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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

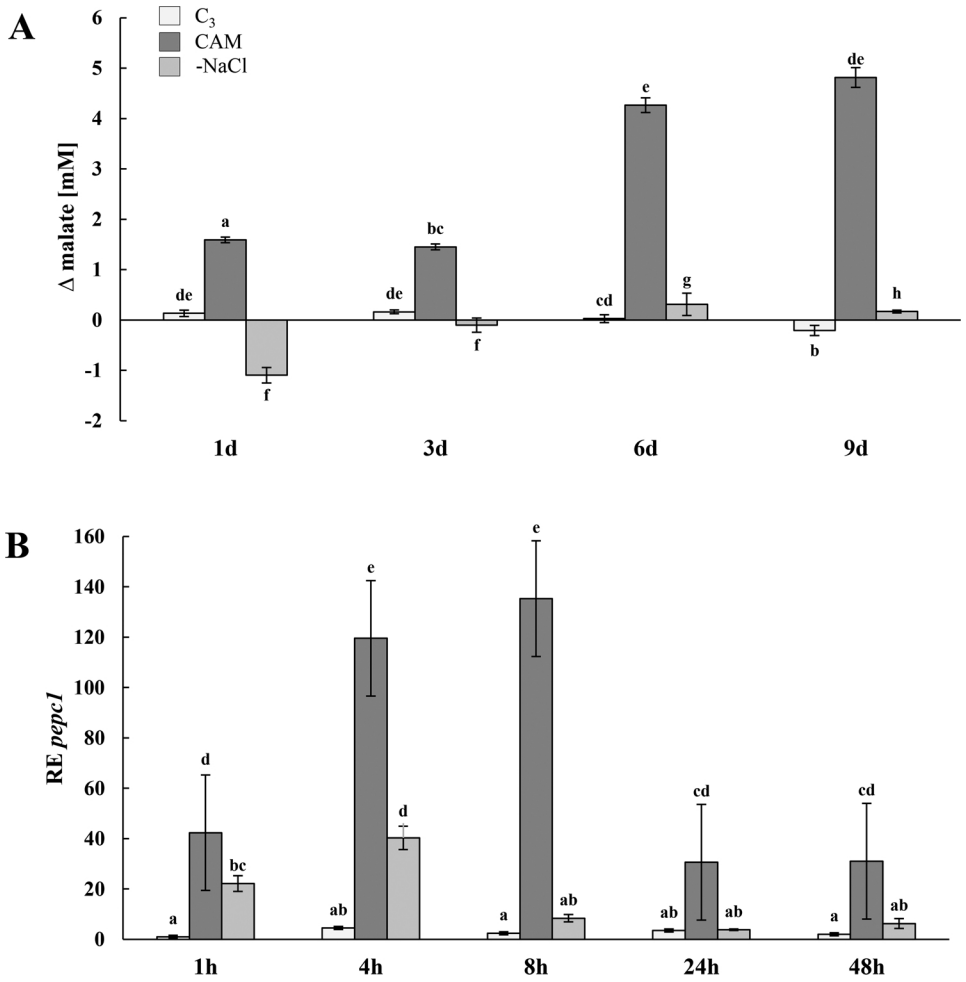

Fig. 1. Δ Malate values (**A**) in C_3, crassulacean acid metabolism (CAM) and desalted CAM (-NaCl) plants measured on the 1st, 3rd, 6th and 9th days of the experiment. (**B**) Relative expression of the phospho*enol*carboxylase1 (*pepc1*) gene in C_3, CAM and -NaCl plants measured at 1, 8, 24, and 48 h past salt removal (psr). The different letters above the bars indicate statistically significant differences according to two-way ANOVA and the Tukey post hoc test (N = 3, P ≤ 0.05, mean value ± SD).

in accordance with the method described by Beauchamp and Fridovich (1971). The gels were incubated in the staining buffer for 30 min, in darkness, at room temperature and then exposed to white light until SOD activity bands became visible. Densitometric analysis of SODs bands was performed with ImageJ 2 (GPL license).

2.7. Statistical analyses

All analyses were performed in triplicate. The results were analyzed with Statistica 12 (Statsoft, USA) statistical software. Two-way ANOVA followed by Tukey's honest significant difference (HSD) multiple range test were used to determine individual treatment effects at P ≤ 0.05.

3. Results

3.1. Removal of salt from the soil affects diurnal PEPC1 gene expression and malate accumulation that is typical for CAM metabolism

The Δ malate value represents the difference in malate concentration between the end of the night period and the beginning of the light phase (Fig. 1A). Compared with the CAM plants, the -NaCl plants presented a significant decrease in Δ malate values during the first day of the experiment. On subsequent days, the Δ malate values in the -NaCl plants were comparable to those in the C_3 plants. In the diurnal part of the experiment the CAM plants exhibited progressive increase of *PEPC1* expression with the greatest accumulation of gene transcript at the end of the light period (8 h psr, 0600 p.m.) (Fig. 1B). Although substantially lower, desalted plants showed similar increase of *PEPC1* expression in diurnal part of the experiment; however, the highest value was reached 4 h psr (0200 p.m.), and at 8 h psr (0600 p.m.) it declined to the values observed in the C_3 plants. At subsequent time points of the experiment (24 and 48 h psr), the pattern with the highest *PEPC1* expression in CAM and equally low in C_3 and -NaCl plants was maintained.

3.2. Desalting alters the gene expression profile and activity of key antioxidative enzymes

In the diurnal part of our experiment, we found that *CAT1* expression was lowest in CAM plants, aside from a single experimental time point, 4 h psr (0200 p.m.) when *CAT1* accumulation was the highest (Fig. 2A). One hour after the stressor was removed (1100 a.m.), the -NaCl plants exhibited a C_3-specific pattern of *CAT1* expression. This pattern was distorted at the end of the light phase of day 1 (8 h psr, 0600 p.m) but re-established at subsequent time points during the experiment, specifically at 24 and 48 h psr.

In the early stage of the light phase (1100 a.m.), the CAT activity was correlated with *CAT1* gene expression; the CAM plants demonstrated significantly lower CAT activity than did both the C_3 and -NaCl plants (Figs. 2A and 3 A). During the next several hours, however, this pattern changed in terms of both enzyme activity and gene expression.

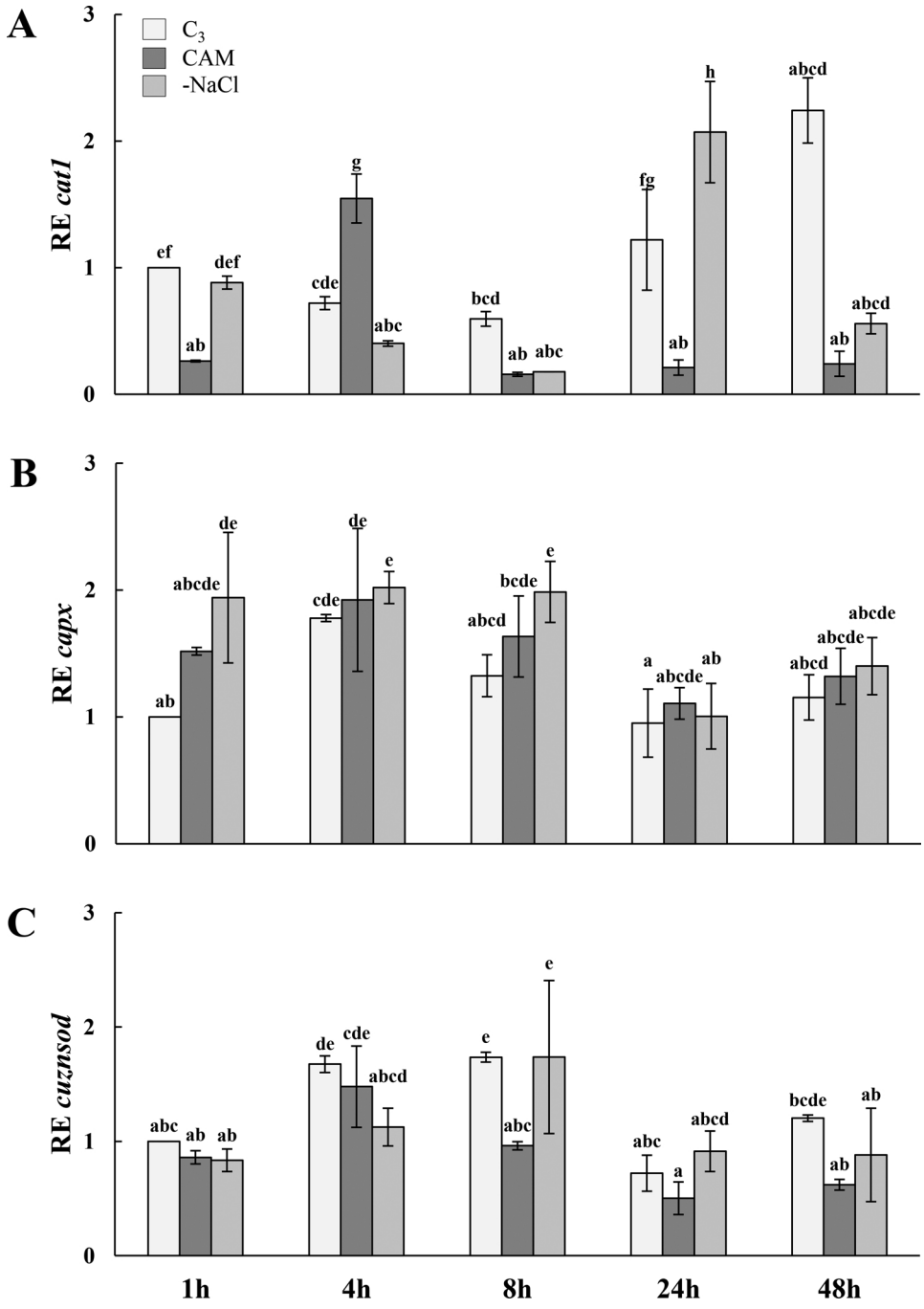

Fig. 2. Relative expression of the leaf catalase (*cat1*) (**A**), cytosolic ascorbate peroxidase (*capx*) (**B**) and copper-zinc superoxide dismutase (*cuznsod*) (**C**) genes in C_3, crassulacean acid metabolism (CAM) and desalted CAM (-NaCl) plants measured at 1, 8, 24, and 48 h past salt removal (psr). The different letters above the bars indicate statistically significant differences according to two-way ANOVA and the Tukey post hoc test (N = 3, P ≤ 0.05, mean value ± SD).

At the end of the light phase on day 1 (0600 p.m.), the CAT activity of the -NaCl plants closely resembled that of the CAM plants.

In addition to CAT, which is the main cellular H_2O_2 scavenger (Scandalios, 1987), the gene expression and protein activity of *cAPX* and APX, respectively, as well as the activity of CuZnSOD was analyzed to describe the effect of desalting on the main enzymatic antioxidants (Fig. 4). The highest expression of *cAPX* occurred at 1 h (1100 a.m.) and 8 h (0600 p.m.) psr in the -NaCl plants, which was similar to the results of the CAM plants. Only at these time points was the enzyme activities in accordance with *cAPX* expressions in all the analyzed plants (Figs. 2B and 3B). No significant differences in *cAPX* expression were observed at subsequent time points between treatments. Similar to the CAT activity, the APX activity changed in response to the removal of the salt stressor. Analysis of the diurnal rhythm of the APX activity revealed substantial fluctuations between experimental variants; APX activities of CAM and -NaCl plants were substantially higher in all time points of diurnal part of experiment than C_3 plants, with the greatest activity measured in the both mentioned variants 4 h psr (0200 a.m.). However, beginning on the 2nd day (24 h), a new pattern of APX activity was established; the highest APX activity occurred in CAM-performing tissues, and significantly lower in both the C_3 and -NaCl plants than in the CAM plants (Fig. 3B).

No significant differences in *CuZnSOD* expression were observed between treatments; the lone exception occurred at 8 h psr

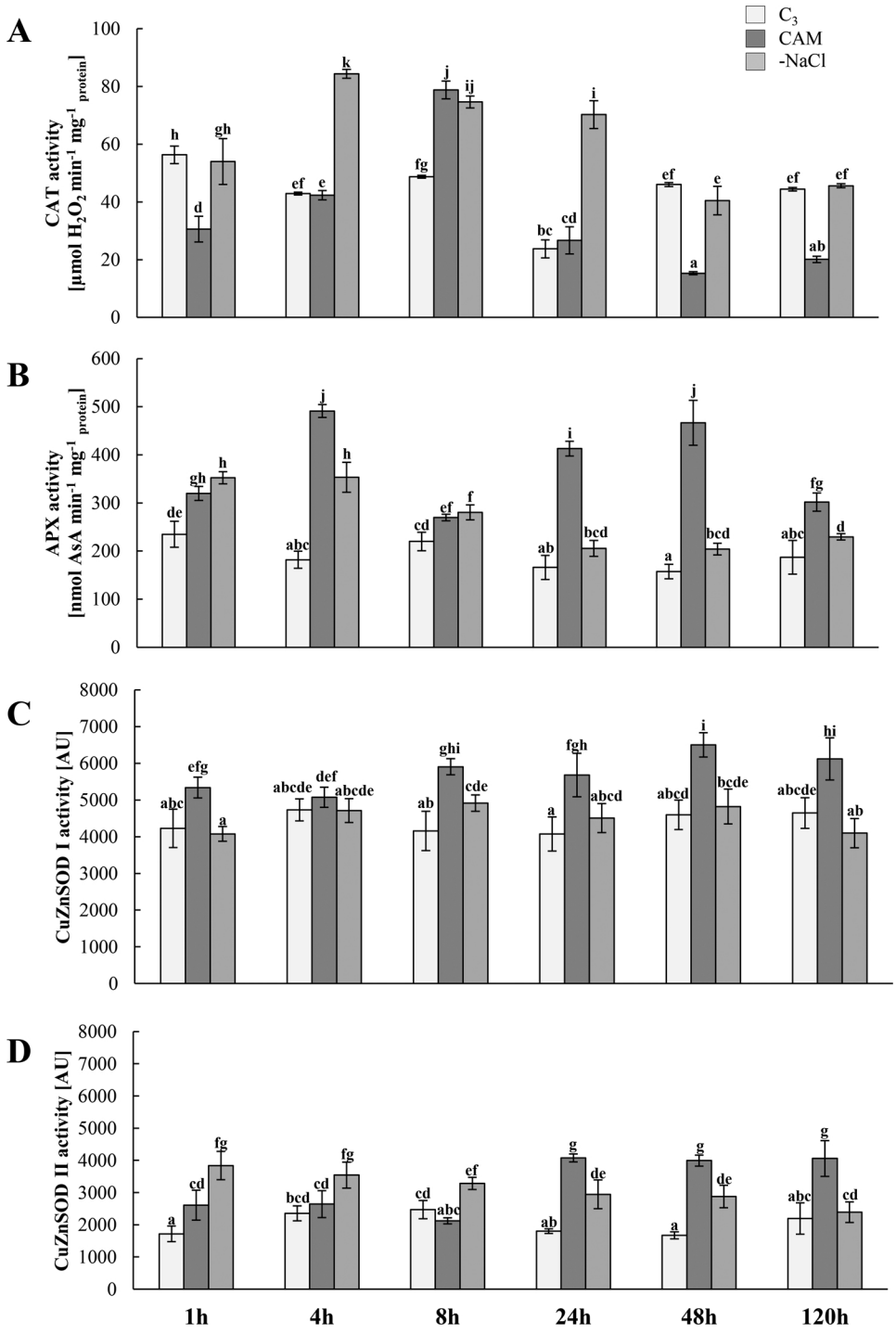

Fig. 3. Total activity of catalase (CAT) (**A**), ascorbate peroxidase (APX) (**B**) and copper-zinc superoxide dismutase (CuZnSOD) isoform I and II (**C, D**) in C_3, crassulacean acid metabolism (CAM) and desalted CAM (-NaCl) plants measured at 1, 8, 24, 48 and 120 h past salt removal (psr). The different letters above the bars indicate statistically significant differences according to two-way ANOVA and the Tukey post hoc test (N = 3, P ≤ 0.05, mean value ± SD).

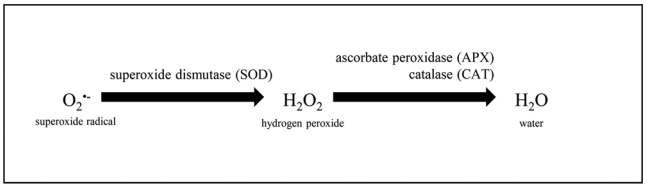

Fig. 4. Role of antioxidative enzyme proteins of respective genes in reactive oxygen species (ROS) metabolism.

(corresponding to the end of the light period) between the C_3 and CAM plants (Fig. 2C). During the early stage of light phase (1 h psr, 1100 a.m.), the activity of CuZnSOD I in the CAM plants was greatest; however, this activity was lower in both the -NaCl and C_3 plants than in the CAM plants (Fig. 3C). Three hours later (4 h psr, 0200 p.m.), no statistically significant differences were observed between plants. However, at the end of the light phase (8 h psr, 0600 p.m.), the CuZnSOD I activity was greater in the -NaCl plants than in the C_3 plants but was significantly lower than that in the CAM plants. During the period from 24 to 120 h, the CAM plants exhibited significantly higher

activity. However, the activity pattern of the second distinguished CuZnSOD isoform differed (Fig. 3D). The CuZnSOD II activity significantly differed between treatments at the majority of the experimental time points, with the exceptions of 4 and 8 h psr. Beginning at 24 h psr, the CuZnSOD II activity of the -NaCl plants was comparable to that of the C_3 plants.

4. Discussion

Previous studies shown that within 2.5 h after the desalting of CAM plants, *PEPC1* expression decreased by 77% (Vernon et al., 1988). Here, the decrease in *PEPC1* (by 48%) was more rapid: it began 1 h after desalting (1100 a.m.), in the early stage of light phase and decreased as time progressed. Decline of *PEPC1* expression in -NaCl plants is even more evident when compared with *PEPC1* diurnal profile found in CAM plants; substantially higher and progressively increasing *PEPC1* content toward the end of light phase (0600 p.m.) seems to be a routine preparation for efficient CO_2 fixation during phase 1 of CAM (Borland and Griffiths, 1997). Additionally, we analyzed a second parameter that, together with *PEPC1* expression, allows the confirmation of functional CAM: Δ malate. Changes in Δ malate values measured in desalted plants were in parallel with the *PEPC1* expression profile during corresponding time points of the experiment. This suggests that, in addition to PEPC1 gene downregulation, malate metabolism was also strongly modified. These results undoubtedly confirm that the removal of the salt stressor induced the withdrawal of functional CAM back to C_3 metabolism and suggest that the CAM→C_3 shift is a transient, reversible response to stress in *M. crystallinum*. This finding shows that the plants can adapt and optimize their carbon metabolism and energy distribution in a very rapid and flexible manner.

Studies have reported a significant decrease in CAT activity associated with salt-dependent CAM induction in *M. crystallinum* (Niewiadomska et al., 1999). Furthermore, the circadian rhythm of CAT activity is different in C_3- and CAM-performing *M. crystallinum*: CAM plants show strong diurnal fluctuations in CAT activity, and the maximum activity occurs at the end of the light period, which relates to CO_2 depletion, increased photorespiration and intensified H_2O_2 generation. Higher *CAT1* expression and CAT activity as well as a lack of circadian rhythm are characteristic features of C_3 metabolism (Niewiadomska et al., 1999). Here, we show that this diurnal expression pattern of *CAT1* is salt-dependent and, with removal of salt stress, this pattern quickly returned to C_3-like pattern. Such a fast downregulation of *CAT1* expression was previously shown in *Arabidopsis thaliana* in response to excess light; the *CAT1* expression started to increase 2 h after light treatment and returned to the level measured in intact plants during the next 24 h (Karpinski et al., 1997). The -NaCl plants re-established and maintained *CAT1* expression at levels observed in untreated C_3 plants during first several hours psr. More time, however, was needed to re-establish enzyme activity. We observed that at the end of light phase of day one (8 h psr, 0600 p.m.) CAT activity of desalted plants resembled that of CAM plants; this suggests that salt removal had no rapid influence and CAT activity, however, only during early hours past stressor removal, is regulated in CAM-like style inducing protein activity for the end of phase III of CAM. This phenomenon leads to the conclusion that higher CAT expression and activity levels characteristic of C_3 plants are preferred in less demanding environmental conditions instead of the CAT activity and expression scheme observed for CAM plants.

In our study, we found no significant differences in *cAPX* content between the CAM and -NaCl plants during the course of the experiment. Among the major enzymatic antioxidants, cytosolic peroxidases, namely *APX1* and *APX2*, undergo the most rapid regulation of gene expression in response to stress and its withdrawal (Karpinski et al., 1997). Here, we showed that *cAPX* expression did not respond to salt stress withdrawal at all. In the context of the diurnal pattern of enzyme activity (1, 4, and 8 h psr) an evident similarity between the CAM and -NaCl plants occurred, however, from 24 h psr until end of the experiment, APX activity of -NaCl resembled C_3. Thus, we propose that like in case of CAT, during early hours past stressor removal, APX activity was regulated in a CAM-like fashion. Moreover, the diurnal fluctuations of APX activity observed in CAM and -NaCl, precisely, increased APX activity up 4 h psr (0200 p.m.) and substantially lower at the end of light phase (0600 p.m, 8 h psr), corresponded to fluctuations of CAT activity found in these variants. This effect was extended at subsequent time points (24, 48 and 120 h) of the experiment and was visible also in C_3 plants. This suggests the compensation effect between APX and CAT action, since both enzymes are involved in removal of the most stable oxygen metabolism by-product, namely H_2O_2. Bonifacio et al. (2011) previously described a compensatory mechanism in rice cAPX double-silenced mutants (APX1/2s) in which minimal *cAPX* expression was compensated by the upregulation of other POD and CAT enzymes. In case of this study, compensation effect could be transferred to the level of protein activity. The APX activity was downregulated upon the removal of salt stress, whereas the CAT activity was upregulated. It is possible that the downregulation of CAT during the CAM→C_3 shift is accompanied by the concomitant upregulation of *cAPX*/APX.

CuZnSOD is an intracellular stress marker. In this study, we showed that stress removal and the resultant CAM→C_3 shift did not affect *CuZnSOD* abundance. This finding confirms previous studies reporting that salt-dependent CAM does not affect *CuZnSOD* gene expression (Ślesak et al., 2002). Moreover, we found no correlation between the expression of *CuZnSOD* and the activity of both identified CuZnSOD isoforms. An analogous lack of correlation between CuZnSOD activity and corresponding mRNA abundance was previously reported for *M. crystallinum* (Ślesak et al., 2002) and other plants (Kliebenstein et al., 1998; Kurepa et al., 1997). However, total CuZnSOD activity was shown to be induced concurrently with salt-dependent CAM (Miszalski et al., 1998). We show that, in response to the removal of the salt stressor, the enzyme activity of both CuZnSOD isoforms significantly decreased and their activity reached values characteristic for C_3 plants; together with no effect from CAM→C_3 shift on *CuZnSOD* abundance, it suggests other than translational regulation of CuZnSOD. Altogether, these results confirm the discrepancies between C_3- and CAM-performing plants regarding regulation of main enzymatic antioxidants. Both group of plants have different characteristics/dynamics of diurnal ROS generation resulting from *e.g.* fluctuating efficiency of photosystems, O_2/CO_2 ratio and photorespiration intensity etc. (Cushman and Bohnert, 1997; Niewiadomska and Miszalski, 2008). These differences are reflected in the specific daily course of antioxidant activity, confirming different requirements for redox homeostasis maintenance in C_3- and CAM-performing plants.

5. Conclusion

As shown in this study, C_3 metabolism in *M. crystallinum* is the preferred mode of carbon assimilation for less demanding environmental conditions, and upon the removal of NaCl from the substrate, a CAM to C_3 shift is initiated. The presence of NaCl is necessary to sustain CAM. CAM withdrawal alters the activity of both APX and CuZnSOD, but does not affect the expression of their respective genes, as it is in the case of CAT. CAT, as well as APX activity, however, only during early hours of CAM withdrawal, are regulated in CAM-like fashion.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2018.07.011.

References

Adams, P., Nelson, D.E., Yamada, S., Chmara, W., Jensen, R.G., Bohnert, H.J., Griffiths, H., 1998. Growth and development of *Mesembryanthemum crystallinum* (Aizoaceae). New Phytol. 138, 171–190.

Aebi, H., 1984. Catalase *in vitro*. Method Enzymol. 105, 121–126.

Alscher, R.G., Erturk, N., Heath, L.S., 2002. Role of superoxide dismutase (SOD) in controlling oxidative stress in plants. J. Exp. Bot. 53, 1331–1341.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.

Bonifacio, A., Fabricio, C., Martins, M., Neto, M., Cunha, J., Ribeiro, C., Margis-Pinheiro, M., Silveira, J., 2011. Silenced rice in both cytosolic ascorbate peroxidases displays pre-acclimation to cope with oxidative stress induced by 3-aminotriazole-inhibited catalase. J. Plant Physiol. 201, 17–27.

Borland, A.M., Griffiths, H., 1997. A comparative study on the regulation of C_3 and C_4 carboxylation processes in the constitutive crassulacean acid metabolism (CAM) plant *Kalanchoë daigremontiana* and the C_3-CAM intermediate *Clusia minor*. Planta 201, 368–378.

Borland, A.M., Griffiths, H., Maxwell, C., Broadmeadow, M.S.J., Griffiths, N.M., Barnes, J.D., 1992. On the ecophysiology of the *Clusiaceae* in Trinidad: expression of CAM in *Clusia minor* L. during the transition from wet to dry season and characterization of three endemic species. New Phytol. 122, 349–357.

Bradford, M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of proteindye binding. Anal. Biochem. 72, 248–254.

Cushman, J.C., Bohnert, H.J., 1997. Molecular genetics of crassulacean acid metabolism. Plant Physiol. 113, 667–676.

Gawronska, K., Niewiadomska, E., 2015. Participation of citric acid and isocitric acid in the diurnal cycle of carboxylation and decarboxylation in the common ice plant. Acta Physiol. Plant 37, 61.

Höfner, R., Vazquez-Moreno, L., Winter, K., Bohnert, H.J., Schmitt, J.M., 1987. Induction of crassulacean acid metabolism in *Mesembryanthemum crystallinum* by high salinity: mass increase and *de novo* synthesis of PEP-carboxylase. Plant Physiol. 83, 915–919.

Hurst, A.C., Grams, T.E.E., Ratajczak, R., 2004. Effects of salinity, high irradiance, ozone, and ethylene on mode of photosynthesis, oxidative stress and oxidative damage in the C_3/CAM intermediate plant *Mesembryanthemum crystallinum* L. Plant Cell Environ. 27, 187–197.

Karpinski, S., Escobar, C., Karpinska, B., Creissen, G., Mullineaux, P.M., 1997. Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress. Plant Cell 9, 627–640.

Kliebenstein, D.J., Monde, R.A., Last, R.L., 1998. Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. Plant Physiol. 118, 637–650.

Kurepa, J., Herouart, D., Van Montagu, M., Inzé, D., 1997. Differential expression of CuZn- and Fe-superoxide dismutase genes of tobacco during development, oxidative stress, and hormonal treatments. Plant Cell Physiol. 38, 463–470.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Libik-Konieczny, M., Surówka, E., Kuźniak, E., Nosek, M., Miszalski, Z., 2011. Effects of *Botrytis cinerea* and *Pseudomonas syringae* infection the antioxidant profile of *Mesembryanthemum crystallinum* C_3/CAM intermediate plant. J. Plant Physiol. 168, 1052–1059.

Libik-Konieczny, M., Surówka, E., Nosek, M., Goraj, S., Miszalski, Z., 2012. Pathogen-induced changes in malate content and NADP-dependent malic enzyme activity in C_3 and CAM performing *Mesembryanthemum crystallinum* L. Plants. Acta Physiol. Plant 34, 1471–1477.

Lüttge, U., 2004. Ecophysiology of crassulacean acid metabolism (CAM). Ann. Bot. 93 (6), 629–652.

Lüttge, U., 2007. *Clusia*. A Woody Neotropical Genus of Remarkable Plasticity and Diversity. Ecological Studies 194. Springer, Berlin.

Miszalski, Z., Ślesak, I., Niewiadomska, E., Baczek-Kwinta, R., Lüttge, U., Ratajczak, R., 1998. Subcellular localization and stress responses of superoxide dismutase isoforms from leaves in the CAM→C_3 intermediate halophyte *Mesembryanthemum crystallinum* L. Plant Cell Environ. 21, 169–179.

Mittler, R., 2004. Reactive oxygen gene network of plants. Trends Plant Sci. 9, 490–498.

Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 5, 867–880.

Niewiadomska, E., Miszalski, Z., 2008. Partial characterization and expression of leaf catalase in the CAM-inducible halophyte *Mesembryanthemum crystallinum* L. Plant Physiol. Biochem. 46, 421–427.

Niewiadomska, E., Miszalski, Z., Ślesak, I., Ratajczak, R., 1999. Catalase activity during CAM→C_3 transition in *Mesembryanthemum crystallinum*. Free Radic. Res. Commun. 31, 251–256.

Nosek, M., Surówka, E., Cebula, S., Libik, A., Goraj, S., Kornas, A., Miszalski, Z., 2011. Distribution pattern of antioxidants in white cabbage heads (*Brassica oleracea* L. var. *capitata* f. *alba*). Acta Physiol. Plant 33, 2125–2134.

Nosek, M., Kornaś, A., Kuźniak, E., Miszalski, Z., 2015a. Plastoquinone redox state modifies plant response to pathogen. Plant Physiol. Biochem. 96, 163–170.

Nosek, M., Rozpądek, P., Kornaś, A., Kuźniak, E., Schmitt, A., Miszalski, Z., 2015b. Veinal-mesophyll interaction under biotic stress. J. Plant Physiol. 185, 52–56.

Osmond, C.B., 1978. *Crassulacean* acid metabolism: a curiosity in context. Annu. Rev. Plant Physiol. 29, 379–414.

Pfaffl, M., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29, 2003–2009.

Ratajczak, R., Richter, J., Lüttge, U., 1994. Adaptation of the tonoplast V-type H^+-ATPase of Mesembryanthemum crystallinum to salt stress, CAM→C_3 transition and plant age. Plant Cell Environ. 17, 1101–1112.

Scandalios, J.G., 1987. The antioxidant enzyme genes CAT and SOD of maize: regulation, functional significance, and molecular biology. Isozymes Curr. Top. Biol. Med. Res. 14, 19–44.

Shameer, S., Baghalian, K., Maurice Cheung, C.Y., Ratcliffe, R.R., Sweetlove, L.J., 2018. Computational analysis of the productivity potential of CAM. Nat. Plants DOI:10/1038/s41477-018-0112-2.

Ślesak, I., Miszalski, Z., Karpinska, B., Niewiadomska, E., Ratajczak, R., Karpinski, S., 2002. Redox control of oxidative stress responses in the C_3–CAM intermediate plant *Mesembryanthemum crystallinum*. Plant Physiol. Biochem. 40, 669–677.

Ślesak, I., Libik, M., Miszalski, Z., 2008. The foliar concentration of hydrogen peroxide during salt-induced CAM→C3 Transition in *Mesembryanthemum crystallinum* L. Acta Biochim Polonica 174, 221–226.

Vernon, D.M., Ostrem, J.A., Schmitt, J.M., Bohnert, H.J., 1988. PEPC transcript levels in *Mesembryanthemum crystallinum* decline rapidly upon relief from salt stress. Plant Physiol. 86, 1002–1004.

Winter, K., Holtum, J.A.M., 2007. Environment or development? Lifetime net CO_2 exchange and control of the expression of crassulacean acid metabolism in *Mesembryanthemum crystallinum*. Plant Physiol. 143 (1), 98–107.

Winter, K., Holtum, J.A.M., 2014. Facultative crassulacean acid metabolism (CAM) plants: powerful tools for unravelling the functional elements of CAM photosynthesis. J. Exp. Bot. 65, 3425–3441.

Winter, K., von Willert, D.J., 1972. NaCl-induzierter Crassulaceen-Säurestoffwechsel bei *Mesembryanthemum crystallinum*. Z. Pflanzenphysiol. 67, 166–170.

Winter, K., Garcia, M., Holtum, J.A.M., 2008. On the nature of facultative and constitutive CAM: environmental and developmental control of CAM expression during early growth of *Clusia*, *Kalanchoë*, and *Opuntia*. J. Exp. Bot. 59, 1829–1840.

Zotz, G., Winter, K., 1993. Short-term regulation of Crassulacean acid metabolism activity in a tropical hemiepiphyte, *Clusia uvitana*. Plant Physiol. 102 (3), 835–841.