Stimulation of CAM Photosynthesis in Kalanchoë blossfeldiana by Transferring to Nitrogen-Deficient Conditions

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ABSTRACT
Kalanchoë blossfeldiana Poelln. cv Hikan plants were grown hydroponically with nutrient solution containing 5 millimolar NO₃⁻ (or NH₄⁺) for 1 to 2 months and then transferred to nutrient solution containing no nitrogen. CO₂ uptake at night, nocturnal increase in titratable acidity, and activity of phosphoenolpyruvate carboxylase increased after the transfer. Thus, transfer to nitrogen-deficient conditions stimulates Crassulacean acid metabolism (CAM photosynthesis) in K. blossfeldiana. The importance of the plant nitrogen status (nitrogen-withdrawal status) for induction and stimulation of CAM photosynthesis is discussed.

CAM photosynthesis is one of the three major pathways of photosynthetic CO₂ assimilation in green plants. The activity of CAM photosynthesis responds to external and internal factors such as plant water relations, photoperiod, temperature, light intensity, leaf age, genotype, and so forth (7, 12). Induction or stimulation of CAM photosynthesis by water stress, photoperiod, and leaf age has been extensively studied in several succulent plants (7, 12, 14, 19, 23). There are some reports of effects of nutrients on CAM photosynthesis (9–11, 21), and the plants grown with lower N were observed to have low activity of CAM photosynthesis (10, 22). But the effect of N on CAM photosynthesis is insufficiently investigated. So in the present study, I investigate the effects of transfer to N-deficient conditions on CAM photosynthesis in Kalanchoë blossfeldiana grown on N-enriched conditions and report the importance of N status for activity of CAM photosynthesis.

MATERIALS AND METHODS

Plant Culture. Cuttings of Kalanchoë blossfeldiana Poelln. cv Hikan having 2 or 3 pairs of leaves were rooted in sand without nutrients and then transferred to solution culture. Three of the plants were held by vermiculite in a plastic basket, and the basket was placed on a 1/5000 arc (3.8 L) Wagner pot which contained 3 L of nutrient solution. Constituents of the nutrient solution were 1 mM KH₂PO₄, 2 mM K₂SO₄, 2 mM MgSO₄·7 H₂O, and 4 mM CaCl₂·2 H₂O for macromolecules, and 10 µM MnSO₄·4 H₂O, 1 µM ZnSO₄·7 H₂O, 1 µM CuCl₂·5 H₂O, 50 µM H₃BO₃, 0.5 µM Na₂MoO₄·2 H₂O, 0.1 mM NaCl, 0.2 µM SO₄·7 H₂O, and 50 µM Fe·Na·EDTA·2 H₂O for microelements. When N was supplied, 5 mM NaNO₃ or 2.5 mM (NH₄)₂SO₄ was added to the solution. The pH of the solution was daily adjusted to 6.8 with 1 N HCl or NaOH, and the solution was renewed once a week. Plants were grown under long day photoperiod (16 h light [6:00–22:00], 25°C; 8 h dark, 20°C; light intensity: 100 W/m²) in an environmental control cabinet.

CO₂ Uptake. CO₂ uptake was measured with instruments in the System for Biological Fixation of Photoenergy in Nagoya University. The upper portion of an attached shoot (5 or 6 leaf pairs from the top) was enclosed in a chamber, and CO₂ uptake was monitored by an infrared gas analyzer every 5 min for 24 h. In measurements of CO₂ uptake (Fig. 1), although the plants grew in the experimental period and new leaves were formed, the enclosed leaf number was maintained constant as much as possible (5 or 6 leaf pairs from the top) in order to avoid the effects of leaf age. The environmental conditions during the measurement were similar to the growth conditions. The curves drawn in the figures were the typical data in repeated experiments. Similar results were obtained in the repeated experiments.

Titratable Acidity. Leaf discs were excised from the 4th and 5th pairs of leaves from the top at 0600 (morning) or 2200 (evening). They were sliced and boiled in distilled water for 10 min and then homogenized and centrifuged at 2000g for 10 min. The supernatant was decanted, and the residue was again similarly extracted with distilled water. The extracts were combined and titrated with 0.01 N NaOH using phenolphthalein to estimate the end point (pH 8.0).

Enzyme, Protein, and Chlorophyll Assays. Enzyme extraction and assay were performed by the method of Holtum and Winter (5). Leaf discs were excised at 0 time from the same leaves as for the determination of titratable acidity. The discs were ground in a chilled mortar and pestle in cold buffer (0–4°C) containing 0.1 mM Hepes-NaOH (pH 8.0), 5 mM DTT, and 0.5% PVP-40. The homogenate was filtered through two layers of Miracloth. Chi was assayed in an aliquot of the filtrate according to Arnon (1). After centrifugation of the filtrate at 10,000g for 5 min, the supernatant was passed through a column of Sephadex G-25 (coarse) equilibrated with buffer containing 25 mM Hepes-NaOH (pH 8.0) and 5 mM DTT and the desalted eluate was used for the assay. Pep carboxylase activity was measured at 25°C by following the change in absorbance at 340 nm in a 3 ml reaction mixture containing 25 mM Bicine, 25 mM Hepes-NaOH (pH 8.0), 2 mM KHCO₃, 5 mM MgCl₂, 0.1 mM NADH, 6 units malate dehydrogenase, 100 µl extract, and 2 mM PEP. Soluble protein content in the extracts was determined by a modified Lowry procedure (2), using BSA as a standard.

RESULTS

Kalanchoë blossfeldiana plants grown on the constant-N solution culture (control plants) had a CO₂ exchange pattern of the weak-CAM type (8), whereas the plants transferred to N-deficient conditions (N-deficient plants) for 4 or 10 weeks had larger net CO₂ uptake during the night and less during the day and had a stronger CAM type pattern (Fig. 1. A–D). Difference of nocturnal CO₂ uptake between N-deficient plants and control

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Abbreviations: PEP, phosphoenolpyruvate; RuBP, ribulose-1,5-bisphosphate.
plants in the 10-week culture was larger than that in the 4-week culture although nocturnal CO₂ uptake rate of control plants grew larger as plants aged (Fig. 1, A–D). Leaves of the plants began to yellow at 4 weeks or more after the transfer, and retardation of growth appeared at 8 weeks or more after the transfer.

When the N supply was resumed at 8 weeks after the transfer to N-deficient conditions, the pattern of diurnal CO₂ uptake exchange recovered to the control level within a week (Fig. 1, E and F). Similar results of the N-deficient culture were observed in the plants supplied with NO₃⁻ as an N source and in the plants with NH₄⁺, although the NH₄⁺-grown plants tended to absorb less CO₂ during the night than the NO₃⁻-grown plants (Fig. 1).

In the experiments depicted in Figure 1, effect of the N-deficient culture appeared slowly (1 month or more after the transfer). So the control plants also had more CAM activity with increasing plant age (Fig. 1, A–D). In this experiment, when the plants were transferred to N-deficient solution from N-enriched solution, the vermiculite which was used for supporting plants was not washed. Thus, remaining N in the vermiculite had to be a reason for the slow appearance of N-deficient effects. Then in the following experiments, the vermiculite was thoroughly washed with water before transfer to N-deficient solution. This procedure brought the similar result of N-deficient culture to the experiment depicted in Figure 1 only in a few weeks of culture. So the plant age effect can be almost excluded. Ten-d culture on N-deficient conditions increased nocturnal CO₂ uptake and decreased daytime CO₂ uptake similarly to the 10-week culture in Figure 1 (Fig. 2).

N-deficient plants showed larger nocturnal increases in titratable acidity and higher activity of PEP carboxylase than control plants (Tables I and II). The ratio of the nocturnal increase in titratable acidity in the N-deficient plants to that in the control plants corresponded with that of the activity of PEP carboxylase in each experiment.

**DISCUSSION**

These results show that the criteria of CAM photosynthesis correspondingly increased in *Kalanchoë blossfeldiana* when the plants were transferred to N-deficient conditions. This indicates that CAM photosynthesis is stimulated by transfer to N-deficient conditions.

CAM photosynthesis is induced or stimulated under drought conditions (7, 12, 19, 23). Although nutrient and water absorption are independent processes in the root, they are closely related because nutrient transport requires available water in both the plant and soil (20). Under drought conditions, nutrient transport in a plant is decreased by low soil-ion availability and the poor ability of roots to absorb and transport nutrients to the shoot (4, 15). Nitrogen assimilation is also decreased by inhibition of nitrate reductase activity at a low water potential (6, 15, 16). Therefore, plants should lack N under drought conditions. Nevertheless, in past studies, the induction or stimulation of CAM photosynthesis under drought conditions has only been investigated and discussed in relation to low water potentials, and the influence of the N status has not yet been considered. On the basis of the results in the present study and these findings, it is suggested that the lack of N is an important factor for stimulation of CAM photosynthesis under drought conditions.

CAM photosynthesis and flowering are controlled by photoperiodism in a similar manner for almost all plants in which the photoperiodic induction of CAM photosynthesis is reported (14). When flowering is induced, N (a mobile element) and other mineral elements redistribute from the leaves to the inflorescences (21). So the same physiological shift must occur when CAM photosynthesis is photoperiodically induced. On the other hand, it is also known that CAM photosynthesis increases with leaf age. The status of the leaves shifts from that of a source of N during leaf development (18, 21). Chang et al. (3) observed that CAM activity increases while NO₃⁻ re-
Fig. 2. Diurnal changes in net CO₂ uptake for a shoot of K. blossfeldiana before and after transfer to N-deficient conditions. Upper, 5 mM NaNO₃ as an N source; lower, 2.5 mM (NH₄)₂SO₄ as an N source. Solid line, 4 d before the transfer; dotted line, 10 d after the transfer. The transfer was carried out after 6 weeks of the culture with the N.

Table I. Effect of Transfer to N-Deficient Conditions on Nocturnal Increase in Titratable Acidity in Leaves of K. blossfeldiana

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Titratable Acidity (μg/g fresh wt)</th>
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<tbody>
<tr>
<td></td>
<td>Morning</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>N-deficient plants</td>
<td>48.7 ± 4.0</td>
</tr>
<tr>
<td>Control plants</td>
<td>23.6 ± 2.6</td>
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<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>N-deficient plants</td>
<td>55.8 ± 11.8</td>
</tr>
<tr>
<td>Control plants</td>
<td>14.5 ± 0.3</td>
</tr>
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that stimulate CAM photosynthesis are accompanied by a status of lowering N level in plant or leaf. Therefore, considering the results in the present study additionally, N-withdrawal status should be an important factor in the stimulation of CAM photosynthesis. In the present study, however, plant age also seemed to have an additive effect on the stimulation of CAM photosynthesis to N-deficient effect (Fig. 1). More research is required to clear up the effect of plant age and the correlation among these factors.

Winter et al. (22) reported that the plants grown on lower NO₃⁻ have lower activity of PEP carboxylase in K. pinnata, and there is no definite change in the ratio of RuBP carboxylase to PEP carboxylase activity with a varying supply of NO₃⁻ during growth. Nobel (10) also reported that lower N levels in the hydroponic solutions led to less amounts of nocturnal acid accumulation in some species of cacti. Furthermore, Sugiyama et al. (17) reported that the activity and protein content of PEP carboxylase decreases as the N level is lowered in the C₄ plant maize. In contrast to these reports, the criteria of CAM photosynthesis including the activity of PEP carboxylase increased under N-deficient conditions in K. blossfeldiana. This discrepancy should be caused by the difference in the experimental conditions to make N lacking. In their experiments, the plants were supplied with different levels of N throughout their growth period. On the contrary, in the present study, K. blossfeldiana plants were grown with rather high N concentrations (5 mM NO₃⁻ or NH₄⁺) for 1 month or more, and the plants grew fairly large and were then transferred to the solution containing no N. So N deficiency in the plants was probably not yet severe for the experimental period, although the leaves slightly yellowed. This view is supported by the fact that the soluble protein content was slightly higher in the leaves of the N-deficient plants than the control plants although the Chl content was lower (Table II). This interpretation of the discrepancy confirms the suggestion that N-withdrawal status is an important factor for the stimulation of CAM photosynthesis, although more detailed experiments are required to certify this interpretation.

Additionally, it was also observed that the plants cultured with NO₃⁻ as a N source performed more CAM photosynthesis than the plants cultured with NH₄⁺ in K. blossfeldiana (Figs. 1 and 2) (13). Thus, N nutrient condition including both quality and quantity of N should be added to the external factors that affect CAM photosynthesis.
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