Are the metabolic components of crassulacean acid metabolism up-regulated in response to an increase in oxidative burden?

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Abstract

In the halophytic species Mesembryanthemum crystallinum, crassulacean acid metabolism (CAM) may be induced by a range of abiotic factors including drought, salinity, high light intensity, low temperature, and anoxia. A key biotic consequence of all these environmental changes is the generation of reactive oxygen species in planta that can elicit potentially damaging oxidative reactions and/or act as signals for engaging mechanisms that alleviate oxidative stress. However, induction of CAM per se also has the potential for increasing the oxidative burden via the enhanced internal O₂ concentrations that develop behind closed stomata during daytime decarboxylation. The aim of this paper was to test two hypotheses. The first one, that reactive oxygen species are key signals for up-regulating the major genes and proteins required for the operation of CAM as part of an integrated strategy for alleviating oxidative burden, was tested using gaseous ozone to increase the oxidative burden at a cellular level. The second hypothesis, that CAM potentially increases oxidative load, was tested using a CAM-deficient mutant of M. crystallinum. The data indicate that ozone, like salinity, elicits an increase in the transcript and protein abundance of myo-inositol o-methyl transferase (a key enzyme of cyclitol synthesis), together with phosphoenolpyruvate carboxylase and other ‘CAM-related’ enzymes. However, ozone, unlike salinity, does not induce functional CAM, implying that the various metabolic components required for CAM respond to different signals. Comparing the activities of different subcellular isoforms of superoxide dismutase in wild-type and CAM-deficient mutants of M. crystallinum suggests that the induction of CAM potentially curtails the oxidative load in planta.

Key words: CAM, cyclitols, Mesembryanthemum crystallinum, reactive oxygen species, starch degradation.

Introduction

The photosynthetic specialization of crassulacean acid metabolism (CAM) encompasses an integrated suite of metabolic traits that conserve CO₂ and/or water in environments that typically restrict the availability of either or both resources. In CAM plants, CO₂ is taken up at night via the enzyme phosphoenolpyruvate carboxylase (PEPC) which is activated via phosphorylation by a dedicated protein kinase (Ppck) that is under circadian control (Nimmo, 2000). The 3-C substrate phosphoenolpyruvate carboxylase (PEP) is produced via the glycolytic breakdown of carbohydrate at night. The organic acids produced via the dark decarboxylation of PEP is produced via the glycolytic breakdown of carbohydrate at night. The organic acids produced via the dark decarboxylation of PEP are stored overnight in a large central vacuole and are subsequently decarboxylated during the day to release CO₂ for fixation by Rubisco behind closed stomata. Phenotypic plasticity in the expression of CAM is manifest as shifts in the amount and proportion of CO₂ taken up over the night and day, which optimizes carbon gain in response to intermittent or longer term...
(seasonal) changes in the environment (Dodd et al., 2002). The high number of independent origins of CAM in over 30 taxonomically diverse plant families implies that the evolution of this water conservation strategy can occur with relative ease and/or is the product of strong selection pressure in certain environments (Raven and Spicer, 1996). The convergent evolution of CAM raises important questions on the extent to which general stress-responsive aspects of C3 metabolism have been recruited as part of this photosynthetic adaptation to water/CO2-limited environments. Moreover, the multiple origins of CAM in extreme environments suggest the possibility that a signal common to all exceptional environmental conditions may be responsible for engaging the CAM phenotype.

The facultative CAM plant *Mesembryanthemum crystallinum* has been the focus of a number of studies investigating the enzymatic (Winter et al., 1982), molecular genetic (Kore-eda et al., 2004), and signalling processes (Taybi and Cushman, 1999) that underpin the induction of CAM. *Ppcl*, the gene that encodes a CAM-specific isoform of PEPC (Cushman et al., 1989), and *Ppck*, the dedicated regulatory kinase, are co-ordinately up-regulated (Taybi et al., 2000), together with genes and proteins associated with decarboxylation, vacuolar transport, chloroplast transport, glycolysis, and starch degradation as CAM is induced (Winter et al., 1982; Haußler et al., 2000), and transcripts of many of these genes show circadian fluctuations in abundance (Dodd et al., 2003; Kore-eda et al., 2004). The signals that result in the convergence of these metabolic components to produce the CAM phenotype in *M. crystallinum* are generated by a range of abiotic factors including salinity, drought, high light intensity, low temperature, and anoxia (Taybi et al., 2002). A key biotic consequence of abiotic stress is the generation of reactive oxygen species (ROS) in planta which can elicit a potentially damaging oxidative burden on cellular constituents and/or act as signals for engaging mechanisms that ameliorate oxidative stress (Foyer and Noctor, 2005). It has been proposed that CAM prevents the production of ROS because the daytime CO2-concentrating effect prevents over-energization of the photosynthetic machinery under water-limited conditions (Griffiths, 1989). Indeed this anti-oxidant and photo-protective role of CAM has been proposed as a major driver for the evolution of the pathway in water-limited environments (Gil, 1986). Such assertions imply a key role for ROS in signalling an up-regulation of the metabolic elements required for CAM.

In addition to CAM, *M. crystallinum* may employ other strategies for alleviating the oxidative burden imposed by abiotic stressors. Cyclitols are known to accumulate to high levels in *M. crystallinum* in response to salinity and have been proposed to act not only as compatible solutes for maintaining osmotic balance, but also to curtail oxidative damage in saline conditions by scavenging ROS (Bohnert et al., 1995). Myo-inositol O-methyl transferase (IMT) is a key enzyme in the cyclitol biosynthetic pathway and the corresponding gene, *Imt*, is transcriptionally induced in response to salinity in *M. crystallinum* (Vernon et al., 1993). Thus, cyclitol accumulation and CAM could represent an integrated strategy for alleviating the oxidative burden imposed by salinity and other abiotic stressors. Other important components of anti-oxidant defence include the various isoforms of superoxide dismutase (SOD) located in different subcellular compartments and which catalyse the disproportionation of superoxide radicals to H2O2 and O2 (Bowler et al., 1994). The induction of CAM in *M. crystallinum* by salinity is accompanied by increased activity of three isoforms of superoxide dismutase, Mn-SOD, Fe-SOD, and Cu/Zn SOD, which are found in the mitochondria, chloroplasts, and cytosol, respectively (Miszalski et al., 1998). However, it is not clear if SOD activity is up-regulated as a direct response to oxidative load imposed by salinity or as a consequence of the increased oxidative burden generated by CAM (Osmond et al., 1999; Lütßge, 2002). In CAM plants, during the day, sustained electron transport behind closed stomata can elevate internal O2 concentrations to around 42% (Spalding et al., 1979), thereby increasing the potential for oxidative stress. Thus, in assessing the role of oxidative burden as a signal for eliciting acclimatory responses in *M. crystallinum*, there is a need to dissect the anti-oxidant requirements imposed by abiotic stress from those that are potentially a consequence of the biotic signals generated by CAM.

The aim of the present work was to test two hypotheses. The first hypothesis that ROS are key signals for up-regulating the major genes and proteins required for cyclitol accumulation and the operation of CAM in *M. crystallinum* was tested using gaseous ozone to increase the oxidative burden at a cellular level (Barnes et al., 1999). Acclimatory responses to salinity were compared with those in plants exposed to an environmentally relevant concentration of ozone (80 ppb for 8 h d−1) over a 15 d period. The efficacy of ozone and salinity as stressors for up-regulating cyclitol accumulation and the various metabolic components of CAM were determined via measurements of key metabolites together with transcript and protein abundance of selected enzymes.

The second hypothesis was that CAM per se increases the oxidative burden in planta. This was tested by using a CAM-deficient mutant of *M. crystallinum*, identified by a deficiency in the leaves of (i) nocturnal acid accumulation, (ii) nocturnal starch degradation, and (iii) net dark CO2 uptake after treatment with 300 mM NaCl for 2 weeks (Branco et al., 2003). Since ROS formation is extremely difficult to measure in plant tissues, induction of the anti-oxidant system is often used as a marker for ROS production in *planta*. The activities of three subcellular isoforms of SOD were compared in both wild type and mutant after exposure to salt. The rationale was that if CAM
enhances oxidative burden, the activity of one or all of the sub-cellular isoforms of SOD would be higher in CAM-performing plants. By contrast, if CAM alleviates oxidative load in salted plants, then SOD activities should be higher in the salted mutant. Overall, the data indicate that whilst ozone does not induce functional CAM, different metabolic components required for the operation of CAM do appear to respond to ozone-enhanced oxidative burden. The possibility is discussed that key metabolic elements required for CAM may have evolved as part of a general strategy for meeting increased respiratory costs and alleviating potential damage generated by increased oxidative load under water/CO$_2$-limited conditions.

**Materials and methods**

**Plant growth and imposition of salinity and ozone treatments**

Seeds of *Mesembryanthemum crystallinum* L. were germinated on soil and 7-10-d-old seedlings were transplanted to soil plugs containing John Innes Little Gem potting compost for a further 7 d to ensure uniformity of growth. Plants were subsequently potted onto 12-cm-diameter pots for continued growth in the same growing medium which was supplemented each week by application of nutrient solution (Phostrogen all-purpose soluble plant food; Phostrogen, Clwyd, UK) diluted according to the manufacturer’s instructions. The plants were grown in a purpose-built growth chamber at a photosynthetic photon flux density (PPFD) at leaf height of 300 mmol photons m$^{-2}$ s$^{-1}$ supplied by fluorescent tube lighting, with a 12 h photoperiod. The chamber temperature was 23/17°C day/night; relative humidity ranged from 55±10% (day) to 70±5% (night).

Five-week-old plants, in which the first four pairs of primary leaves had emerged, were subsequently transferred into controlled environment chambers. Charcoal/Parafilm$^\text{TM}$-filtered air (CFA) was supplied to each of the chambers via an air-conditioning system (Air Supply Ltd, Swindon, UK) under computer control. The concentration of ozone—well-watered plants were exposed to gaseous ozone for up to 70 mol m$^{-2}$ s$^{-1}$ at plant height of 300 mmol photons m$^{-2}$ s$^{-1}$ supplied by fluorescent tube lighting, with a 12 h photoperiod. The chamber temperature was 23/17°C day/night; relative humidity ranged from 55±10% (day) to 70±5% (night).

For the comparison of wild-type and CAM-deficient mutants, seeds were germinated and plants potted on as described above. Plants were grown at a PPFD at leaf height of 500 mmol photons m$^{-2}$ s$^{-1}$ supplied by fluorescent tube lighting, with a 12 h photoperiod. Chamber temperature was 23/17°C day/night; relative humidity ranged from 55±10% (day) to 70±5% (night). After reaching the 5 week growth stage (primary leaf 4 emerged) plants were maintained under well-watered conditions or watered with 300 mol m$^{-3}$ NaCl for 14 d before sampling.

**Metabolite analyses**

For the extraction of leaf metabolites, four leaves (primary leaf 4) were sampled at the end of the photoperiod, powdered in liquid N$_2$, and stored at −80°C for subsequent analysis. Soluble metabolites (including titratable acids and cyclitol) were extracted from 2.5 cm$^2$ frozen and powdered leaf discs into 80% methanol according to the method described by Christopher and Holtum (1996). The remaining insoluble pellets were subjected to enzymatic hydrolysis via amylase and amyloglucosidase, to liberate glucose equivalents from starch (Borland, 1996), which were subsequently analysed using the colorimetric phenol-sulphuric acid test of Dubois et al. (1956).

The expression of CAM was determined as the difference in titratable acid content in leaves sampled at the start and end of the dark period. Methanol extracts were titrated against 1 mol m$^{-3}$ NaOH to a neutral endpoint, as indicated by phenolphthalein.

Leaf cyclitol contents were determined via HPLC using pulsed amperometric detection (Dionex, Cambridge UK) as described by Adams et al. (1992). The methanol extracts were dried down, taken up in nanopure water then desalted by passage through ion exchange columns in series (Dowex AG50W and Amberlite IRA; Sigma, UK). Desalted extracts were separated on a Carbopac PA-100 column (Dionex, UK) via isocratic elution using 150 mol m$^{-3}$ NaOH at a flow rate of 1 cm$^3$ min$^{-1}$.

**Abundance of IMT and PEPC proteins**

Leaves (primary leaf 4) were sampled at the end of the photoperiod, powdered in liquid N$_2$, and stored at −80°C. Approximately 150 mg of powdered tissue was mixed with 250 μl of a buffer containing 100 mol m$^{-3}$ TRIS, pH 8.3 at 4°C, 10 mol m$^{-3}$ NaCl, 5 mol m$^{-3}$ ethylenediamine tetraacetic acid (EDTA), 10 mol m$^{-3}$ dithiothreitol (DTT) plus 4 μg leupeptin, 4 μg E-64, 2 mol m$^{-3}$ phenylmethylsulphonyl fluoride (PMSF), and 20 μl of a plant protease inhibitor cocktail (all protease inhibitors from Sigma). The extract was centrifuged at 4°C for 10 min at 12 000 g and the supernatant was mixed thoroughly with glycerol (10% final volume). Protein contents were determined as described by Bradford (1976). Exactly 15 μg protein from each treatment was resolved on 12% polyacrylamide gels, blotted onto polyvinylidene fluoride membranes (Sigma) and anti-PEPC or anti-IMT polyclonal antibodies used to identify respective protein bands. Detection was achieved using the enhanced chemiluminescence system (Amersham, Bucks, UK) following the manufacturer’s instructions.

**Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was purified from 250 mg of powdered leaf tissue bulked from leaves of four individual plants using Tri-Reagent (Helena Biosciences, UK) as described by Taybi and Cushman (1999). Exactly 5 μl of RNA extract was treated with DNase I (Life Technologies, Paisley, UK), to prevent amplification of genomic DNA. *Fnr1* (ferredoxin NADP$^+$ reductase, GenBank accession M22528) was used as a positive PCR control since expression levels of this gene remain constant in leaves from well-watered and salted plants of *M. crystallinum* (Taybi and Cushman, 1999; Dodd et al., 2003). Primers for *Fnr1* and the other genes of interest are given in Table 1. RT-PCR was conducted as a single-tube reaction and cDNA synthesis was promoted using the reverse primer. Each 25 μl reaction contained 10× PCR reaction buffer, 10 mol m$^{-3}$ DTT, 2.5 mol m$^{-3}$ MgCl$_2$, 0.25 mol m$^{-3}$ dNTPs (Biolite Scientific, London, UK), 400 nM each of forward and reverse primers, 12 U RNase Out (Life
Table 1. Primers used for reverse transcription and amplification of selected genes by PCR in Mesembryanthemum crystallinum

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’ . . . 3’</th>
<th>Reverse primer 5’ . . . 3’</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fnr (ferredoxin NADPH reductase)</td>
<td>ATTGGCAGAGGCCCTTG</td>
<td>GAAACCAGTAATCACATCT</td>
<td>M22528</td>
</tr>
<tr>
<td>Ppc1 (phosphoenol-pyruvate carboxylase)</td>
<td>CACTTAAACATGCTGTTGAG</td>
<td>TAGCTTCACAGCTCACG</td>
<td>X13660</td>
</tr>
<tr>
<td>Int (inositol methyl transferase)</td>
<td>TTGAGTCGATGCTCCCT</td>
<td>ACCGAAAACTGATCCATTG</td>
<td>M87340</td>
</tr>
<tr>
<td>Sfp (chloroplastic starch phosphorylase)</td>
<td>CAGGACTGAGGAGAGAG</td>
<td>ACCTGTTAACCAAGGAGT</td>
<td>AF158091</td>
</tr>
<tr>
<td>bamy (β-amylase)</td>
<td>GAAATGACGCTCAGGAG</td>
<td>TTTGCTCGGATATCTG</td>
<td>A026304</td>
</tr>
<tr>
<td>bnxy (b-amylose)</td>
<td>TCTTGACGGTTATGAGG</td>
<td>CATTGTTGTGGTTGCTTC</td>
<td>AA962939</td>
</tr>
</tbody>
</table>

Technologies, UK), 0.5 U Taq DNA polymerase (Bioline Scientific), 30 U Superscript II reverse transcriptase (Life Technologies), and 100 ng total RNA. RNA input amount and cycle number were individually optimized for each gene, which ensured a linear relationship between total RNA input and product quantity when separated on a 1.2 % (w/v) agarose gel. The amplification of each cDNA was perfectly specific.

Starch-degrading enzymes

The activities of amylase and starch phosphorylase isozymes were measured using protein samples separated via native polyacrylamide gel electrophoresis (PAGE), as modified from the method of Zeeman et al. (1998). Protein extracts were prepared by homogenizing 250 mg frozen, powdered leaf tissue in 300 mol m⁻³ HEPES (hydroxymethyl piperazine-1-ethanesulfonic acid) pH 7.4, 20 mol m⁻³ MgCl₂, 1 mol m⁻³ EDTA, 2 mol m⁻³ DTT, 4 mol m⁻³ benzamidine, 1 mol m⁻³ PMSF, and 0.1% Triton X-100. After centrifugation for 10 min at 4 °C and 12 000 g, the supernatant was brought to 45% (v/v) glycerol. Protein contents were determined via the Bradford (1976) method. Exactly 15 μg of proteins from each treatment was loaded onto discontinuous acrylamide gels (4% stacking, 7.5% resolving) that contained either 0.1% potato amylopectin degradation were visualized by staining with a tetrazolium, according to Beauchamps and Fridovich (1971). After incubation, the gels were illuminated on a light box until the SOD activity bands became visible. The relative activities of the different isoforms in watered (control) and salted plants were assessed via scanning laser densitometry. SOD activities in salted plants (four replicates) were expressed as a percentage increase over those in control plants (four replicates).

Results

Impacts of ozone and salinity on cyclitol accumulation

Exposure of M. crystallinum to 80 ppb O₃ for 8 h d⁻¹ elicited an increase in the transcript abundance of inositol methyl transferase (Int) after 4 d that was similar to the response noted in salted plants (Fig. 1a). Uniform transcript abundance of constitutively expressed Fnr demonstrates that the total RNA input was similar for all reactions (Fig. 1b). An increase in the amount of IMT protein was first observed in salted plants after 4 d, but by days 8–15, IMT abundance was similar in salted and ozone-treated (80 ppb) plants (Fig. 1c). Well-watered control plants showed no change in transcript abundance of Int or protein content of IMT over the course of the experiment (data not shown) and cyclitols did not accumulate in control plants over the 15 d time-course (Fig. 2). In plants exposed to salt and ozone, the time-courses of changes in IMT abundance were paralleled by changes in the content of leaf cyclitols (Fig. 2). After 15 d, cyclitol contents were similar in leaves from both the salt and 80 ppb ozone treatments (Fig. 2). There was a clear dose–response relationship between leaf cyclitol content and O₃ exposure with cyclitol content of plants exposed to 80 ppb ozone, almost double that of plants exposed to 40 ppb ozone (Fig. 2).

Impacts of ozone and salinity on PEPC and CAM expression

After 4 d of exposure to ozone, an increase in transcript abundance of Ppc1, the CAM-associated isoform of PEPC, was apparent and was comparable with that observed in plants exposed to 500 mM NaCl for 4 d (Fig. 3a; plants were sampled at the end of the photoperiod). Uniform transcript abundance of constitutively expressed Fnr demonstrates that the total RNA input was similar for all reactions (Fig. 3b). The increase in abundance of PEPC protein in plants exposed to ozone was somewhat delayed as compared with plants treated with NaCl (Fig. 3c). However, after 15 d, levels of Ppc1 transcripts and PEPC...
protein were similar in plants exposed to both treatments. Well-watered control plants showed no change in the abundance of Ppc1 transcripts or PEPC protein over the course of the experiment (data not shown) and CAM was not induced, as evidenced by the lack of overnight acid accumulation in control plants (Fig. 4). Ozone-treated plants, despite the increase in PEPC protein, did not express CAM, as indicated by the lack of an overnight accumulation of acidity (Fig. 4). In salted plants, CAM expression was noted after 4 d and increased further with extended salinity.

In order to examine further which metabolic components might be limiting functional expression of the CAM pathway under ozone, the levels of starch (a potentially limiting substrate for PEPC-mediated CO2 uptake) were compared in leaves from control, salted, and ozone-treated plants, sampled at the end of the photoperiod (Fig. 5a). Salinity resulted in a substantial reduction in leaf starch content and after 8–15 d, starch content declined to <50% of that in the control plants. By contrast, in plants exposed to ozone, leaf starch content was substantially increased compared with controls. After 8–15 d in ozone, the starch content had increased by >100% compared with that in control plants (Fig. 5a).

To establish if the differences in starch mobilization between salt and ozone-treated plants could be attributed to the activity of any starch-degrading enzymes, native
Page was used to separate various isoforms of hydrolytic enzymes (Fig. 5b) and chloroplastic and cytosolic isoforms of starch phosphorylase (Fig. 5c). Both salt and ozone resulted in the induction of several amylase isoforms after 4 d (Fig. 5b). The induced isoforms persisted over the 15 d salt treatment, but declined in activity with extended exposure in ozone. Salt and ozone also stimulated the activity of chloroplastic starch phosphorylase, but the effect was more marked in the salted plants (Fig. 5c).

Day/night patterns of transcript abundance in plants exposed to salinity or ozone

In CAM-performing plants, dark CO2 uptake is regulated via the phosphorylation of PEPC by a dedicated PEPC kinase (Ppck) which is usually more highly expressed at night. To examine the possibility that the lack of functional CAM in ozone-treated plants was due to a disruption in Ppck expression, the day/night patterns of transcript abundance of both Ppc1 and Ppck were compared in plants treated with 500 mol m\(^{-3}\) NaCl or 80 ppb ozone for 8 d. Confirming the results shown in Fig. 3a, both salt and ozone elicited an increase in transcript abundance of Ppc1 compared with controls. Day/night patterns of transcript abundance were similar in plants exposed to the two treatments (Fig. 6a), although during the dark period transcript abundance of Ppc1 was lower in the ozone-treated plants compared with that in salted plants (Fig. 6a). Ozone elicited an increase in the abundance of Ppck transcripts compared with controls (Fig. 6b), and transcript abundance for this gene peaked at a similar level towards the end of the dark period in plants exposed to salt or ozone. However, Ppck transcripts appeared earlier in the photoperiod in salted plants and persisted for longer into the photoperiod, as compared with ozone-treated plants (Fig. 6b). Uniform transcript abundance of constitutively expressed Fnr across the light/dark cycle demonstrates that the total RNA input was similar for all reactions (Fig. 6c).

To establish if plants exposed to ozone showed altered day/night patterns of expression of genes implicated in starch degradation, transcript abundance of the genes encoding b-amylase and chloroplastic starch (a-glucan) phosphorylase were examined (Fig. 7). Transcript abundance of b-amylase was strongly up-regulated by ozone and salt, but in ozone-treated plants, transcripts were up-regulated earlier in the photoperiod (Fig. 7a). By contrast, transcripts encoding chloroplastic starch phosphorylase were more strongly up-regulated and earlier in the photoperiod by salt compared with ozone (Fig. 7b).

Comparing potential oxidative load in wild-type and CAM-deficient mutants

Exposure to 300 mol m\(^{-3}\) NaCl for 14 d induced substantially more CAM activity in wild-type plants compared with the mutant as indicated by measurements of leaf
titratable acidity at the start (07.00 h) and end (19.00 h) of the photoperiod (Fig. 8a). The salted wild-type plants accumulated >3-fold higher levels of acidity overnight compared with the salted mutants. Previous investigations have shown that the mutants have barely detectable levels of leaf starch (Branco et al., 2003). Feeding detached leaves of the mutant with 300 mM glucose restores nocturnal acid accumulation (T Taybi, AM Borland, unpublished observation), suggesting that the CAM deficiency is due to substrate limitation of dark CO₂ uptake. In order to determine if the magnitude of CAM expression influenced oxidative load in salted plants, the activities of different subcellular isoforms of SOD were compared in the wild-type and CAM-deficient mutants after 14 d of the salinity treatment (Fig. 8b). Of the three SOD isoforms, mitochondrial MnSOD showed the greatest increase in activity in response to salinity. However, the salinity-induced increase in activity of all three SOD isoforms was most marked in the CAM-deficient mutant, indicating potentially higher oxidative load compared with the wild-type.

Fig. 6. The influence of salinity (500 mol m⁻³ NaCl) and ozone (80 ppb for 8 h d⁻¹) administered for 8 d on day/night changes in transcript abundance of (a) Ppc1, the CAM-specific isoform of PEPC, (b) Ppck (PEPC kinase), and (c) Fnr (ferredoxin NADP-reductase: PCR control). Solid bars at the top of each composite indicate the period of darkness.

Fig. 7. The influence of salinity (500 mol m⁻³ NaCl) and ozone (80 ppb for 8 h d⁻¹) administered for 8 d on day/night changes in transcript abundance of (a) β-amylase and (b) chloroplastic starch phosphorylase. Solid bars at the top of each composite indicate the period of darkness.

Fig. 8. (a) Leaf titratable acidities measured at the start (07.00 h) and end (19.00 h) of the photoperiod in leaves sampled from well-watered (control) or salted (300 mM NaCl for 14 d) wild-type or CAM-deficient mutant plants. Each bar is the mean of four replicates ± standard error. (b, c) Native page activity gels of different subcellular isoforms of superoxide dismutase (SOD) with two representative replicates (of four) are shown from leaves of wild-type (b) and CAM-deficient mutants (c) either well-watered (H₂O) or subjected to 300 mol m⁻³ NaCl for 14 d. Extracts were prepared from leaves sampled in the middle of the photoperiod. Numbers in brackets are the percentage increase ± standard error of the mean in activity of isoforms from salted plants relative to the watered controls.
Discussion

Metabolic responses to increased oxidative burden

In Mesembryanthemum crystallinum, ozone, in common with salinity, elicited an increase in both transcript (Imt) and protein (IMT) abundance of myo-inositol o-methyl transferase, a key enzyme of cyclitol synthesis. The exposure–response relationship between the administered concentration of ozone and cyclitol content implies a functional response of cyclitol metabolism to an increase in oxidative burden. This observation supports previous suggestions that cyclitols could serve as scavengers of ROS (Orthen et al., 1994).

By contrast, and consistent with other findings (Niewiadomska et al., 2002; Hurst et al., 2004), ozone failed to induce functional CAM expression. However, whilst ozone did not induce net dark CO₂ uptake, as shown by the lack of nocturnal acidification, the present data indicate that some metabolic components associated with dark CO₂ uptake (Ppc1, and Ppck transcripts and PEPC protein) were up-regulated in response to this gaseous oxidant. Previously, Niewiadomska et al. (2002) reported that ozone stimulated the activity of decarboxylating enzymes (NAD and NADP-malic enzymes) and fumarase in M. crystallinum. In the absence of CAM expression, such ozone-induced metabolic responses indicate an enhanced capacity for providing reducing power necessary for detoxification, as well as energy and carbon skeletons required for repair processes (Sehmer et al., 1998; Casati et al., 1999). Ozone is known to increase plant maintenance respiration and this has been attributed to the ‘costs’ associated with detoxification of the pollutant and the repair of damaged membranes and proteins (Amthor, 1988). Given that PEPC is the major anapleurotic enzyme in higher plant tissues that replenishes metabolites within the tricarboxylic acid (TCA) cycle, the up-regulation of PEPC abundance by ozone (and indeed salinity) could represent a core component of an energy-generating and repair strategy for plants exposed to abiotic stressors that increase the cellular oxidative burden. The recapture of respiratory CO₂ by PEPC has been proposed as an initial step in the evolution of CAM from C₃ photosynthesis (Griffiths, 1989). By analogy to the ozone-induced shifts in metabolism described above, it is conceivable that key metabolic components required for CAM may have evolved as part of a general strategy for meeting increased respiratory costs and curtailing damage potentially generated by the increased oxidative load that can develop under water/CO₂-limited conditions.

Ozone elicited an increase in the transcript abundance and activity of several starch-degrading enzymes which have also been implicated as important metabolic components for the operation of CAM in M. crystallinum. As the major source of C skeletons required for the nocturnal production of PEP, starch is a key limiting factor for the magnitude of CAM expression in M. crystallinum (Dodd et al., 2002). Salinity is known to elicit substantial increases in a range of enzymes implicated in starch degradation in M. crystallinum and, together with the reported increase in enzymes of glycolysis (Winter et al., 1982), this has been proposed to satisfy the demand for substrate for PEPC-mediated dark CO₂ uptake as CAM is induced (Paul et al., 1993). However, the salinity-induced increase in activity of several starch-degrading enzymes has been shown to precede the induction of CAM by several days (Paul et al., 1993). The present results indicate that salinity elicits a substantial mobilization of leaf starch reserves in M. crystallinum so, in addition to a CAM-specific role, it appears that starch degradation has a more general role in terms of salinity acclimation by providing substrates for respiratory repair and for the synthesis of compounds responsible for osmo-regulation. By contrast to salinity, ozone did not elicit appreciable mobilization of leaf starch and this could be a key factor that limited CAM expression in ozonated plants. However, despite the elevated levels of starch relative to control plants, exposure to ozone did result in an increase in transcript abundance and activity of some starch-degrading enzymes, including chloroplastic α-glucan (starch) phosphorylase. Chloroplastic starch phosphorylase has been proposed to play an important role in stress tolerance in Arabidopsis by providing hexose phosphates as substrates for the oxidative pentose phosphate pathway (Zeeman et al., 2004). The oxidative pentose phosphate pathway generates reducing power for a range of biosynthetic reactions and also plays an important role in controlling the levels of ROS (Scheibe, 2004). An increase in the activity of chloroplastic starch phosphorylase in Arabidopsis thaliana after exposure to 100 ppb ozone for 7 d has been observed recently (C Callaghan, J Barnes, A Borland, unpublished observation) implying a link between the activity of this enzyme and increased oxidative burden. Thus, the increase in transcript abundance and activity of chloroplastic starch phosphorylase in M. crystallinum that was elicited by both salinity and ozone is in keeping with the general hypothesis that key metabolic elements associated with the operation of CAM may have evolved as part of a general strategy for meeting increased respiratory costs and curtailing damage potentially generated by oxidative stress.

CAM curtails oxidative burden in planta

In order to establish if CAM potentially promotes or curtails the oxidative burden in planta, the activities of three isoforms of SOD were compared as markers for ROS production in different subcellular compartments in wild-type and CAM-deficient mutants of M. crystallinum. The data indicated that the higher CAM expression in wild-type plants after 2 weeks of salinity was accompanied by a potentially lower oxidative load in chloroplasts, mitochondria,
and cytosol compared with that detected in the CAM-deficient mutants. Such observations support previous assertions that the daytime regeneration of CO$_2$ from malate decarboxylation curtails over-reduction of the electron transport chain under water-limited or saline conditions (Adams and Osmond, 1988; Griffiths, 1989). The SOD activities described in the present work reflect a relatively long-term (2 weeks) adjustment of anti-oxidant responses to salinity, and comparing SOD activities in wild-type and mutant after only 7 d of salinity failed to show a clear influence of CAM on oxidative load (data not shown). Previously it was shown in *M. crystallinum* that increased chloroplastic Fe-SOD activity was a fast (4 d) and somewhat transitory response to salinity, whilst an increase in cytosolic CuZn-SOD and mitochondrial Mn-SOD activities paralleled CAM expression after 9–12 d salinity (Miszalski *et al.*, 1998). In the present work, mitochondrial Mn-SOD showed the greatest salt-induced increase in activity out of the three SOD isoforms in both wild type and CAM-deficient mutant. Increased Mn-SOD activity is usually found in tissues with elevated respiratory activity and may indicate a high reduction state of the mitochondrial electron transport chain (Van Camp *et al.* 1996). That salinity *per se* increases respiratory burden in *M. crystallinum* is consistent with the notion discussed above that the increased expression of certain genes associated with CAM may constitute an energy-generating and repair strategy for plants growing in water/CO$_2$-limited environments. Experiments are currently in progress to establish how wild-type CAM-performing *M. crystallinum* curtail the production of ROS in the mitochondrial electron transport chain as compared with the CAM-deficient mutant.

In conclusion, the present findings lend credence to the idea of CAM as a syndrome of various metabolic components that can respond to diverse abiotic and biotic signals yet ultimately converge to produce the CAM phenotype in water- and/or CO$_2$-limited environments. Key metabolic elements required for CAM may have evolved as part of a general strategy for meeting increased respiratory costs and alleviating potential damage imposed by the increased oxidative burden that accompanies abiotic stress. Moreover, CAM appears to curtail the oxidative burden in plants exposed to extended periods of salinity. Further comparative studies of wild-type and CAM-deficient mutants of *M. crystallinum* will elucidate the various anti-oxidant strategies that are engaged as essential components for the operation of this photosynthetic syndrome in a changing environment.

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