Malate Accumulation in Different Organs of *Mesembryanthemum crystallinum* L. Following Age-dependent or Salinity-triggered CAM Metabolism

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Different organs of *Mesembryanthemum crystallinum* exhibit differing levels of CAM (Crassulacean acid metabolism), identifiable by quantification of nocturnal malate accumulation. Shoots and also basal parts of young leaves were observed to accumulate high concentrations of malate. It was typically found in mature leaves and especially prominent in plants subjected to salt stress. Small amount of nocturnal malate accumulation was found in roots of *M. crystallinum* plants following age-dependent or salinity-triggered CAM. This is an indication that malate can be also stored in non-photosynthetic tissue.

Measurements of catalase activity did not produce evidence of the correlation between activity of this enzyme and the level of malate accumulation in different organs of *M. crystallinum* although catalase activity also appeared to be dependent on the photoperiod. In all material collected at dusk catalase activity was greater than it was observed in the organs harvested at dawn.

Key words: Mesembryanthemum crystallinum, CAM, Catalase, Oxidative Stress

Introduction

Mesembryanthemum crystallinum in its native habitat (Namibian Desert of southern Africa) germinates in the short rainy season and changes its mode of photosynthesis from C_3 to Crassulacean acid metabolism (CAM) in the dry season. Its further development is strictly influenced by progressive drought stress coupled with increasing salinity (Winter *et al*., 1978). Such conditions have resulted in the evolution of acclimatory processes which can be defined in terms of anatomical, physiological, biochemical and molecular processes (Kluge and Ting, 1978; Lüttge, 1993; Adams *et al*., 1998).

CAM is a typical ecophysiological adaptation of plants to arid conditions (Lüttge, 1993; Grams and Thiel, 2002). Plants exhibiting CAM usually fix CO_2 during the night with a concomitant synthesis of malic acid, which is stored in the vacuole. During daytime the accumulated malic acid is released and decarboxylated to provide CO_2 for use via the Calvin cycle (Lüttge, 1993; Dodd *et al*., 2002). The CAM induction has been studied in considerable detail, but little is known about the common process induced from different stress factors that can stimulate CAM induction. In recent years *M. crystallinum*, a C_3-CAM intermediate plant, has become a model for the investigation of involvement of stress factors including high salinity, osmotic stress, excess light and the exogenous application of ABA in C_3/CAM transition (Chu *et al*., 1990; Taybi and Cushman, 1999; Ślesak *et al*., 2002). At the physiological level the consequence of these abiotic stresses have much in common. It seems that their common denominator could be a water

Abbreviations: ABA, abscisic acid; AOS, active oxygen species; APX, ascorbate peroxidase (EC 1.11.1.11); BSA, bovine serum albumin; CAM, Crassulacean acid metabolism; CAT, catalase (EC 1.11.1.6); DDT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; GR, glutathion reductase (EC 1.11.1.9); NAD-ME, NAD-malic enzyme (EC 1.1.1.38); NADP-ME, NADP-malic enzyme (EC 1.1.1.40); PAGE, polyacrylamide gel electrophoresis; PEPC, phospho*enol*pyruvate carboxylase (EC 4.1.1.31); PEPCK, phospho*enol*pyruvate carboxykinase (EC 4.1.1.32); Rubisco, ribulosebisphosphate carboxylase/oxygenase (EC 4.1.1.39); SOD, superoxide dismutase (EC 1.15.1.1); Tricine, *N*-tris(hydroxymethyl)-methylglycine; Tris, tris(hydroxymethyl)aminomethane.

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stress component. Some molecular studies have shown that numerous stresses including osmotic stress, low temperature and exposure to ABA, can have a similar effect on the expression of CAM specific genes (Chu *et al*., 1990). Thus, it was possible to conclude that similar responses to differing environmental challenges could be due to the existence of a general plant response mechanism (Vernon *et al*., 1993).

In the laboratory conditions, CAM metabolism can be readily induced by irrigating plants with a solution containing 0.1–0.4 M NaCl (Cheng and Edwards, 1991). It is well documented that salt treatment apart from the osmotic stress, causes an increase of the level of active oxygen species (AOS) resulting in oxidative stress (Miszalski *et al*., 1998; Ślesak *et al*., 2003). In *M. crystallinum* and, similarly in other C_3-CAM intermediate plants (*e.g. Sedum album*) the process of CAM induction (age-dependent or stress-triggered) is linked with the oxidative stress (Castillo, 1996; Miszalski *et al*., 1998).

Apart from its negative role AOS can function as secondary messenger in plants (Vranova *et al.*, 2002). Generally, AOS are formed by partial reduction of molecular oxygen as a consequence of aerobic life. Because of the highly cytotoxic and reactive nature of AOS, their accumulation must be under tight control to prevent cellular damage. Plants posses a very efficient antioxidant system that helps to regulate the level of AOS (Foyer and Noctor, 2001; Vranova *et al*., 2002). During different biotic or abiotic stresses the balance between AOS and their scavengers is disturbed. High concentration of AOS can lead to cell death and induction of some genes whereas low concentration induces the expression of other genes and adaptive response dependently on the particular stimulus. Thus, besides the role in cellular damage, AOS are considered to act as ubiquitous signaling molecules in plants. To allow for this dual function, cellular level of AOS is controlled by some antioxidative enzymes for example SOD, CAT, APX (Castillo, 1996; Miszalski *et al*., 1998, Ślesak *et al*., 2002). There is a relationship between relative tolerance to oxidative stress and the capacity of organisms to increase their level of AOS scavengers (Bowler *et al*., 1992).

The CAM cycle displays a series of distinct features that occur at specific points during the circadian rhythm (Dodd *et al*., 2002). *M. crystallinum*, like other CAM plants, exhibits a diurnal pattern of expression and regulation of some enzymes engaged in carbon fixation (*e.g.* PEPC, PEPCK, Rubisco, NADP-ME and NAD-ME). From previous published works on *M. crystallinum* leaves it is known that also the activity of some antioxidative enzymes changes during the day (Niewiadomska *et al*., 1999; Miszalski *et al*., 2001). These oscillations can be regarded as an additional indicator of the functioning of CAM photosynthesis, however, the mechanisms of regulation of antioxidant enzymes are still poorly understood (Ślesak *et al*., 2002).

The changes in the morphology, anatomy, physiology, biochemistry and also molecular processes has been described previously for the life cycle of *M. crystallinum* (Baur *et al*., 1992; Cushman and Bohnert, 1997; Adams *et al*., 1998). However, it is not known if expression of CAM and oxidative stress linked with CAM induction is uniform throughout the plant. The aim of this experiment was to investigate the C_3-CAM transition including the ability of the different parts of the plant to accumulate malate. This work also focused on the levels of CAT activity in different organs during CAM induction.

Material and Methods

Plant material and culture conditions

Plants of *Mesembryanthemum crystallinum* were grown from seeds in phytotron growth chambers in soil culture, under photoperiod 12/12 h, temperature 25/17 °C (day/night), 60/80% relative humidity, and irradiance of 250–300 μmol quanta $m^{-2}s^{-1}$ (PAR; λ = 400–700 nm). Two types of plants were used for the experiment: 7 weeks-old plants irrigated with water (age-dependent CAM) and 7 weeks-old plants irrigated after 3 weeks with 0.4 M NaCl (salinity-triggered CAM). Plants were separated into different organs or their parts and they were frozen in liquid nitrogen to await further analysis:

- mature leaves (first and second leaf pair) separated into: apical part (Ml-Ap), basal part (Ml-Ba)
- mature leaves (first and second leaf pair) separated into: blade (Ml-Bl), rib (Ml-Ri)
- young leaves (third and fourth leaf pair) separated into: apical part (Yl-Ap), basal part (Yl-Ba)
- shoots (Sh)
- roots (Rt).

Determination of malate concentration

The differences in malate concentration (*Δ*-malate) at the beginning (6.00 a.m.) and at the end of the light period (6.00 p.m.) were measured in the cell sap of all samples as a basic indicator of CAM metabolism. For isolation of cell sap samples were frozen at −22 °C, thawed and centrifuged for 3 min at 12000 × *g*. The supernatant (cell sap) was used for determination of the malate concentration following the enzymatic method described by Möllering (1985).

Protein determination

To isolate fractions of soluble proteins, leaf material (1 g fresh weight) was homogenized at 4 °C using a pestle and mortar in 2.5 cm^3 of extraction buffer (100 mM Tricine, adjusted with Tris to pH 8.0, containing 3 mM $MgSO_4$, 1 mM DDT, 3 mM EDTA) at 4 °C. Non-soluble material was removed by centrifugation for 1 min at 12000 × *g*.

Protein concentration was determined according to Bradford (1976), using the BioRad protein assay, with BSA as a standard. Soluble protein fractions were stored at −40 °C until further use.

Biochemical analysis of CAT

The activity of CAT was measured spectrophotometrically, according to Aebi (1984), by monitoring the disappearance of H_2O_2 at 240 nm, in 50 mM phosphate buffer, pH 7.0, containing initially 13 mM H_2O_2. Enzyme activity was defined as an amount of H_2O_2 [mmol] degraded by 1 mg of protein during 1 minute.

Results

The level of nocturnal malate accumulation differed strongly throughout the whole plant (Table I). In salinity-triggered CAM plants the organs most active in diurnal malate accumulation were shoots (15.9 mM) and basal parts of young leaves (20.5 mM). In these plants malate was also accumulated in roots, reaching the level 1.8 mM. In age-dependent CAM plants the highest amount of malate was also observed in shoots (8.0 mM). However, similar to results obtained in young leaves of salinity-triggered plants, basal parts of the leaves accumulated more malate (3.2 mM) than apical parts (1.0 mM). In age-dependent CAM plants a small accumulation of malate was also found in roots (0.1 mM).

Table I. Diurnal oscillation of malate concentration (*Δ*-malate) [mM] in *Mesembryanthemum crystallinum* organs during age-dependent and salinity-triggered CAM (n = 3).

Samples	Age-dependent CAM	Salinity-triggered CAM
Roots (Rt)	0.1 ± 0.06	1.8 ± 0.09
Shoots (Sh)	8.0*	15.9*
Mature leaves		
Apical part (Ml-Ap)	2.9*	
Basal part (Ml-Ba)	1.7*	12.3 ± 0.02
		11.8*
Mature leaves		
Blade (Ml-Bl)	3.0 ± 0.09	12.7*
Rib (Ml-Ri)	2.0*	11.0*
Young leaves		
Apical part (Yl-Ap)	1.0*	13.0 ± 0.07
Basal part (Yl-Ba)	3.2*	20.5 ± 0.02

* SD values were omitted when lower than 0.01.

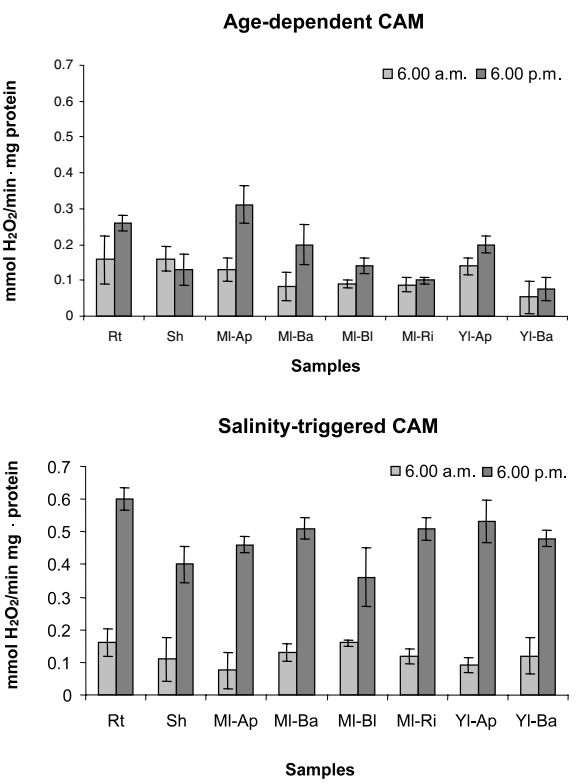

Fig. 1. CAT activity in different organs collected at 6.00 a.m. and 6.00 p.m. from age-dependent and salinity-triggered *Mesembryanthemum crystallinum* plants.

In age-dependant CAM material, harvested at 6.00 a.m. CAT activity ranged in different parts of plant from 0.053 to 0.16 mmol H_2O_2/min/mg protein and in plants harvested at 6.00 p.m. from 0.076 to 0.31 mmol H_2O_2/min mg protein (Fig. 1). In the salinity-triggered CAM plants harvested at 6.00 a.m. the CAT activity ranged from 0.075 to 0.16 mmol H_2O_2/min/mg protein (Fig. 1). A low level of CAT activity in salinity-triggered CAM plants collected at 6 a.m. is not sufficient to be detectable on the native activity stained gels in contrast to the CAT activity in the salt treated material harvested at 6.00 p.m., where it oscillated between 0.36 to 0.6 mmol H_2O_2/min/mg protein (Fig. 1) which was visible on native gels (data not shown).

Discussion

Measurements of differences in malate concentration between morning and evening hours (Δ-malate) presented in this paper confirmed previous findings that salt applied to the root medium can stimulate the development of CAM in *M. crystallinum* (Lüttge, 1993; Cushman and Bohnert, 1997; Adams *et al.*, 1998). In NaCl treated material (salinity-triggered CAM plants) large amounts of malate stored during the night were measured. Also in the plant tissues not treated with NaCl (age-dependent CAM plants) accumulation of malate was shown, suggesting that CAM metabolism was also induced.

Day/night malate oscillations were also detectable in shoots of both age-dependent CAM and salinity-triggered CAM plants. It indicates that shoot tissues are also capable of CAM mode of photosynthesis. This result corresponds with results observed in many other succulent plants, for example cactus where CAM is performed just in shoots (Winter and Smith, 1996).

Using malate accumulation as an indicator it was observed that CAM is not induced with the same intensity in different organs of *M. crystallinum* (Table I). Similarly in the dicotyledonous tree *Clusia minor* it has been demonstrated that CAM can be induced only in the leaves exposed to the stress regime (dry air or high light) while the other leaves continue to show C_3 metabolism (Dodd *et al.*, 2002; Grams and Thiel, 2002).

Different parts of *M. crystallinum* leaves showed only small differences in malate concentration. In young leaves, the concentration of malate was higher in the basal part of the leaf than in the apical part in contrast to old leaves where these values were reverse (Table I). Those differences can be due to the process of leaf growth and development. Older leaves had their blades in the apical part well unfolded and exposed to high light, while young leaves had their blades still partially folded. It has been already established that to evolve day/night differences in malate concentration high light as well as salinity is required because it stimulates some CAM related enzymes (Miszalski *et al.*, 2001). In young leaves higher concentration of malate in the basal region may result only from influence of the salt presence transported from the roots.

Detectable amounts of Δ-malate was observed in roots of plants exposed to salinity stress (Table I). This is evidence that malate can be also stored in non-photosynthetic cells. The possibility of malate storage in non-photosynthethic tissues was mentioned by Holtum (2001).

However, we do not know if malate is synthesized in these tissues or transported there from other organs. This information together with the observation that shoot tissue is very active in malate accumulation might indicate that salt or other signals coming from roots can effectively induce CAM. Malate is involved in different pathways depending on plant organs. Malate can function as an intermediate in Krebs-cycle, as an osmoticum for the regulation of pH and transport ions in the roots (Casati *et al.*, 1999). In our experiments the high volume accumulated periodically during the dark period indicates that its main role is to store carbon for later breakdown and use CO_2 in the Calvin cycle.

CAT activity present in the cells of different parts of the plants growing in different conditions was measured by spectrophotometric analysis and visualized by CAT activity staining on native gels (data not shown). As it is known that catalase shows diurnal fluctuation of the activity (Niewiadomska *et al.*, 1999) therefore, the extracts were prepared from plants collected at the beginning of the light period (6.00 a.m.) and at the end of the light period (6.00 p.m.). CAT activity in age-dependent CAM plants (low malate accumulation) showed in most samples small increase in activity during afternoon. This result is similar to measurements of CAT activity in well-watered plants (not treated with salt) presented by Niewiadomska *et al.* (1999). Much higher CAT activity mainly in

salinity-triggered CAM plants in samples collected at 6.00 p.m. can be explained in terms of the age of plants used in this experiment. They could exhibit processes of senescence.

It is prominent that in root samples, non-photosynthetic tissue, this photorespiratory enzyme showed high activity (Fig. 1). This was also confirmed in native-PAGE experiments (data not shown). In salt-triggered CAM, CAT activity in all tested samples was higher in plant material harvested during the afternoon (Fig. 1B). A similar result was also observed in experiments with root extracts where CAT activity was higher than observed in leaf extracts. This indicates that catalase, a photosensitive enzyme, is protected in root tissues (Streb *et al*., 1998). Strong day/night oscillation in activity of CAT in root extracts suggests that this enzyme is involved in CAM and even in the roots CAT activity can protect against oxidative stress. If this assumption is correct, then it might indicate that in *M. crystallinum* some photorespiratory processes can take place also in non-photosynthetic tissue.

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