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Effects of exogenously applied hydrogen peroxide on antioxidant and osmoprotectant profiles and the C_3-CAM shift in the halophyte *Mesembryanthemum crystallinum* L.

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ABSTRACT

Exogenously applied H_2O_2 (50, 100 and 200 mM) to *Mesembryanthemum crystallinum* root medium induced a transition from C_3 to Crassulacean Acid Metabolism (CAM), as evaluated by diurnal malate (Δmal) fluctuations. A very high concentration of H_2O_2 (400 mM) reduced Δmal below the value measured in control plants. An increase of malate content during the night in 400 mM H_2O_2-treated plants might suggest that malate decarboxylation is crucial for CAM functioning. We conclude that malate plays a dual role: i) a protective and signaling function before CAM expression, and ii) a storage form of CO_2 in plants performing CAM. A slight stimulation of photosystem II (PSII) photochemistry and net photosynthesis observed during the C_3-CAM shift indicated that neither photoinhibition nor reduction of the photosynthetic rate were prerequisites for CAM. Moreover, CAM induction corresponded to a decrease of catalase activity. In CAM-performing plants, α-tocopherol, polyamines (putrescine and spermidine) and proline showed daily alterations and the content of α-tocopherol and polyamines was lower at the end of the day. In contrast, the proline concentration correlated with the applied H_2O_2 concentration and was higher at the end of the day in treated plants. The dynamic changes of antioxidant and osmolyte levels suggest their active role in preventing oxidative damage, stress acclimation mechanisms and involvement in metabolic regulation and/or signal transduction cascades.

1. Introduction

Facultative CAM plants provide a convenient experimental system to study changes in plant metabolism upon CAM induction. Among the well-known facultative species, described as C_3-CAM intermediate plants, *Mesembryanthemum crystallinum* is a predominant experimental species due to its physiological and biochemical features and the comparatively small size of the genome (about 350,000 kb) (Bohnert and Cushman, 2001). CAM expression is revealed by nocturnal malate accumulation, as the result of dark uptake and fixation of CO_2 (Phase I), followed by its daytime decarboxylation, resulting in an elevated internal CO_2 concentration (day/night malate oscillation; Δmal) behind closed stomata (Phase III). Carboxylation processes are flanked by periods of net CO_2 uptake at the beginning (Phase II) and at the end of the day (Phase IV), respectively (Lüttge, 2002).

However, little is known about the factors (necessary and sufficient) leading to a C_3-CAM shift. It has been shown that up-regulation of the CAM pathway is primarily controlled by the environment and stress factors, such as salinity, drought, an increased level of ABA or low temperature, which are *per se* effective in CAM induction acceleration (Winter and Holtum, 2007). While high light is not able to induce CAM as a single factor (Gawronska et al., 2013; Kornas et al., 2010), it leads to the highest and most predictable degrees of CAM development (Broetto et al., 2006;

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylene glycol-bis(α -aminoethyl ether)-N,N,N',N'-tetraacetic acid tetrasodium salt; PAR, photosynthetically active radiation; RH, relative humidity.

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Miszalski et al., 2001; Ślesak et al., 2003). Moreover, Winter and Holtum (2007) excluded leaf water deficit alone as a sufficient factor leading to CAM, while some abiotic factors such as ozone, sulphur dioxide or ethylene are able to induce only certain symptoms indicative of the CAM mode of carbon assimilation (Borland et al., 2006; Hurst et al., 2004; Niewiadomska et al., 2002; Surówka et al., 2007). Since factors that lead to CAM expression are responsible for ROS (Reactive Oxygen Species) generation at the cellular level, we focused on the possible influence of exogenously applied ROS in the C_3-CAM shift.

ROS are oxygen-derived small molecules, including oxygen radicals, e.g. superoxide ($O_2^{\bullet -}$) and hydroxyl ($^{\bullet}OH$), as well as non-radicals that are either oxidizing agents and/or are easily converted into radicals, such as singlet oxygen ($^1O_2$) or hydrogen peroxide (H_2O_2). At the cellular level, ROS are generated as by-products of cell processes, but can also be "deliberately" generated by ROS-producing enzymatic complexes (e.g. NADPH oxidase) (Circu and Aw, 2010, Wittmann et al., 2012). The localization and content of ROS in plant tissues is not homogeneous (Fryer et al., 2002). Among ROS, hydrogen peroxide is one of the most abundant and stable ROS molecules and the regulation of its concentration can occur on the enzymatic as well as molecular levels in terms of H_2O_2 stability (Baxter et al., 2014; Circu and Aw, 2010). The diffusion of H_2O_2 through the plasma membrane and intracellular membranes of plant cells is determined by cellular aquaporin water channels (AQPs; Bienert and Chaumont, 2014; Borisova (Mubarakshina) et al., 2012). H_2O_2 is able to influence the transport of water and many solutes between cells and alter plasmodesmata permeability (Rutschow et al., 2011). H_2O_2 can either directly trigger chemical reactions and affect responsive targets, such as metabolites or proteins, or it can act as a signaling molecule both inside and outside organelles of its origin (Wittmann et al., 2012).

The impact of ROS is strongly dependent on the extent to which the potent antioxidative system allows its accumulation (Baxter et al., 2014; Wittmann et al., 2012). Catalase (CAT; EC 1.11.1.6) is known to be an integral part of the plant antioxidative system and this enzyme is responsible for the removal of high H_2O_2 concentrations. Different CAT isoforms are localized in peroxisomes, cytosol and mitochondria (Heyno et al., 2014). Peroxisomal CAT acts as a regulator fine-tuning redox signaling processes in connection with carbon flux through photorespiration or fatty acid β-oxidation (Queval et al., 2012). Catalase activity in CAM-performing *M. crystallinum* plants presents clear diel fluctuations (Niewiadomska et al., 1999). Other low molecular antioxidants, like tocopherols and proline, play also a role of information-rich redox buffers that interact with biomembrane-related compartments (Rejeb et al., 2014). Among tocopherols, α-tocopherol (α-TOC) is the most abundant form and shows the highest biological activity. α-TOC deactivates photosynthesis-derived ROS (mainly $^1O_2$ and $^{\bullet}OH$), and prevents the propagation of lipid peroxidation in the thylakoid membranes (Mène-Saffrané and DellaPenna, 2010; Surówka et al., 2009). Proline is an amino acid that plays multiple functions in osmoregulation, signaling and ROS scavenging (Hayat et al., 2012; Rejeb et al., 2014). Sanada et al. (1995) showed that proline accumulation preceded the shift of CAM in *M. crystallinum* plants, and this plant was shown to accumulate up to about 8 mM proline (in the leaf sap) under salinity in comparison to 0.25 mM in control plants (Demmig and Winter, 1986; Thomas et al., 1992).

Aliphatic polyamines (PAs) such as agmatine (Agm), putrescine (Put), spermidine (Spd), and spermine (Spm) are also involved in the salt tolerance of halophytes. They are known to control cellular metabolism through lowering ROS oxidant activity and affecting signaling cascades, but on the other hand, their catabolism/interconversion contributes to *in vivo* ROS generation, particularly H_2O_2 (Alcázar et al., 2010; Minocha et al., 2014). The accumulation of PAs in *M. crystallinum* is an organ-specific process. Shevyakova et al. (2006) showed that the dynamics of Spm accumulation resembled that of phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) in leaves of *M. crystallinum*.

Stress tolerance mechanisms during the C_3-CAM shift in *M. crystallinum* are also reflected in photochemistry. Broetto et al. (2006) showed that the photosynthetic capacity was reduced without causing chronic photoinhibition. Ślesak et al. (2003) pointed out that applying excess light led to changes in redox events in the vicinity of PSII and/or PSI, which are involved in the regulation of the C_3-CAM transition in *M. crystallinum*. They also found that the photo-produced H_2O_2 plays a role in the regulation of the C_3-CAM shift. However, the presented day/night changes of malate were not comparable to those determined under salinity or drought stresses. Moreover, it was not demonstrated that exogenously applied ROS *per se* can result in CAM induction. We tested the effect of prolonged (12 days) exogenous H_2O_2 application to the root medium on the C_3-CAM shift. We analyzed not only Δmal, but also malate content at the beginning and at the end of the day. The second aim was to determine whether alterations in PSII photochemistry and net photosynthesis are involved in the C_3-CAM shift. Moreover, we tested the influence of exogenously applied H_2O_2 on the pattern of ROS scavenging compounds, such as catalase, α-tocopherol, proline and polyamines, described previously as involved in plant signaling pathways.

2. Materials and methods

2.1. Plant material

M. crystallinum L. (the common ice plant; *Aizoaceae*) plants were grown in a soil culture in a phytotron chamber/greenhouse, under 12/12 h photoperiod, irradiance about 250–300 μmol m^{-2} s^{-1} (PAR range), temperature 25/18°C (day/night) and 50–70% RH regime. For biochemical analysis, the pooled samples of leaves obtained from at least three plants were used and two independent experiments were performed. Three-week-old plants were watered at the end of the day with tap water (control), while another set of plants was watered with 50, 100, 200 and 400 mM H_2O_2 solution for 12 days. The third leaf pairs were collected at the beginning and at the end of the light period on day 0 (before the onset of the H_2O_2 treatment), 4, 8 and 12 of the experiment. Then the samples were frozen in liquid nitrogen ground with a mortar and stored at −70 °C for further analysis.

2.2. Isolation and determination of soluble protein content

To isolate the fractions of soluble proteins, plant material was homogenized with a mortar 0.1 g fresh weight of plant material per 1 mL of extraction buffer (3 mM $MgSO_4$, 1 mM DTT, 3 mM EDTA, 100 mM Tricine pH 8.0). Non-soluble material was removed by a 3-min centrifugation at 10000*g*. The protein concentration was determined using the BioRad protein assay with BSA as a standard. Protein fractions were used directly for measurements or stored at −70°C until further use.

2.3. Spectrophotometric analysis of catalase (CAT; EC 1.11.1.6.) activity

CAT activity was measured using a spectrophotometric method described by Aebi (1984), based on the monitoring of H_2O_2 disappearance at 240 nm, in 50 mM phosphate buffer pH 7.0, initially containing 13 mM H_2O_2. Enzyme activity was determined in units [U] defined as 1 [mmol] of H_2O_2 degraded in 1 min per mg of protein. Leaves were collected at the end of the light period.