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 C₃ photosynthesis, Crassulacean acid metabolism (CAM), as characterized by CO₂ 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 amount about a week after NaCl treatment. It was found that PPDK existed in C₃-*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ビルピン酸正リン酸ジキナーゼの活性及びレベルの変化: 野村和幸・中村保典**・川添芳信***・松倉信**・鯨島宗明**・細和一（九州大学農学部・**農業生物資源研究所）

要 旨 C₃ 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 CO₂ を吸収するようになり、光合成型が C₃ 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酸酸含量の増加の推移はホスホエノールビルピン酸カルボキシラーゼ（PEPCase）、NADP-リン酸酵素（NADP-ME）及びビルピ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、C₃ 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、ビルピン酸正リン酸ジキナーゼ、ホスホエノールビルピン酸カルボキシラーゼ、*Mesembryanthemum crystallinum*.

The halophilic species *Mesembryanthemum crystallinum* L. shifts the photosynthetic CO₂ fixation from C₃ plant type to CAM plant type, which exhibits substantial CO₂ uptake and malate synthesis in the dark by exposure to high salinity in the nutrient medium. 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. The increase in PEPCase activity is accounted for by an accumulation of the enzyme protein and mRNA. However, little is known about whether pyruvate, orthophosphate dikinase (PPDK) (EC 2.7.9.1), which is one of the key enzymes of photosynthetic CO₂ fixation in C₄ plants and some CAM plants, is newly synthesized during the induction of CAM.

PPDK was thought to be absent from C₃ plants and was reported to be absent from C₃-*M. crystallinum*. However, the presence of PPDK in leaves of some C₃ plants and mRNA for PPDK in C₃-*M. crystallinum* has been documented.

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...
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 H₂BO₄, 1 μM MnSO₄, 1 μM ZnSO₄, 0.25 μM CuSO₄, 0.03 μM (NH₄)₂MoO₄, 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, Musical 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), 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.

When NADP malic enzyme (NADP-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 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 PEPC at a final concentration of 4 mM²⁴.

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-HCl (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 μM²⁴.

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-
diergenase, 5 mM DTT and 100 μL enzyme extract. The reaction was initiated by adding ATP at a final concentration of 1.25 mM\(^{21}\).

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 Arnon\(^5\).

6. Malate content Leaf disks were harvested from third foliar leaves at the end of the dark period. Malate was measured according to Möllering\(^{20}\).

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 system\(^{18}\). Proteins were transferred electrophoretically onto nitrocellulose membranes using a semidy 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% thimearosal and either anti-PEPCase or anti-PPDK antibody. The nitrocellulose membranes were washed in TBS containing 0.05% Tween 20 (3 × 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 × 10 min) and rinsed in distilled water. The protein-antibody complexes were visualized using a solution containing 3,3'-diaminobenzidine and cobalt\(^{19}\).

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 C₄- 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
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\(^8,13,25\).

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 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 denoted 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. However, the presence of mRNA of PPDK in C₃- M. crystallinum was reported. 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 Bassham 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 night. Cushman et al. reported that two distinct

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<th>Enzyme</th>
<th>C₃</th>
<th>CAM*</th>
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<tr>
<td>PEP carboxylase</td>
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<tr>
<td>(µmol min⁻¹ cm⁻¹)</td>
<td>0.05</td>
<td>0.61</td>
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<tr>
<td>(µmol min⁻¹ mg⁻¹ chlorophyll)</td>
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<td>8.03</td>
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<td>(µmol min⁻¹ mg⁻¹ protein)</td>
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<td>0.50</td>
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<td>PPDK</td>
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<tr>
<td>(µmol min⁻¹ cm⁻¹)</td>
<td>0.01</td>
<td>0.04</td>
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<tr>
<td>(µmol min⁻¹ mg⁻¹ chlorophyll)</td>
<td>0.17</td>
<td>0.50</td>
</tr>
<tr>
<td>(µmol min⁻¹ mg⁻¹ protein)</td>
<td>0.01</td>
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*: The treated plant on the ninth day after NaCl treatment.
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 (C3) and the treated plant with 400 mM NaCl on the ninth day (CAM). The extracts had the same leaf area. Protein contents were 10 µg for the non-treated plant and 14 µg 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 C3 plants.

Acknowledgment

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References

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