Induction of a Crassulacean Acid-like Metabolism in the C₄ Succulent Plant, *Portulaca oleracea* L.: Study of Enzymes Involved in Carbon Fixation and Carbohydrate Metabolism

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The *C₄* succulent plant *Portulaca oleracea* shifts its photosynthetic metabolism to crassulacean acid metabolism (CAM) after 23 d of withholding water. This is accounted by diurnal acid fluctuation, net nocturnal but not day CO₂ uptake and drastic changes in phosphoenolpyruvate carboxylase (PEPC) kinetic and regulatory properties [Lara et al. (2003) *Photosynth. Res.* 77: 241]. The goal of the present work was to characterize the CAM activity in leaves of *P. oleracea* during water stress through the study of enzymes involved in carbon fixation and carbohydrate metabolism. After drought stress, a general decrease in the net CO₂ fixation and in the activity of enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase, PEPC, pyruvate orthophosphate dikinase, phosphoenolpyruvate carboxykinase and NAD-malic enzyme was observed. We also found changes in the day/night activities and level of immunoreactive protein of some of these enzymes which were correlated to night CO₂ fixation, as occurs under CAM metabolism. Based on the results obtained, including those from in situ immunolocalization studies, we propose a scheme for the possible CO₂ fixation pathways used by *P. oleracea* under conditions of sufficient and limiting water supply.

Keywords: C₄ metabolism — Crassulacean acid metabolism — Drought stress — NAD-malic enzyme — Phosphoenolpyruvate carboxylase — *Portulaca oleracea*.

Abbreviations: BSC, bundle sheath cell; CAM, crassulacean acid metabolism; G3PDH, glyceraldehyde-3-Phosphate dehydrogenase; GAPDH, nonphosphorylating glyceraldehyde-3-Ph dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; MC, mesophyll cell; NAD-MDH, NAD-malate dehydrogenase; NADP-MDH, NADP-malate dehydrogenase; NAD-ME, NAD-malic enzyme; NADP-ME, NADP-malic enzyme; PEPC, phosphoenolpyruvate carboxylase; PEPC, phosphoenolpyruvate carboxykinase; PFK, ATP-dependent phosphofructokinase; PPDK, pyruvate orthophosphate dikinase; RuBisCO, ribulose biphosphate carboxylase-oxygenase; TPI, triosephosphateisomerase; WSC, water storage cell.

Introduction

Crassulacean acid metabolism (CAM) is one of the three major variations in the over-all photosynthetic CO₂ assimilation pathways along with the so-called C₃ and C₄ pathways, and probable the least well understood of the three. Stomata are closed during much of the day and open at night. Malic acid is accumulated in the vacuoles of mesophyll cells (MC) at night as the result of CO₂ fixation by phosphoenolpyruvate carboxylase (PEPC). During the day, malic acid is decarboxylated and the released CO₂ is refixed in the C₃ cycle (Holtum and Winter 1982). In *C₄* plants, atmospheric CO₂ is first incorporated into C-4 acids in the MC by PEPC and then transported to bundle sheath cells (BSC) where they are decarboxylated and the released CO₂ is incorporated into the C₃ cycle (Edwards and Walker 1983).

Switches between C₃ and CAM photosynthesis are well known in some species, *Mesembryanthemum crystallinum* being a model for such transitions. Many criteria are used to confirm the set up of CAM, such as nocturnal CO₂ uptake, diurnal acid fluctuation, changes in malate content and increases in PEPC activity (Cushman and Bohnert 1997). In contrast, *Portulaca* is the only genus known to have C₄ species, which can exhibit some kind of CAM metabolism (Koch and Kennedy 1980, Ku et al. 1981, Kraybill and Martin 1996, Guralnick et al. 2002, Lara et al. 2003). Specifically, we recently studied the induction of a CAM-like metabolism in the *C₄* succulent plant *Portulaca oleracea* L. after 23 d of drought stress (Lara et al. 2003). The transition was characterized by changes in the CO₂ exchange pattern, malic acid content and titratable acidity during the day–night cycle, in addition to changes in PEPC: while in watered plants it had the characteristics of a C₄-type enzyme, this carboxylase resembled those of CAM plants under water stress (Lara et al. 2003). On the other hand, Guralnick et al. (2002) working with a closely related species (*Portulaca grandiflora*) found the up-regulation of the CAM pathway after water stress. In this case, they observed that after 8 d of water stress the leaves presented CAM-cycling activity and suggested that this feature may occur completely in water storage tissue. Nevertheless, what is certain is that it is impossible for C₄ and CAM photosynthesis to occur at the same time in the same tissue (Sage 2002). In this way, it seems that different metabolic modifications can be induced in *Portulaca* due to water stress: transition to a CAM-like metabolism (as in *P. oleracea*) or induction of a CAM-cycling metabolism compartmentalized in a different cell type while the C₄ pathway is also operating (*P. grandiflora*).
Characterization of CAM metabolism in *P. oleracea*

Carbohydrate turnover is a central component in determining the magnitude of CAM expression from C₃ species due to diurnal change in the flux of carbon from synthesis of storage carbohydrate via gluconeogenic pathway in the light to formation of malic acid via glycolytic pathway in the dark (Holtum and Winter 1982). As the induction of a CAM metabolism in *P. oleracea* occurs from a different enzymatic background (the one present in C₄ species) it is of great interest the study of enzymes related to the carbohydrate metabolism in this species. On the other hand, as the function of a CAM metabolism in a C₄ species implies a different spatial and temporal participation of the enzymes involved in the CO₂ assimilation and fixation, we also investigated if the drought stress affects the expression of the decarboxylating enzymes NAD-malic enzyme (NAD-ME), NADP-malic enzyme (NADP-ME) and PEP carboxykinase (PEPCK), along with the enzyme pyruvate orthophosphate dikinase (PPDK). Moreover, in order to establish the cellular and subcellular localization of the principal photosynthetic enzymes, we used immunogold labelling in samples performing either C₃ or CAM metabolism. These studies lead us to suggest a possible scheme for the CAM-like metabolism functioning in a C₄ species like *P. oleracea*.

### Results

*P. oleracea* leaves from well-watered plants were named as controls. Leaves of plants from which water was withheld for 21–23 d and which exhibited no CO₂ assimilation in the light and CO₂ up-take in the night (such as group 2 and 3 in Lara et al. 2003) were selected and named as stressed samples. These samples were used for further analysis when the set up of a CAM-like metabolism in the plants was confirmed by titratable acidity diurnal fluctuation, accounted by malic acid, as previously reported (Lara et al. 2003).

#### Activity measurement of the decarboxylating systems

*P. oleracea* is classified as a NAD-ME subtype C₄ plant. This is in agreement with the specific activity measured in day-control plants, which was higher than the maximum activities of NADP-ME and PECK. NAD-ME specific activity was almost three times higher for control samples taken in the day than taken in the night (Table 1). Surprisingly, when water-stressed samples were assayed for NAD-ME, 16% of the specific activity measured in the control day plants was found. This value was practically invariant during the day and night in water-stressed plants (Table 1).

Apart from a slight decrease of the NADP-ME activity in control day samples, the specific activity of this enzyme remained practically unchanged in samples taken in the light or in the dark in both control and stressed plants (Table 1). In addition, the specific activity of NADP-ME found in *P. oleracea* leaves resembles the value found in C₃ plants.

The activity of the third decarboxylating enzyme, PECK, changed reciprocally in stressed plants with respect to control plants. In control night samples, the activity of this enzyme decreased 36% under the levels measured in the day (Table 1). Nevertheless, after 23 d of water stress treatment, PECK spe-

### Table 1  Decarboxylating systems operating in *P. oleracea* control and water stressed plants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th><em>P. oleracea</em> control</th>
<th><em>P. oleracea</em> stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Night time</td>
<td>Day time</td>
</tr>
<tr>
<td>NAD-ME</td>
<td>0.036±0.002</td>
<td>0.107±0.009</td>
</tr>
<tr>
<td>NADP-ME</td>
<td>0.042±0.011</td>
<td>0.035±0.002</td>
</tr>
<tr>
<td>PEPCK</td>
<td>0.060±0.009</td>
<td>0.081±0.024</td>
</tr>
</tbody>
</table>

Samples were taken after 5 h into the light and dark periods in both groups of plants. Values represent the mean of at least six independent determinations in different control and stressed groups of plants ± SD.
pecific activity, besides a small decrease relative to values found in well-watered plants, presented an opposite activity between day and night samples with a small increase of activity during the night (Table 1). That is, even though control plants showed the highest values during the day, stressed samples showed the highest values during the night.

Western blot analysis of the decarboxylating systems

Fig. 1A shows a typical result obtained when antibodies against NAD-ME α subunit were used. In this case, a decrease in the intensity of the immunoreactive band is observed after drought stress. This is in agreement with the decrease in the enzyme activity measured in stressed samples (Table 1). In addition, in control samples, a more intense reaction was observed in leaves collected during the day, indicating a correlation between enzyme activity and immunoreactive protein levels (Table 1).

When Western blot analysis was conducted using antibodies against maize NADP-ME (Fig. 1B), only one immunoreactive band with a molecular mass of 72 kDa was observed in all cases. An isoform of NADP-ME of the same molecular mass is the only form described in leaves of C₄ terrestrial species like wheat, C₃ Flaveria species and Chenopodium album (Drinco-vich et al. 2001). No changes in the level of this enzyme were detected when the same amount of total protein was loaded in each lane (Fig. 1B). The low specific activity found in crude extracts together with the molecular mass of this enzyme suggest that NADP-ME could possibly have an anaplerotic function rather than a photosynthetic role in P. oleracea control and stressed plants.

Finally, when antibodies against PEPCK were used, an immunoreactive band with the same molecular mass as the enzyme from maize was observed in all the samples analyzed. The results obtained (Fig. 1C) indicate that changes in the specific activity of PEPCK in control samples (Table 1) are correlated with changes in the level of the immunoreactive band of 74 kDa. In contrast, in stressed plants the levels of immunoreactive protein remained almost constant during the day and night, and similar to those observed in control samples taken during the night (Fig. 1C).

Western blot analysis of RuBisCO and PPDK

In contrast to the PPDK found in maize leaves (Fig. 1D, lane 1), two immunoreactive bands were detected in P. oler-aeeea leaves (Fig. 1D). In control plants, a higher level of immunoreactive protein was observed with respect to stressed plants, with the lower molecular mass isoform being the more abundant (Fig. 1D, lanes 2 and 3). This band clearly diminishes when plants are withheld water, being more intense the higher molecular mass immunoreactive band in this condition in relation to control plants. (In other words, the relation between higher and lower molecular mass immunoreactive bands is higher in stressed samples.) Moreover, a higher reaction is observed in the lower molecular mass enzyme when stressed samples are collected in the light (Fig. 1D).

Western blot analysis using antibodies against RuBisCO large subunit shows an immunoreactive band of 55 kDa in all samples whose level decreases after the drought stress (Fig. 1E). No changes are observed between samples collected at night or day.

In situ immunolocalization of PEPC, RuBisCO and NAD-ME

RuBisCO is exclusively localized in chloroplasts of BSC in leaves of well-watered and drought-stressed plants (Fig. 2A, B). However, a higher density of immunogold particles was detected in control samples (Fig. 2A), in agreement with Western blot studies revealed with the same antibodies (Fig. 1E). No labeling was observed in WSC.

When antibodies raised against NAD-ME α subunit were used, immunogold particles were detected only in BSC in samples of control as well as stressed plants (Fig. 2C, D). As electronic microscopy studies in this species indicate the presence of chloroplasts associated with prominent mitochondria (Edwards and Walker 1983), the occurrence of immunogold particles in the boundaries of the chloroplasts suggests a mitochondrial localization of this enzyme (Fig. 2C, D). As in Western blot analysis (Fig. 1A), a higher density of the signal was found in samples from control plants (Fig. 2C).

In control as well as in stressed plants, immunolocalization studies using anti-PEPC shows a strong labeling in the cytosol of MC and lower labeling in the cytosol of WSC (Fig. 2E, 3F). No labeling was observed in the nucleus or the chloroplasts of these cells. BSC showed no labeling either in control or in water-stressed samples. A greater ratio of labeling WSC/MC was observed in stressed samples with respect to control plants.

Background labeling with pre-immune serum was very low in all cases (data not shown). Immunolocalization studies were also performed using anti-maize NADP-ME (not shown). In this case, as the protein content level was very low, the density of immunogold particles was not enough so as to properly establish the localization of this enzyme within the tissue.

Activity of carbohydrate metabolism-related enzymes

The activity of two enzymes, PFK and PFP, that catalyze reactions of the first part of the glycolytic pathway were studied. PFP specific activity was similar in control plants collected in the day or night. In stressed plants, the value obtained in the night was similar to that obtained for control plants, while the activity in the day increased 1.8 times (Table 2). PFK specific activity in control plants was 2.6 times higher during the day, in contrast to stressed plants, in which an increase of 1.2 times was found during the night (Table 2).

Aldolase specific activity in control samples taken in the dark was more than twice the value obtained in the light. While the specific activity remained almost constant in stressed samples collected in the light in comparison to the value from control plants, the specific activity measured in the dark decreased 70% (Table 2).
Higher fructose-1,6-bisphosphatase (FBP) activity (two times) was measured in control samples collected in the light with respect to those collected in the dark. No important changes were observed in stressed samples collected in the day with respect to those taken in the dark. These values were smaller than those found in control light samples (Table 2).
Table 2: Carbohydrate metabolism-related enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (U mg⁻¹)</th>
<th>P. oleracea control</th>
<th>P. oleracea stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Night time</td>
<td>Day time</td>
<td>Night time</td>
</tr>
<tr>
<td>PFP</td>
<td>0.017±0.001</td>
<td>0.016±0.002</td>
<td>0.016±0.003</td>
</tr>
<tr>
<td>PFK</td>
<td>0.021±0.002</td>
<td>0.055±0.005</td>
<td>0.037±0.003</td>
</tr>
<tr>
<td>Aldolase</td>
<td>4.68±0.23</td>
<td>2.20±0.35</td>
<td>1.40±0.12</td>
</tr>
<tr>
<td>FBP</td>
<td>0.019±0.002</td>
<td>0.038±0.003</td>
<td>0.018±0.002</td>
</tr>
<tr>
<td>NADP-MDH</td>
<td>0.309±0.024</td>
<td>0.309±0.004</td>
<td>0.440±0.030</td>
</tr>
<tr>
<td>NAD-MDH</td>
<td>84.2±5.5</td>
<td>85.7±8.6</td>
<td>116.7±8.6</td>
</tr>
<tr>
<td>G6PDH</td>
<td>0.370±0.030</td>
<td>0.351±0.050</td>
<td>0.295±0.011</td>
</tr>
<tr>
<td>GAPDHnP</td>
<td>0.034±0.003</td>
<td>0.021±0.011</td>
<td>0.036±0.004</td>
</tr>
</tbody>
</table>

Samples were taken after 5 h into the light and dark periods in both groups of plants. Values represent the mean of at least six independent determinations in different control and stressed groups of plants ± SD.

Table 2 shows increased MDH specific activity in stressed samples with respect to control samples. In the case of NADP-MDH, increases of 1.3 and 1.5 times were found for stressed samples collected in the dark or in the day, respectively, with respect to control plants. A similar pattern was observed for NAD-MDH, with increases of 1.4 and 1.6 times.

Finally, two enzymes that provide reducing power in the form of NADPH were analyzed. G6PDH specific activity decreased in stressed samples (20% and 44% decrease in samples collected in the night and day, respectively). In contrast, GAPDHnP activity remained practically constant when control and stressed plants were compared, with higher values for samples collected during the night (Table 2).

Western blot analysis of carbohydrate metabolism-related enzymes

Western blot analysis with antibodies against chloroplastic FBP shows a higher level of immunoreactive protein in control samples collected in the day, the same quantity of protein in stressed samples taken in the dark or in the light, and the lowest level of reaction (a slight decrease with respect to levels in stressed plants) for control samples collected during the night (Fig. 3A).

Using antibodies against NADP- or NAD-MDH a correspondence between enzyme activity and immunoreactive protein level was found (Fig. 3B, C). Taking into account the correlation obtained between NAD-MDH activity (Table 2) and immunoreactive protein levels when using antibodies against cytosolic A. comosus NAD-MDH (Fig. 3B), as well as the conditions used for activity measurements, it is likely that the activity determined in P. oleracea represents that of the cytosolic NAD-MDH isoform. NADP-MDH from P. oleracea leaves not only shows cross-reaction with the antibodies raised against the maize enzyme but also presents a similar molecular mass (43 kDa, Fig. 3C).

Discussion

In C₄ mode, P. oleracea preferably uses NAD-ME as decarboxylating enzyme, which is up-regulated by light, showing increased activity and protein levels (Table 1, Fig. 1A). In contrast, in CAM-mode, both activity and specific activity show a great decrease (Table 1, Fig. 1). The other decarboxylase enzyme that changes after drought stress is PEPCK, which is also up-regulated during the day in control plants (Table 1, Fig. 1C). In other malic enzyme-C₄ species (Z. mays), a specific BSC PEPCK of 74 kDa was observed, where its mRNA level was higher during the day (Furumoto et al. 1999, Lea et al. 2001). A similar scheme may occur in P. oleracea performing C₄ photosynthesis and; thus, this enzyme may contribute, along with NAD-ME, to the CO₂ concentrating mechanism. In contrast to PEPCK-CAM plants in which the enzyme is more
Characterization of CAM metabolism in *P. oleracea*

**Fig. 4** Proposed CO₂ fixation schemes in *P. oleracea* leaves. (A) Well-watered conditions: C₄ photosynthesis, spatial separation of CO₂ primary fixation and secondary assimilation. (B) Water-stressed conditions: CAM-like metabolism, spatial and temporal separation of fixation by PEPC and RuBisCO. During the night, PEPC catalyses CO₂ condensation with glycolytic PEP. Then, malate is transitory accumulated in the vacuole. In the following light period, CO₂ is released for fixation and pyruvate and PEP generated by the decarboxylases are transformed through gluconeogenesis to sugars-P, which are stored in the cytosol as soluble sugars or in the chloroplast as starch. Numbers indicate the enzymes involved: 0, carbonic anhydrase; 1, PEPC; 2, NADP-MDH; 3, NAD-MDH; 4, PPDK; 5, NAD-ME; 6, aspartate-amidotransferase; 7, alanine aminotransferase; 8, PEPCK. Abbreviations: αKG, α-ketoglutarate; PCR, photosynthetic carbon reduction cycle; Pyr, pyruvate; mal, malate.
Characterization of CAM metabolism in *P. oleracea*

active during the day by phosphorylation, *P. oleracea* presented a small increase in PEPCK activity during the night after drought stress (Table 1). Nevertheless, the same amount of PEPCK immunoreactive band is observed by Western blot analysis (Fig. 1C). In this way, the differences in PEPCK activity in day and night samples in the CAM-mode may be due to alteration in the phosphorylation cascade after drought stress, as observed in the case of PEPC (Lara et al. 2003), or merely due to the high instability of this enzyme (Walker et al. 1997).

With regards to PPDK, significant differences were observed in control and stressed plants. In C₄ mode, the more abundant isoform (Fig. 1D) is the lower molecular mass immunoreactive band similar to that of maize (94 kDa) (Sugiyama 1973). In C₃ species, the photosynthetic PPDK is localized in MC chloroplasts; thus, the lower molecular mass band may be the MC chloroplastic enzyme involved in C₃ photosynthesis. On the other hand, in plants under drought stress, the higher molecular mass immunoreactive band is more intense with respect to that from control plants (Fig. 1D). In MC of NAD(P)-ME CAM plants, two PPDK isoforms have been described; their intracellular localization, chloroplastic and/or cytosolic, varies depending on the species (Kondo et al. 2000). In this way, it could be proposed that in *P. oleracea* under CAM mode, the higher molecular mass isoform could represent a cytosolic protein exclusively involved in CAM. In C₄ plants, chloroplastic PPDK is light up-regulated by dephosphorylation (Edwards et al. 1985). Nevertheless, PPDK regulation is less well characterized in CAM plants in which PPDK activity increases upon light exposure (Sugiyama and Laetsch 1975). So, the finding of higher levels of the lower molecular mass PPDK in day stressed samples it is not surprising. It is also worth mentioning that a cytosolic PPDK has been found in non-photosynthetic organs of C₃ and C₄ plants (Aoyagi and Chua 1988), which is induced by drought stress in rice roots (Moons et al. 1998).

Different assimilate partitioning patterns are described among CAM species. For example, *M. crystallinum* shows increased glycolytic activity when CAM is induced (Holtum and Winter 1982). In stressed *P. oleracea* while PFK activity increased in leaves taken in the dark, probably up-regulating nocturnal glycolysis, PFP activity remained constant. In contrast, PFP activity increased during daytime in stressed *P. oleracea* leaves (Table 2). As this enzyme can act in a gluconeogenic direction, it can contribute to the production of sugars during malic acid decarboxylation in the early light period. Aldolase activity decreased in *P. oleracea* stressed samples collected in the dark. However, as this enzyme catalyses a reversible reaction, and participates in both glycolysis and gluconeogenesis, the fluxes through both pathways may be regulated, at this point, by the amounts of substrates rather than by enzyme level.

G6PDH activity, which provides NADPH in the cytosol through the pentose pathway, decreased in stressed plants with respect to the control group (Table 2). Thus, under CAM-like metabolism the enzyme may be down-regulated by the low levels of carbon and high levels of NADPH, which probably occurs as consequence of lower CO₂ fixation and constant photosynthesis in the light (Lara et al. 2003). Another enzyme studied which provides NADPH in the cytosol was GAPDHnP. However, under drought stress conditions neither activity nor immunoreactive protein changed with respect to control plants (Table 2, Fig. 3).

Finally, NAD- and NADP-MDH increased their protein and specific activity levels (Fig. 3C, D, Table 2) under drought stress, as was the case for the induction of CAM in the ice plant (Holtum and Winter 1982). Both activities could be of great importance in the establishment of CAM in *P. oleracea*, as these enzymes are involved in malate metabolism. The increase of NAD-MDH activity along with the decrease in NAD-ME under CAM, suggests that the CO₂ releasing system may change from malate to pyruvate via NAD-ME in the C₄ mode to malate/OAA/PEP via NAD-MDH/PEPCK in the CAM mode.

A model for the photosynthetic pathway in *P. oleracea* can be suggested based on specific in situ immunolocalization of RuBisCO, NAD-ME and PEPC as well as activity measurements and PEPC kinetic and regulatory properties (Lara et al. 2003). During periods of enough water supply, BSC and MC cooperate to fix CO₂ in a C₄ photosynthetic pathway, with NAD-ME as the main (Fig. 4A), and PEPC as the minor decarboxylating enzymes (Fig. 1A), and PEPC activity increased during daytime in stressed *P. oleracea* leaves. CO₂ exchange patterns and periodic organic acid accumulation, in the form of malate, support the concept of temporal separation (Lara et al. 2003). Differential localization of RuBisCO, NAD-ME and PEPC indicates that, as in control plants, PEPC primary CO₂ assimilation and RuBisCO final CO₂ fixation take place in different cell types (Fig. 2B, D, F). Thus, during the night, CO₂ would be initially fixed as OAA by cytosolic PEPC in the MC and stored as malate. In the following light period, malate would be transported to BSC and decarboxylated, to provide CO₂ to RuBisCO. As under drought stress higher levels of PEPC immunoreactive protein in WSC are found (Fig. 2D), an enhanced activity of these cells, generating and storing malate, could take place under this condition (Fig. 2B). Although WSC are localized near the epidermis and the CO₂ diffusion pathway is shorter than in MC, at present we cannot confirm the role of WSC in photosynthesis.
In summary, the results obtained in the present work show relevant differences in several enzymes related to CO₂ assimilation or carbohydrate metabolism, which support the postulated transition to a CAM-metabolism from a C₄ status. In this respect, the occurrence of a CAM-like metabolism, operating throughout MC and BSC, could be the consequence of the expression of an ancestral CAM with the more evolved Kranz anatomy, as previously suggested by Sage (2002). In this way, the plasticity shown by \emph{P. oleracea} with respect to its natural habitats would be just a feature of its flexibility with respect to its metabolism.

Materials and Methods

Plant material

\emph{P. oleracea} L. plants were grown from seeds in a compost : sand : perlite mixture (2 : 1 : 1 by volume) and kept in a greenhouse (30/18°C day/night thermoperiod, 12 h photoperiod, 350 µmol m⁻² s⁻¹ light fluency). Plants were divided in two groups: one was watered daily (control) and water was withheld from the other (stressed). Each group contained at least 10 plants for each set of experiments. Day and night samples were taken 5 h into the day and night periods, respectively, and were composed of young leaves of the same age.

Light microscopy and in situ immunolocalization

Samples were prepared and treated using Protein A-gold as in Maurino et al. (1997). Slides were incubated 2 h with 1 : 1,000 serum against spinach RuBiCO large subunit (Dr. A. Viale, National University of Rosario, Argentina) or 4 h with the following affinity purified antibodies: anti-zea leaf NADP-ME IgG (Maurino et al. 1997); anti-PEPC IgG (Colombo et al. 1998) or anti-\emph{A. hypochondriacus} αNAD-ME IgG (Dr. J.O. Berry, Buffalo, New York University, U.S.A.). Finally, slides were stained with 0.25% (w/v) Safranin O and photographed using a Nikon Eclipse E-800 or a Zeiss Axioplan2 LSM5 Pascal microscope.

Protein extraction

Total protein was extracted, determined and prepared for SDS-PAGE as in Lara et al. (2003). In NAD-ME enzyme extraction buffer 0.5% Triton X-100 was also included.

Enzyme assay

Enzyme activity was measured in a 1 ml final volume at 30°C and 340 nm using a UNICAM Helios β spectrophotometer (Cambridge, U.K.). One unit of enzyme activity is defined as the amount of enzyme resulting in the production or consumption of 1 µmol of NAD(P)H min⁻¹. The reaction mixtures used for each enzyme were as follows.

Aldolase: 50 mM Tris-HCl, pH 7.7; with 5 mM MgCl₂; 4 mM fru-1,6-P₂; 0.15 mM NADH and 10 U of triosephosphate isomerase (TPI).

Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GDPHnP): 50 mM Tricine, pH 8.5; with 0.4 mM NADP; 1.2 mM fru-1,6-P₂; and 1 U of aldolase.

Glucose-6-phosphate dehydrogenase (G6PDH): 50 mM Tris-HCl, pH 7.5 assay media containing 2 mM glucose-6-P₂ and 0.2 mM NADP.

Fruuctose-1,6-bisphosphatase (FBP): Tris-HCl, pH 7.7; 5 mM MgCl₂; 50 mM fru-1,6-P₂; 0.15 mM NADP; 1 U phosphoglucoisomerase and 1 U G6PDH. As control, non-specific phosphatase activity was measured in the media without MgCl₂ and adding 1 mM EDTA.


NADP-malate dehydrogenase (NADP-MDH): as in Maurino et al. (1997). Samples were preincubated in 100 mM dithiothreitol (DTT) at room temperature.

NAD-ME: 50 mM HEPES, pH 7.3; 2 mM NAD; 5 mM L-malate; 5 mM DTT; 75 µM CoA; 10 mM MgCl₂; 10 U of MDH. Activity was assay after MDH reached the equilibrium.

NADP-ME: as in Maurino et al. (1997). PEPCK: 50 mM HEPES, pH 7.3; 4 mM PEP; 10 mM NaHCO₃; 2.5 mM MgCl₂; 2.5 mM MnCl₂; 0.15 mM NADH; 10 U of MDH and 3 mM ADP.

ATP-dependent phosphofructokinase (PFK): 50 mM Tris-HCl, pH 8; with 5 mM MgCl₂; 1 mM ATP; 2 mM fru-6-P; 0.15 mM NADH; 1 U aldolase; 10 U TPI and 1 U glyceral-3-P dehydrogenase (G3PDH).

PPD-dependent phosphofructokinase (PFP): 50 mM Tris-HCl, pH 7.7; with 5 mM MgCl₂; 2 mM NaPP; 2 mM Fru-6-P; 2 µM fru-2,6-P₂; 0.15 mM NADH; 1 U aldolase; 10 U TPI and 1 U G3PDH, starting the reaction with NaPP₇.

Gel electrophoresis

SDS-PAGE and Western blot analysis were performed in 8% or 10% (w/v) polyacrylamide gels (Lara et al. 2003) using 1 : 200 anti-\emph{Anaranthus viridis} PEPC; 1 : 200 anti-maize 62 kDa NADP-ME; 1 : 100 anti-\emph{A. comosus} PEPCK (Dr. F.E. Podestá, National University of Rosario, Argentina), 1 : 500 anti-recombinant chloroplastic oil seed rape FBP (Dr. R. Wolosuï, University of Buenos Aires, Argentina); and anti-\emph{A. comosus} NAD-MDH (Cuevas and Podestá 2000). Serum (1 : 1,000) against \emph{Amaranthus hypochondriacus} αNAD-ME (Long et al. 1994); 1 : 1,000 against maize PPDK (Dr. R. Chollet, University of Nebraska, U.S.A.); 1 : 10,000 against spinach RuBiCO large subunit and 1 : 500 against spinach NADP-MDH (Dr. R. Scheibe, Osnabrück University, Germany) also were used.

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References


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