Nocturnal Accumulation of Malic Acid Occurs in Mesophyll Tissue without Proton Transport to Epidermal Tissue in the Inducible Crassulacean Acid Metabolism Plant Mesembryanthemum crystallinum

EVIDENCE AGAINST A PREVIOUS HYPOTHESIS

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ABSTRACT

The inducible Crassulacean acid metabolism plant, Mesembryanthemum crystallinum, accumulates malic acid, i.e., equivalent amounts of malate anions and protons in the mesophyll cells at night. Levels of malate and titratable acidity are low in the epidermal tissue and do not change significantly during the day/night cycle. This result is in contrast to a recent report (Bloom 1979 Plant Physiol 64: 919-923) that the synthesis of malic acid during dark CO2 fixation is associated with an equivalent exchange of inorganic cations from epidermal tissue with protons in the mesophyll cells.

It has recently been proposed by Bloom (1) that the development of CAM in the halophilic, inducible CAM plant Mesembryanthemum crystallinum (19) requires exposure of plants to high salinity levels and a substantial accumulation of inorganic cations by the shoots. It has also been proposed by the same author (2) that inorganic cations such as Na+ and K+ cycle between mesophyll and epidermal tissue in this species to balance nocturnal malate accumulation and to produce stomatal opening in the dark. Protons, derived from malic acid synthesis at night, could exchange with inorganic cations in the epidermis. If this hypothesis were correct, day/night fluctuations of the malate anion should be restricted to the mesophyll, whereas, fluctuations in acidity should be restricted to the epidermis and should not occur in the mesophyll. Paradoxically, Bloom’s hypothesis is based exclusively on the observation of a nocturnal increase of positively charged ions in the mesophyll, i.e. H+ (titratable acidity), Na+, and K+. No measurements were made on the isolated epidermis. To clarify this issue, day/night changes in malate anion and titratable acidity were determined in mesophyll and epidermis of leaves of M. crystallinum exhibiting CAM in response to high NaCl salinity.

MATERIALS AND METHODS

Eight- to 10-week-old plants of M. crystallinum L. were used in the experiments. Plants were cultivated in either soil or half-strength modified Johnson nutrient solution (12). Four treatments consisted of different combinations of NaCl in the root medium and of different degrees of water stress, all of which led to the development of CAM in the experimental plants. Treatments are specified in the “Results and Discussion” section (legend to Fig. 1). Plants were kept in a growth cabinet under a 12-h light (25 C)/12-h dark (15 C) cycle. Irradiance was 600 µE m-2 s-1 in the experiment of Figure 1D. In three other experiments (Figs. 1A-C), irradiance was 200 µE m-2 s-1 for the first 3 h of the light period, 430 µE m-2 s-1 for the next 6 h, and 200 µE m-2 s-1 for the last 3 h of the light period. Discs were punched from fully expanded leaves with a corkborer at the end of the light and the dark periods. The upper and lower epidermis (including the epidermal bladder cells) were stripped off and pooled. The mesophyll was completely freed from epidermal tissue, whereas, minor contamination of the epidermis, particularly the lower one, by mesophyll sap was unavoidable. Epidermis and mesophyll were extracted for 15 min with 20% boiling ethanol. Aliquots of the extracts were used for determination of L-(−)-malate after Hohorst (6). Titratable acidity was measured by titration of extracts with 2 or 5 N NaOH to a pH of 6.5.

For determination of organic acids by HPLC, discs were extracted in 80% (v/v) boiling methanol for 30 min. After centrifugation at 20,000g, the supernatant (5 ml) was lyophilized and resuspended in 1 ml 10% (v/v) isopropanol. Aliquots were loaded onto a Bio-Rad (Bio-Rad Laboratories, Richmond, CA) Aminex HPX-87 column (300 mm x 7.8 mm), and organic acids were separated using 0.013 N H2SO4 as a solvent at 900 p.s.i.

RESULTS AND DISCUSSION

Plants from all treatments showed CAM as judged from the increase in malate content and titratable acidity in the dark (Fig. 1). In all experiments, the mesophyll of M. crystallinum accumulated equivalent amounts of malate anions and protons, i.e, malic acid at night (Fig. 1), a result which is similar to whole-leaf extracts of this and other CAM species (8-10). Titratable acidity in the epidermis was low throughout the day/night cycle. The slight increase in malate and titratable acidity in the epidermis at the end of the dark period is likely due to contamination from the mesophyll. These results demonstrate that nocturnal increase in
Table I. Organic Anion Content in Mesophyll and Epidermis (Upper Plus Lower)

<table>
<thead>
<tr>
<th>Anion</th>
<th>Oxalate</th>
<th>Citrate Plus Isocitrate</th>
<th>cis-Aconitate</th>
<th>Malate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesophyll, L*</td>
<td>19.7</td>
<td>20.4</td>
<td>0.7</td>
<td>ND</td>
</tr>
<tr>
<td>Mesophyll, D</td>
<td>18.6</td>
<td>21.2</td>
<td>Trace</td>
<td>16.5</td>
</tr>
<tr>
<td>Epidermis, L</td>
<td>2.1</td>
<td>0.6</td>
<td>0.3</td>
<td>ND</td>
</tr>
<tr>
<td>Epidermis, D</td>
<td>2.3</td>
<td>0.8</td>
<td>0.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

* L, 12-h Light period; D, 12-h dark period.
ND, Not detected.

the concentration of inorganic cations in the mesophyll, especially to balance malate\(^{2-}\), as has been suggested by Bloom (2), does not occur. In fact, Bloom's finding of a nocturnal increase in titratable acidity in the mesophyll of _M. crystallinum_ is in agreement with this view and contradicts his own hypothesis (2).

In the experiment of Figure 1B (growth conditions similar to those in Bloom's study [2]), possible fluctuations of organic acids other than malate in mesophyll and epidermis which could require charge balance by inorganic cations were examined. HPLC revealed substantial amounts of oxalate and citrate plus isocitrate and small amounts of aconitate in the leaf mesophyll, levels of which did not fluctuate between end of the light and end of the dark periods (Table I). The levels of these organic acids were low in the epidermal tissue (Table I). Since titratable acidity in leaves of _M. crystallinum_ is only sufficient to balance malate\(^{2-}\) levels, the negative charges of oxalate, citrate plus isocitrate, and aconitate are probably balanced by inorganic cations.

The possibility of transport of certain cations (e.g. Na\(^+\) and K\(^+\)) between epidermis and mesophyll tissue during CAM has not been excluded in the present study. However, the above data indicate that an exchange of protons and inorganic cations between tissues would be insignificant and secondary relative to the total level of acidification.

A recent study on the CAM plant _Kalanchoë daigremontiana_ revealed a close relationship between organic anions and inorganic cation levels in deacidified leaves at the end of the light period (11). The results obtained under these conditions, however, must be clearly separated from the day/night fluctuations in organic

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FIG. 1. Malate (Ma) and titratable acidity (Ac) per cm\(^2\) of leaf in mesophyll and epidermis (upper plus lower) from _M. crystallinum_ performing CAM at the end of the 12-h light (L) and the 12-h dark (D) periods. SD is indicated by bars and is based on 3 to 4 samples. In columns without bars, SD was ±0.1 µeq cm\(^{-2}\) or less. A, Plants were 9 weeks old, grown in soil, and watered twice prior to onset of the experiment with nutrient solution containing 100 mM NaCl. Each salt treatment was followed by a 1-week drying cycle. Discs were taken from the third foliar leaf pair (expanded) at the end of the second drying cycle. B, Plants were 10 weeks old, grown in soil, and watered three times prior to onset of the experiment with nutrient solution containing 100 mM NaCl. Each treatment was followed by a 1-week drying cycle. Discs were taken from the fourth foliar leaf pair (expanded) at the end of the third drying cycle. C, Plants were 8 weeks old and grown in culture solution containing 400 mM NaCl 2 weeks prior to onset of the experiment. Discs were taken from the fourth foliar leaf pair (expanded). D, Plants were 8 weeks old, grown in soil, and watered with nutrient solution containing 400 mM NaCl at 3-day intervals for 3 weeks prior to onset of the experiment. Discs were taken from the third foliar leaf pair (expanded).
acids associated with CAM.

Not only the data of Figure 1, but also anatomical considerations, fail to support Bloom’s hypothesis (2). Examination of freshly prepared leaf cross-sections showed that the ratio volume mesophyll to volume epidermis (excluding the bladder cells which do not participate in CAM) is at least 10:1. Nocturnal malic acid synthesis on a total leaf water basis may be 100 μeq or more. If the protons from malic acid concentrate in the epidermis and are balanced by Cl\textsuperscript{-}, as proposed by Bloom (2), a highly improbable epidermal concentration of at least 1 N HCl would result. In contrast to Bloom’s anatomical description of M. crystallinum (2), the leaf epidermis of this species consists not of a double, but of a single, cell layer (5, 7). Furthermore, the epidermal bladder cells are not positioned on top of the epidermis, as indicated by Bloom, but represent enlarged epidermal cells (5, 7).

Statements that M. crystallinum exhibits CAM only in response to high salinity (1) ignore earlier investigations (13–15, 17), which show the induction of CAM in plants grown in cooled or nonaerated nutrient solutions without added NaCl. Thus, reduced water availability, not an ion-specific effect, is probably the trigger for the induction of CAM in M. crystallinum. Lower net dark CO\textsubscript{2} fixation rates under stress conditions without special NaCl treatment in comparison to growth at high salinity levels may reflect the halophilic properties of this species. M. crystallinum shows optimum growth only when cultivated in nutrient solutions containing 5 to 100 mM NaCl (15, 17; Winter, unpublished data). Contrary to Bloom’s conclusions (1), plants with CAM generally do not occupy saline habitats or accumulate high levels of inorganic salts typical of halophytes (16, 18). It should also be noted that sodium-deficient, nonhalophilic CAM plants, such as Bryophyllum tubiflorum, may show reduced growth (4) but still exhibit all features of CAM (3). Therefore, implications that Na\textsuperscript{+} is a prerequisite for the onset of CAM (1) are misleading.

LITERATURE CITED