PPDK) 活性の増加の推移とほぼ一致しており、それらは NaCl 処理後約 1 週間で最大に達した。本研究では、 C_3 型 *M. crystallinum* の葉身にも PPDK が存在していることを見出し、CAM 型光合成へのシフトにともなう PEPCase 及び PPDK の活性の増加は、これらの酵素タンパク質の新たな合成によって達成されていることが明らかとなった。

キーワード : ベンケイソウ型酸代謝誘導, ピルビン酸正リン酸ジキナーゼ, ホスホエノールピルビン酸カルボキシラーゼ, *Mesembryanthemum crystallinum*.

The halophilic species *Mesembryanthemum crystallinum* L. shifts the photosynthetic CO_2 fixation from C_3 plant type to CAM plant type, which exhibits substantial CO_2 uptake and malate synthesis in the dark by exposure to high salinity in the nutrient medium^{9,28}. The increases in the activities of phosphoenolpyruvate carboxylase (PEPCase) (EC 4.1.1.31) and other enzymes associated with CAM with high NaCl treatment have been documented^{8,9,12,13,25}. The increase in PEPCase activity is accounted for by an accumulation of the enzyme protein and

mRNA^{8,12,19,22,23}). However, little is known about whether pyruvate, orthophosphate dikinase (PPDK) (EC 2.7.9.1), which is one of the key enzymes of photosynthetic CO_2 fixation in C_4 plants¹⁰ and some CAM plants¹⁴, is newly synthesized during the induction of CAM.

PPDK was thought to be absent from C_3 plants⁷) and was reported to be absent from C_3 -*M. crystallinum*^{13,27}). However, the presence of PPDK in leaves of some C_3 plants^{3,4,11,18}) and mRNA for PPDK in C_3 -*M. crystallinum* has been documented²³).

In this study, changes in activities and levels of PPDK during the induction of CAM in *M. crystallinum* were examined.

Materials and Methods

1. *Plant materials* *Mesembryanthemum crystallinum* L. was grown from seed (collected from

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a natural population at Guerrero Negro in Baja, California) in soil until plants were approximately 5 weeks of age and transferred in culture solution containing 3 mM KNO₃, 2 mM Ca(NO₃)₂, 0.5 mM MgSO₄, 0.5 mM NH₄H₂PO₄, 0.5 mM (NH₄)₂HPO₄, 12.5 μM H₃BO₄, 1 μM MnSO₄, 1 μM ZnSO₄, 0.25 μM CuSO₄, 0.03 μM (NH₄)₆Mo₇O₂₄, 10 μM Fe-EDTA. Five plants were grown in each pot which contained 4L of culture solution. The culture solution was changed every seven days. All plants were maintained in a growth cabinet under a 12 hr light (20°C, 70% relative humidity, 12,000 Lux) and 12 hr dark (15°C, 70% relative humidity) cycle. When the plants became 4.3 leaf age, the NaCl concentration of the nutrient solution was brought to 400 mM NaCl by steps of 100 mM every day.

2. *CO₂ exchange and transpiration measurements* Rates of CO₂ assimilation and water transpiration were simultaneously measured by using a climate-controlled chamber. Details of gas exchange system and methods have been described previously²⁾. Light was provided by metal-halide lamps (Toshiba, Modal D-400) filtered through approximately 15 cm of running water.

3. *Extraction methods* The third foliar leaves which fully expanded prior to increasing the NaCl concentration were used for the preparation of the enzyme extracts. The disks with a total area of 0.8 cm² were excised from the leaves using a cork borer in the second half of the light period and ground with a teflon homogenizer in 650 μL of ice-cold solution containing 150 mM Tricine-NaOH (pH 8.1), 5 mM dithiothreitol (DTT), 10 mM MgCl₂, 1 mM ethylenediaminetetraacetate (Na₂-EDTA) and 0.5% (w/v) polyvinylpyrrolidone (PVP). After centrifugation at 13,000 rpm and 4°C for 1 min, the supernatant was immediately used for enzyme assays.

When NAD malic enzyme (NAD-ME) (EC 1.1.1.38) was measured, non-centrifuged crude extracts were incubated with 0.5% triton X-100 for 5 min at room temperature. After centrifugation at 13,000 rpm for 1 min, the extracts were maintained at room temperature for 30 min prior to assay.

When PPDK was measured, the disks with a total leaf area of 0.8 cm² were ground, after illumination, in 650 μL of ice-cold solution

containing 150 mM Tricine-NaOH (pH 8.1), 2.5 mM pyruvate, 5 mM DTT, 1 mM Na₂-EDTA, 10 mM MgCl₂ and 0.5% (w/v) PVP. After centrifugation at 13,000 rpm for 1 min at room temperature, the supernatant was immediately used for enzyme assays and SDS gel electrophoresis.

4. *Enzyme assays* All enzymes were measured at 25°C by following the change in absorbance of 340 nm in a 1 mL reaction mixture as described below. The assay conditions for all enzymes tested were modified from the referred sources to give optimum activities in the extracts from *M. crystallinum*.

The assay mixture of ribulose-1,5-bisphosphate carboxylase (RuBPCase) (EC 4.1.1.39) contained 100 mM Hepes-NaOH (pH 8.0), 10 mM KCl, 1 mM Na₂-EDTA, 30 mM MgCl₂, 5 mM ATP, 20 mM NaHCO₃, 5 mM phosphocreatine, 13.5 IU phosphoglycerate kinase, 13.5 IU NAD glyceraldehyde-3-phosphate dehydrogenase, 10 IU creatine phosphokinase, 0.2 mM NADH, 1 mM DTT and 20 μL enzyme extract. The reaction was initiated by adding RuBP at a final concentration of 0.5 mM²⁴⁾.

The assay mixture of PEPCase contained 25 mM Bicine-NaOH (pH 8.0), 100 mM Hepes-NaOH (pH 8.0), 5 mM MgCl₂, 10 mM NaHCO₃, 5 mM DTT, 0.12 mM NADH, 6 IU NAD malate dehydrogenase and 20 μL enzyme extract. The reaction was initiated by adding PEP at a final concentration of 4 mM¹⁵⁾.

The assay mixture of NADP malic enzyme (NADP-ME) (EC 1.1.1.40) contained 100 mM Hepes-NaOH (pH 8.0), 5 mM Na₂-EDTA, 0.5 mM NADP, 5 mM malate, 5 mM DTT and 25 μL enzyme extract. The reaction was initiated by adding MgCl₂ at a final concentration of 22.5 mM²⁴⁾.

The assay mixture of NAD-ME contained 100 mM Hepes-HaOH (pH 7.4), 0.2 mM Na₂-EDTA, 2 mM NAD, 5 mM malate, 5 mM DTT and 50 μL enzyme extract. The reaction was initiated by adding MnCl₂ at a final concentration of 5 mM and coenzyme A at a final concentration of 75 μM¹³⁾.

The assay mixture of PPDK contained 100 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.1 mM Na₂-EDTA, 1.25 mM pyruvate, 2.5 mM K₂HPO₄, 0.2 mM NADH, 50 mM NaHCO₃, 2 IU PEPCase, 2 IU NAD malate dehy-

drogenase, 5 mM DTT and 100 μ L enzyme extract. The reaction was initiated by adding ATP at a final concentration of 1.25 mM²¹⁾.

5. Soluble protein and chlorophyll content The soluble protein content of the extracts was estimated by the method of Lowry et al.¹⁷⁾ after precipitating protein with 10% (w/v) trichloroacetic acid. Bovine serum albumin was used as the reference protein. Chlorophyll was extracted with 80% acetone, and assayed according to Arnon⁵⁾.

6. Malate content Leaf disks were harvested from third foliar leaves at the end of the dark period. Malate was measured according to Möllering²⁰⁾.

7. SDS gel electrophoresis (SDS-PAGE) and immunoblot analysis The samples were prepared for SDS-PAGE by mixing a three-fold volume of the protein solutions with SDS sample buffer and boiling immediately for 3 min. Proteins were resolved by SDS-PAGE using a discontinuous buffer system¹⁶⁾. Proteins were transferred electrophoretically onto nitrocellulose membranes using a semidry blotter (Saltorius, West Germany). The nitrocellulose membranes were incubated 30 min at room temperature in 20 mM Tris-HCl (pH 7.4), 0.5 mM NaCl (TBS) containing 3% (w/v) gelatin. Antibodies raised against maize leaf PEPCase and PPDK used in this report were the generous gift of Professor T. Sugiyama of Nagoya University. Immunoblots were carried out by incubating the nitrocellulose membranes overnight at room temperature in TBS containing 1% (w/v) gelatin, 0.01% thimerosal and either anti-PEPCase or anti-PPDK antibody. The nitrocellulose membranes were washed in TBS containing 0.05% Tween 20 (3 \times 10 min) and incubated with horse-radish peroxidase-conjugated goat anti-(rabbit) IgG antibodies (Bio-Rad) for 1 hr at room temperature. The nitrocellulose membranes were then washed in TBS containing 0.05% Tween 20 (3 \times 10 min) and rinsed in distilled water. The protein-antibody complexes were visualized using a solution containing 3,3'-diaminobenzidine and cobalt¹⁾.

Results

Diurnal change of the CO₂ exchange rate were measured during the induction of CAM after NaCl treatment (Fig. 1 A). The net CO₂ exchange rate before NaCl treatment was

almost constant in the light and dark periods. On the second day after NaCl treatment, the net CO₂ uptake in the light period, especially around noon, decreased, while the net CO₂ release in the dark period was constant. On the fourth day, the net CO₂ uptake in the light period decreased to half of that before NaCl treatment. It was also noticed that the net CO₂ release in the dark period (at about 21 h) decreased remarkably. After a week, the net CO₂ exchange rate showed a typical pattern of CAM, that is, a drastic depression of net CO₂ uptake in the light and an appearance of that in the dark.

The leaf conductance in the light period decreased rapidly after NaCl treatment (Fig. 1 B). Changes in leaf conductance in the light period were similar to those in the net CO₂ exchange rate during the induction of CAM.

The activities of five major photosynthetic enzymes changed with increasing NaCl concentration in the nutrient solution (Figs. 2 and 3). The activities of RuBPCase, PEPCase, NADP-ME, NAD-ME and PPDK, which were extracted from the leaves of the non-treated plant on the fifteenth day, did not show any change from those before NaCl treatment. The RuBPCase activity before NaCl treatment was eight-fold that of the PEPCase activity. When the NaCl concentration increased to 400 mM, the activity of RuBPCase per unit of leaf area decreased gradually to half of the original level on the sixteenth day. On the other hand, the activity of PEPCase continued to increase remarkably until the sixth day and it remained constant thereafter. As a consequence, the PEPCase activity in the treated plant was thirteen-fold that in the non-treated plant on the sixteenth day. Hence, it was higher than RuBPCase activity. The NADP-ME activity was similarly enhanced by the NaCl treatment to approximately three-fold that of the non-treated plant. By contrast, the NAD-ME activity of the treated plant was slightly higher than that of the non-treated plant on the sixteenth day.

The PPDK activity in *C₃-M. crystallinum* was very low, but it was enhanced by the NaCl treatment on the fifteenth day to approximately five-fold that of the non-treated plant (Fig. 3).

Accumulation of malate in the leaves increased at the end of the dark period by the

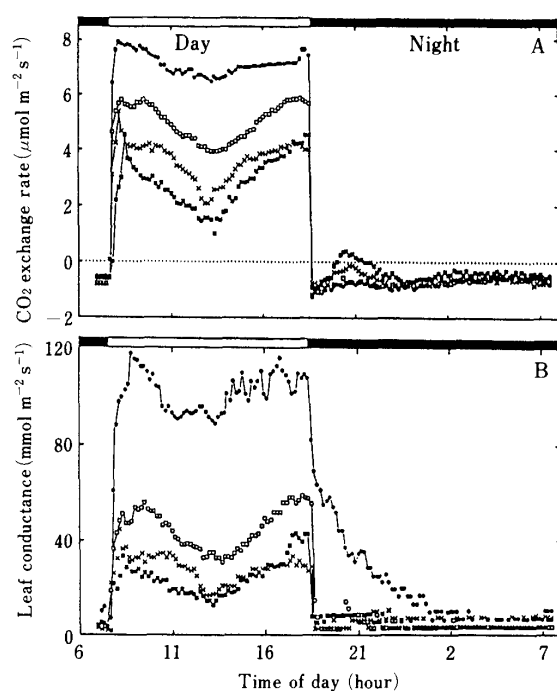


Fig. 1. Diurnal patterns of the CO_2 exchange rate (A) and the leaf conductance (B) in *M. crystallinum* during the induction of CAM with adding NaCl in the culture solution. ●, Before NaCl treatment; ○, on the second day after NaCl treatment; ×, on the fourth day; ■, on the seventh day.

high NaCl treatment, and it became fifty-three fold as compared with that before the NaCl treatment on the ninth day (Fig. 3). There was a close relationship between accumulation of malate at the end of the dark period and the activity of PEPCase. This result was similar to that observed in the earlier experiments^{9,13,25}.

The activities of PEPCase and PPDK in the extracts from the leaves of the non-treated plant (C_3 - *M. crystallinum*) and the treated plant on the ninth day (CAM- *M. crystallinum*) were expressed per unit of leaf area, on a chlorophyll basis and on a protein basis in Table 1. The increase in the enzyme activities with the NaCl treatment was independent of the basis.

The extracts from the leaves of the non-treated plant (C_3 - *M. crystallinum*) and the treated plant on the ninth day (CAM- *M. crystallinum*), having the same leaf area, were subjected to SDS-PAGE and analyzed by the anti-PEPCase antibody and the anti-PPDK antibody (Fig. 4). The intensity of staining increased in several regions of the gel. When

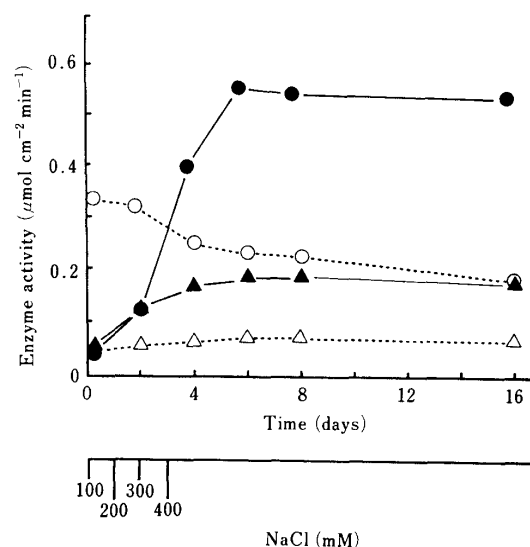


Fig. 2. Changes in the activity of PEPCase, RuBPCase, NADP-ME and NAD-ME with adding NaCl in the culture solution. ●, PEPCase; ○, RuBPCase; ▲, NADP-ME; △, NAD-ME.

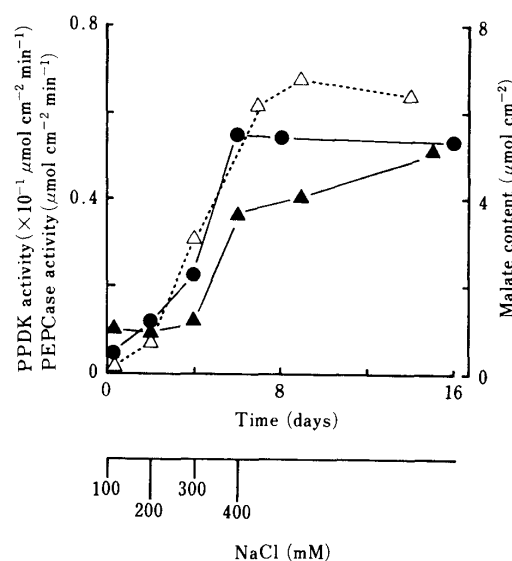


Fig. 3. Changes in the activity of PEPCase and PPDK and the content of malate at the end of the dark period with adding NaCl in the culture solution. ●, PEPCase; ▲, PPDK; △, malate.

the bands obtained from CAM- *M. crystallinum* were compared with corresponding bands from C_3 - *M. crystallinum*, remarkable increases in the quantity of the polypeptides denoted by the band numbers 1 to 4 appeared on the Coomassie-stained gel.

In the extracts from the leaves of both C_3 - and CAM- *M. crystallinum*, the bands denoted by the numbers 1 (ca. 114 kD) and 2 (ca. 106

kD) reacted with the anti-PEPCase antibody (Fig. 4 B). The intensity of the bands increased with the NaCl treatment. The increase in PEPCase activity was accompanied by the increase in PEPCase protein.

In *C₃- M. crystallinum*, an immunoreactive protein with the anti-PPDK antibody was detected as a faint band and had a molecular weight of about 98 kD (Fig. 4 C). The intensity of the PPDK band increased with the NaCl treatment. The increase in PPDK activity was also accompanied by the increase in PPDK protein.

The amount of soluble protein per unit of leaf area increased until the second day after NaCl treatment and it remained constant thereafter (Fig. 5). Fluctuation of the chlorophyll content with the NaCl treatment was small.

Discussion

The pattern of net CO₂ exchange rate indicated a typical feature of CAM with a remarkable depression of net CO₂ uptake during the light period and an appearance of that in the dark period at a week after NaCl treatment (Fig. 1). Accumulation of malate at the end of the dark period reached a maximum about a week after NaCl treatment (Fig. 3). The activities of PEPCase, NADP-ME and PPDK, which were active in CAM photosynthesis, also reached a maximum about a week after NaCl treatment (Figs. 2 and 3). These results indicated that the induction of CAM in *M. crystallinum* was caused by the increase in the activity of CAM photosynthesis.

Whether the increase in the activities of CAM photosynthetic enzymes are caused by activation of existing enzyme proteins or by synthesis of new enzyme proteins is important in relation to cellular control of the enzymes and the development of CAM.

As the activities of PEPCase and PPDK which were key enzymes in CAM photosynthesis increased remarkably with the NaCl treatment, the levels of these enzyme proteins were studied by immunological analysis of proteins from crude extracts (Fig. 4). The two bands denoted by the numbers 1 and 2 reacted with the anti-PEPCase antibody in *C₃-* or *CAM- M. crystallinum*. In leaves of *CAM- M. crystallinum*, the band denoted by the number 2 was stained more intensely than that denot-

Table 1. PEPCase and PPDK activities in the leaves of *C₃-* and *CAM- M. crystallinum*.

Enzyme	<i>C₃</i>	<i>CAM</i> *
PEP carboxylase		
($\mu\text{mol min}^{-1}\text{cm}^{-1}$)	0.05	0.61
($\mu\text{mol min}^{-1}\text{mg}^{-1}\text{chlorophyll}$)	0.69	8.03
($\mu\text{mol min}^{-1}\text{mg}^{-1}\text{protein}$)	0.06	0.50
PPDK		
($\mu\text{mol min}^{-1}\text{cm}^{-1}$)	0.01	0.04
($\mu\text{mol min}^{-1}\text{mg}^{-1}\text{chlorophyll}$)	0.17	0.50
($\mu\text{mol min}^{-1}\text{mg}^{-1}\text{protein}$)	0.01	0.03

* : The treated plant on the ninth day after NaCl treatment.

ed by the number 1. The PEPCase bands was stained more intensely in the extract from *CAM- M. crystallinum* than that from *C₃- M. crystallinum*. This result indicated that the increase in the activity of PEPCase in *M. crystallinum* was accompanied by the increase in PEPCase protein.

The band denoted by the number 3 in *C₃-* or *CAM- M. crystallinum* reacted with the anti-PPDK antibody (Fig. 4). The PPDK band was stained more intensely in the extract from *CAM- M. crystallinum* than that from *C₃- M. crystallinum*. This result indicated that the increase in the activity of PPDK in *M. crystallinum* was accompanied by the increase in PPDK protein.

PPDK was reported to be absent from *C₃- M. crystallinum*^{13,27)}. However, the presence of mRNA of PPDK in *C₃- M. crystallinum* was reported²³⁾. We were able to measure low PPDK activity in the extracts from *C₃- M. crystallinum* (Table 1) and to detect a faint PPDK protein by immunoblot analysis (Fig. 4 C). Therefore, we could show the enzymes required for C₄ acid metabolism were present in *C₃- M. crystallinum*, although the amount was much less.

Aoyagi and Bassham⁴⁾ proposed that C₄ intracellular carbon transport was concerned with photosynthesis. If the C₄ intracellular carbon transport is present, PEPCase must be active during the light period in *C₃- M. crystallinum*. In CAM plants, PEPCase is inactive during the day time, but active at night²⁶⁾. Cushman et al.⁶⁾ reported that two distinct

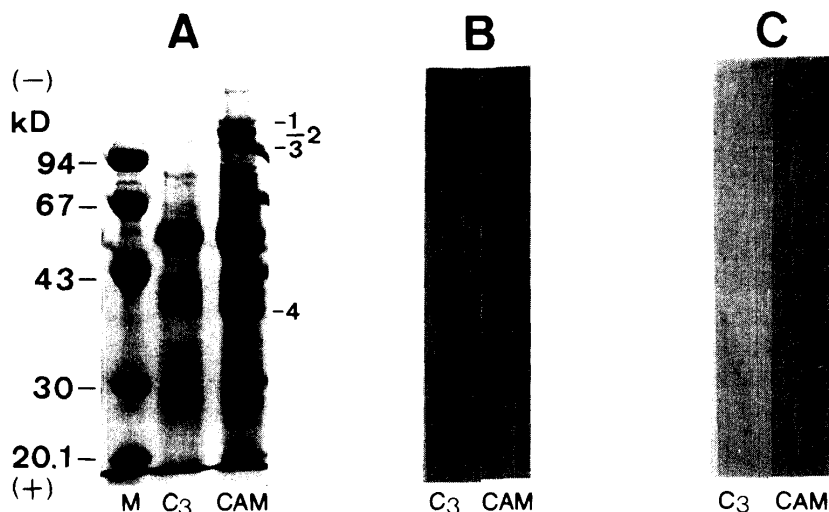


Fig. 4. Coomassie-stained SDS polyacrylamide slab gel (A), immunoblot analysis with anti-PEPCase antibody (B) and with anti-PPDK antibody (C) in the extracts from the leaves of the non-treated plant (C_3) and the treated plant with 400 mM NaCl on the ninth day (CAM). The extracts had the same leaf area. Protein contents were 10 μ g for the non-treated plant and 14 μ g for the treated plant. M, mass markers.

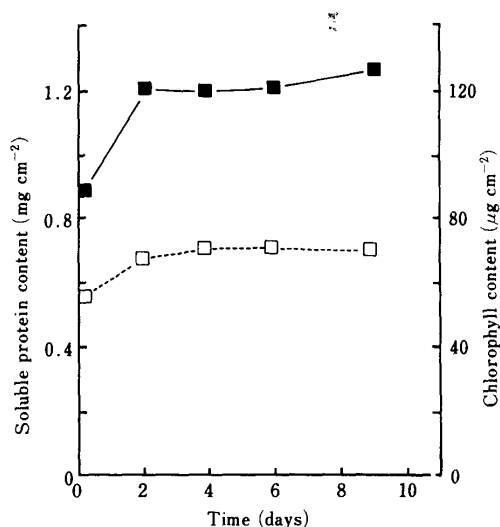


Fig. 5. Changes in soluble protein and chlorophyll content in the leaves of *M. crystallinum*. ■, Soluble protein; □, chlorophyll.

genomic PEPCase genes that differ markedly in their expression during CAM induction were present in *M. crystallinum*. Therefore, it will be interesting to see whether the different regulation of PEPCase is present between in the different PEPCase isoforms or not. Knowledge of the properties of PEPCase isoforms in *M. crystallinum* could help in planning future plant breeding or even molecular genetic engineering designed to impart CAM characteristics to C_3 plants.

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