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Short communication

Withdrawal from functional *Crassulacean acid metabolism* (CAM) is accompanied by changes in both gene expression and activity of antioxidative enzymes

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ABSTRACT

In *Mesembryanthemum crystallinum*, crassulacean acid metabolism (CAM) is seemingly reversible, but unequivocal evidence for functional CAM withdrawal has yet to be shown. In this study, we confirmed the rapid downregulation of *PEPC1* expression just 1 h after the removal of NaCl from the plant growth media. At the same time, the Δ malate values in desalted plants rapidly (1 d) re-established to values typical for C_3 plants. This phenomenon allowed us to confirm functional CAM withdrawal in the desalted plants. Desalting altered the expression of the genes of the main antioxidative enzymes and/or the activity of their respective proteins; for catalase (CAT), both gene expression and protein activity were restored to levels observed in C_3 plants in response to desalting, while for cooper-zinc superoxide dismutase (CuZnSOD) and ascorbate peroxidase (APX), only protein activity was restored. Therefore, we conclude that during the C_3→CAM transition the CAM-specific antioxidative enzyme activity profile constitutes a transient and fully reversible response to abiotic stress.

1. Introduction

During their evolution, plants have developed a vast number of adaptations allowing them to grow and develop in even most inhospitable environments. Among such features, crassulacean acid metabolism (CAM) seems to be essential for survival in hot and dry habitats with minimal water availability. While C_3 plants gather CO_2 mostly during the day, plants performing CAM fix carbon dioxide during the night with phospho*enol*puryvate carboxalase (PEPC) and store it as malate in mesophyll cells' vacuoles. After night-time, malate is decarboxylated in chloroplasts, and released CO_2 is fixed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). This simple mechanism allows CAM plants to perform photosynthesis the following day while their stomata are closed. Plants with mature photosynthetic tissues expressing permanent CAM are referred to as constitutive/obligate CAM species; those with CAM as an option, usually turned on in response to environmental changes, are known as facultative CAM species or C_3/CAM intermediates (Winter et al., 2008). The main advantage of CAM is a significant reduction in water use during photosynthesis. This advantage, however, is achieved at a high cost of energy consumption resulting from the actions of CO_2 primary fixation, transformation/storage of the fixed products, etc. On the other hand, recent studies suggest that introducing CAM photosynthesis in C_3 crops could increase water-use efficiency without significant losses to productivity (Shameer et al., 2018). Although CAM is a substantial burden, full withdrawal from this photosynthesis was observed only in facultative CAM plants (Vernon et al., 1988; Ratajczak et al., 1994; Winter and Holtum, 2014). For many species, CAM is rapidly induced in response to temperature, high light or drought stress (Borland et al., 1992; Zotz and Winter, 1993; Lüttge, 2004, 2007). A widely studied facultative CAM species is *Mesembryanthemum crystallinum*. It is an annual halophyte and native to the Namib Desert (southern Africa); currently, this species is widely distributed in seasonally arid habitats worldwide. In natural environments with Mediterranean-type climates, a gradual C_3 to CAM transition coincides with seasonal water availability (Adams et al., 1998). The ability of *M. crystallinum* to switch from C_3 metabolism to CAM in response to high soil salinity was first described by Winter and von Willert (1972). Early reports indicating

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that the shift to CAM in *M. crystallinum* is a pre-programmed developmental process (Osmond, 1978) were dispelled when Winter and Holtum (2007) demonstrated that plants of this species grown under non-saline and well-watered conditions undergo their entire life cycle as C_3 plants. This discovery once again confirmed the facultative character of CAM for this species.

An enzymatic indicator of CAM metabolism is PEPC. Compared with that in C_3 plants, its activity in CAM plants increases by approximately 40-fold (Höfner et al., 1987). Vernon et al. (1988) demonstrated that the induction of both PEPC activity and *PEPC1* gene expression in juvenile *M. crystallinum* is a reversible response to environmental conditions (e.g., to osmotic stress) rather than an irreversibly expressed adaptation. Later, a decrease in Δ malate values, which is another hallmark of CAM, in response to salt removal was reported (Ratajczak et al., 1994). These reports have not received adequate attention, and while CAM reversibility in *M. crystallinum* has been noted (Adams et al., 1998; Winter and Holtum, 2014), no original studies have yet concentrated on this phenomenon.

One of the natural consequences of CAM occurrence is intracellular fluctuations of CO_2 concentration and CO_2/O_2 ratio resulting in diurnal fluctuations of photorespiration (Cushman and Bohnert, 1997). As a result, the scheme of reactive oxygen species (ROS) generation during light phase is altered (Niewiadomska et al., 1999). Redox homeostasis of plant cells is maintained with a complex interplay of non-enzymatic and enzymatic components interacting with ROS; among the latter group, a cytosolic form of superoxide dismutase (CuZnSOD, EC 1.15.1.1) responsible for enzymatic dismutation of superoxide radical ($O_2^{\cdot -}$) generated in cytosol (Alscher et al., 2002), catalase (CAT, EC 1.11.1.6) and peroxidases (class I – ascorbate peroxidase, APX, EC 1.11.1.11; class III peroxidases, POD, EC 1.11.1.7) involved in neutralization of hydrogen peroxide (H_2O_2) were distinguished as the major antioxidative agents (Mittler, 2004). The $C_3 \rightarrow$CAM transition leaves fingerprints of the expression/activity of major enzymatic antioxidants, e.g. CuZnSOD, CAT, APX, POD, and of H_2O_2 concentrations. On the other hand, *CuZnSOD* and cytosolic ascorbate peroxidase (*cAPX*) expression is upregulated during salt-induced CAM (Hurst et al., 2004; Nosek et al., 2015b), whereas CAT and POD activities are downregulated (Niewiadomska et al., 1999; Libik-Konieczny et al., 2011, 2012). Furthermore, H_2O_2 concentrations increase during the $C_3 \rightarrow$CAM transition (Ślesak et al., 2008). Together, these results confirm that CAM induction is related to both the substantial reorganization of the antioxidative system and the establishment of a new oxidative status in the cell.

The aim of this study was to verify whether functional CAM of *M.crystallinum* is reversible and how transient this process is. Rapid reversibility of this phenomenon would suggest that, in response to stress, plants undergo transient changes in their metabolism and revert to steady-state conditions after stress withdrawal. Consequently, alterations in ROS metabolism accompanying the CAM→C_3 shift, namely, the expression and activity of antioxidative enzymes, would constitute an independent response to salt stress rather than a part of the transition. To better understand this phenomenon, we tested how the return from functional CAM to C_3 metabolism alters both the expression of major enzymatic antioxidative genes and the activity of their proteins.

2. Materials and methods

2.1. Plant material

Common ice plants (*M. crystallinum* L.) were grown in pots in a greenhouse under 250–300 μmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR), a 12-h photoperiod (25/17 °C day/night temperature) and 60/80% relative humidity (RH). After 6 weeks, the plants were divided into two groups: the first group was irrigated with tap water (C_3 plants), and the second group was irrigated with 0.4 M NaCl to induce CAM (CAM plants). After 14 days, CAM was confirmed in the NaCl-treated plants by measuring the diurnal Δ malate in the leaf cell sap in accordance with the spectrophotometric protocol described in Gawronska and Niewiadomska (2015). Half of the CAM plants were subsequently subjected to desalting; it was initiated at 0800 a.m. CET, 2 h past the beginning of the light phase, by continuous rinsing of the soil substrate with tap water for 2 h (desalted CAM plants (−NaCl)). The leaves of the C_3, CAM and −NaCl plants were collected at 1 (1100 a.m.), 4 (0200 p.m.), 8 (0600 p.m.), 24 (2nd day, 1000 a.m.), 48 (3rd day, 1000 a.m.) and 120 h (6th day, 1000 a.m.) past salt removal (psr) for gene expression and enzyme activity analyses; samples from 4 plants were pooled per treatment. The Δ malate was analyzed using the material collected on the 1st, 3rd, 6th and 9th days of experiment past desalting. The plant material was immediately frozen, ground to a fine powder in liquid nitrogen and then stored at −80 °C until further analysis.

2.2. RNA preparation and gene expression

The total RNA was isolated with the Bio-Rad® Aurum™ Total RNA Mini Kit according to the manufacturer's instructions, and *q*PCR was performed in accordance with the methods described in Nosek et al. (2015b); polyubiquitin (gi|327492448) served as a housekeeping reference gene. The reaction efficiency was tested by serial dilutions of cDNAs with gene specific primers (Supplementary material). All samples were analyzed in triplicate. The expression was calculated as described by Pfaffl (2001); C_3 plants after 1 h served as internal control, and all analyses were performed in triplicate.

2.3. Protein extraction and quantification

To analyze the superoxide dismutase (SOD) and CAT activity, crude protein was extracted in accordance with the procedure described in Nosek et al. (2015a). To determine the ascorbate peroxidase (APX) activity, the crude protein was extracted in accordance with the procedure described in Nosek et al. (2011). Briefly, 0.5 g of frozen tissue was homogenized in 50 mM phosphate buffer (pH 7.0) that contained 1 mM ascorbic acid (AsA) and 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 12,000*g* and 4 °C for 4 min. The protein content in the supernatant was subsequently quantified in accordance with the Bradford (1976) method; bovine serum albumin (BSA) served as a standard.

2.4. CAT activity assays

The CAT activity was measured in accordance with the method described by Aebi (1984). One unit of enzyme activity was defined as 1 μmol of H_2O_2 decomposed during minute. Absorbance coefficient $\varepsilon^{240} = 43$ $(\mu mol/L)^{-1}$ cm^{-1} was used for calculation.

2.5. APX activity assay

The APX activity was spectrophotometrically assayed in accordance with the method described by Nakano and Asada (1981). Ascorbate peroxide activity was determined via the loss of the ascorbate concentration in a reaction mixture containing pH 7.0 phosphate buffer (KH_2PO_4/K_2HPO_4), 1 mM EDTA, 15 mM L-AsA and 40 mM H_2O_2 at a wavelength of 240 nm. Enzyme, activity was defined as 1 mmol of AsA decomposed by 1 mg of protein per minute. For calculation, an AsA absorbance coefficient of 2.8 $(mmol/L)^{-1}$ cm^{-1} was used.

2.6. Analysis of SOD by native PAGE

The separation of soluble protein fractions was performed according to the procedure described by Laemmli (1970); native (without sodium dodecyl sulphate) PAGE at 4 °C and 180 V was used. Visualization of SOD bands was performed on discontinuous 12% polyacrylamide gels