

RESEARCH PAPER

A CAM- and starch-deficient mutant of the facultative CAM species *Mesembryanthemum crystallinum* reconciles sink demands by repartitioning carbon during acclimation to salinity

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Abstract

In the halophytic species *Mesembryanthemum crystallinum*, the induction of crassulacean acid metabolism (CAM) by salinity requires a substantial investment of resources in storage carbohydrates to provide substrate for nocturnal CO₂ uptake. Acclimation to salinity also requires the synthesis and accumulation of cyclitols as compatible solutes, maintenance of root respiration, and nitrate assimilation. This study assessed the hierarchy and coordination of sinks for carbohydrate in leaves and roots during acclimation to salinity in *M. crystallinum*. By comparing wild type and a CAM-/starch-deficient mutant of this species, it was sought to determine if other metabolic sinks could compensate for a curtailment in CAM and enable acclimation to salinity. Under salinity, CAM deficiency reduced 24 h photosynthetic carbon gain by >50%. Cyclitols were accumulated to comparable levels in leaves and roots of both the wild type and mutant, but represented only 5% of 24 h carbon balance. Dark respiration of leaves and roots was a stronger sink for carbohydrate in the mutant compared with the wild type and implied higher maintenance costs for the metabolic processes underpinning acclimation to salinity when CAM was curtailed. CAM required the nocturnal mobilization of >70% of primary carbohydrate in the wild type and >85% of carbohydrate in the mutant. The substantial allocation of carbohydrate to CAM limited the export of sugars to roots, and the root:shoot ratio declined under salinity. The data suggest a key role for the vacuole in regulating the supply and demand for carbohydrate over the day/night cycle in the starch-/CAM-deficient mutant.

Key words: Carbon partitioning, Crassulacean acid metabolism, cyclitols, salinity, starch.

Introduction

Mesembryanthemum crystallinum (L.), the common ice plant, is an annual halophyte, native to the Namibian desert in Southern Africa, that has been introduced around the arid coastal regions of the Mediterranean, the Red Sea and Arabian Gulf, the western USA and Australia, Mexico, Chile, and the Caribbean (Bohnert and Cushman, 2000). The photosynthetic specialization of crassulacean acid metabolism (CAM) is known to be induced in *M. crystallinum* in response to salinity and water deficits (Winter and Holtum, 2007). By enabling partial or predominant uptake of CO₂ at

night and minimizing water loss, CAM appears to be critical to the ecological success of *M. crystallinum* by ensuring seed set under conditions of drought and in saline soils (Winter and Zeigler, 1992). CAM requires a substantial investment of resources in storage carbohydrates to conduct nocturnal CO₂ uptake, which inevitably curtails carbohydrate partitioning to other metabolic activities, including dark respiration, nitrogen assimilation, and growth (Borland and Dodd, 2002). Understanding how *M. crystallinum* prioritizes allocation of carbohydrates to competing sinks under changing environmental

conditions will help to highlight the metabolic traits that underpin the competitive ability of CAM species as well as their potential for sustainable productivity on marginal lands (Borland *et al.*, 2009, 2011).

The nocturnal uptake of CO₂ in CAM plants is sustained by degradation of carbohydrate reserves to produce phosphoenolpyruvate (PEP) as substrate for PEP carboxylase (PEPC), and up to 20% of leaf biomass can be allocated to carbohydrates for this purpose. A CAM-deficient phenotype in mutants of *M. crystallinum* was linked to a deficiency in leaf starch and plastidic phosphoglucomutase (PGM), thereby indicating the role of starch in providing substrate for nocturnal carboxylation in this species (Cushman *et al.*, 2008). Previous studies have illustrated that during acclimation to salinity in *M. crystallinum*, the carbon allocated to starch is maintained at the expense of the soluble sugar pool (Borland and Dodd, 2002), which might imply that other sinks for carbon have a lower priority than CAM. Cyclitols (e.g. myo-inositol, ononitol, and pinitol) are known to accumulate in leaves and roots of salt-treated *M. crystallinum* where they act as osmolytes, facilitating retention of water in the cytoplasm, allowing sodium sequestration to the vacuole or apoplast, and protecting against oxidative stress (Vernon and Bohnert, 1992; Bohnert *et al.*, 1995; Adams *et al.*, 1998; Borland *et al.*, 2006). Cyclitols are synthesized from glucose-6-phosphate, so a reserve supply of carbohydrates might be required for continued sugar alcohol synthesis, particularly as net CO₂ assimilation declines in the face of increased salinity. Such considerations imply possible competition between CAM and cyclitol synthesis for carbohydrate during acclimation to salinity. The severe curtailment in CAM expression noted in starch-deficient mutants of *M. crystallinum* poses the question as to whether cyclitol synthesis is curtailed to a comparable extent in the mutants or whether cyclitols assume priority as a sink for carbon and ensure continued growth of the mutants under salinity without the water-conserving, carbon acquisition, and photoprotective properties of CAM (Borland *et al.*, 2006; Sunagawa *et al.*, 2010).

Continued growth of a halophyte in saline conditions also requires that a threshold rate of root respiration be maintained. Root respiration maintains root growth, together with the uptake of ions (including Na⁺ and Cl⁻) as well as essential nutrients. A reduction in carbohydrate supply to the root for an extended time can lead to a reduction in root growth and root respiration (Lambers, 1979; Williams and Farrar, 1990). If CAM becomes a major sink for carbohydrate in the shoot, this could place constraints on root respiration, and subsequently root growth. In general, plant shoot growth is usually more inhibited than root growth under salinity (Munns and Termaat, 1986; Flowers and Colmer, 2008). However, for many halophytes and for several CAM species, root:shoot ratios have been found to decrease during prolonged water and/or salt stress (Nobel and North, 1996; Shabala and MacKay, 2011), and a decrease in root biomass in response to salinity was also observed in *M. crystallinum* (Kholodova *et al.*, 2002). Such observations suggest that in CAM plants,

root respiration might be especially sensitive to salinity or water stress. Nitrogen assimilation is also an important sink for carbon, and changing sink demands, including the diversion of photoassimilates to CAM, might limit the rate of nitrate assimilation. These observations pose the question of whether or not root respiration and nitrogen assimilation might be maintained better under saline conditions if CAM were curtailed as a sink for carbohydrate.

The aim of the present study was to assess the hierarchy and coordination of sinks for carbohydrates in leaves and roots during acclimation to salinity in *M. crystallinum*. By comparing the wild type and a starch-/CAM-deficient mutant of *M. crystallinum*, the aim of the study was to determine: (i) if and how other metabolic sinks for carbon could enable acclimation to salinity, maintain growth, and compensate for a curtailment in CAM; and (ii) the metabolic traits that determine CAM activity under contrasting conditions of carbon supply and demand.

Materials and methods

Plant growth conditions

Sterilized seeds of both the wild type and a CAM-/starch-deficient mutant (line 351, deficient in chloroplastic PGM; Cushman *et al.*, 2008) were germinated on agar containing Murashige and Skoog (MS) salts (4.3 l⁻¹), Gamborg's vitamins (1 ml l⁻¹), and 3% sucrose. Seedlings were maintained in a growth room with a 12 h photoperiod, photon flux density (PFD) of 250 μmol photons m⁻² s⁻¹ at leaf height, and day/night temperature regime of 25 °C/17 °C. Relative humidity ranged from 55±10% during the day to 70±5% during the night.

Hydroponic culture and imposition of salinity

Some 7–10 d after germination, seedlings were transplanted to acid-washed sand and received half-strength Hoagland's nutrient solution for 3 weeks. The seedlings were then removed from sand, roots were washed, and seedlings were transferred to 300 ml pots containing half-strength Hoagland's solution. Plants were maintained without aeration for 1 week until vigorous root growth was observed (Adams *et al.*, 1998). The hydroponic nutrient solution was subsequently aerated throughout the duration of the experiment. Solutions were exchanged once weekly, and the hydroponic reservoirs were topped up with nutrient solution on a daily basis. When the plants were 7 weeks old, the imposition of salinity in hydroponic culture was achieved by replacing the solution with half-strength Hoagland's solution plus 300 mM NaCl.

Leaf gas exchange measurements

Net CO₂ exchange of primary leaf 5 (PL5, numbered from the base) was monitored over 24 h light/dark cycles using a Walz CMS-400 compact mini-cuvette system equipped with a BINOS-100 infra-red gas analyser (IRGA; H. Walz GmbH, Effeltrich, Germany). The sample leaf was clamped into a well-stirred gas exchange cuvette that tracked PFD and temperature conditions within the plant growth chamber. Relative humidity was adjusted to ~50% during the light period, and ~70% during the dark period. Data logging occurred at 15 min intervals, and the data collected for any given interval represented the mean value within that interval. After every 10 data collection periods, the instrument automatically redirected the reference gas stream simultaneously through reference and analytical IRGAs to enable compensation for small drifts in the zero balance between the two IRGAs. Gas

exchange parameters were calculated using the DIAGAS software package (H. Walz GmbH). Each 24 h gas exchange curve presented was representative of at least three independent runs on different plants.

Sampling of leaves and roots for metabolite extraction

Primary leaves (leaf number 5 from the base of the shoot) and whole roots were sampled from four individual plants of both the wild type and mutant at each time point [i.e. at the start (day 0) and after 4, 8, and 14 d of salinity treatment with 300 mM NaCl]. Control plants were also sampled after 14 d without salinity. The two opposite leaves were sampled alternatively at the start (dawn) and end (dusk) of the photoperiod, whereas the roots were sampled at the end of the photoperiod. The roots were blotted quickly with tissue paper and, along with leaf samples, were frozen in liquid nitrogen and kept at -80°C for further analysis. Metabolite extraction was conducted by placing 1.54 cm^2 of frozen leaf tissue or 115 mg of frozen root tissue in 5 ml of 80% methanol. The samples were heated at 70°C for 30–40 min, allowed to cool, and made up to the required volume of 5 ml with 80% methanol.

Determination of total soluble sugars and starch

Aliquots of the methanol extracts were assayed for total soluble sugars using the phenol–sulphuric acid colorimetric method of Dubois *et al.* (1956). The remaining insoluble plant material was rinsed several times in distilled water, ground in 0.1 M acetate buffer (pH 4.5), and boiled for 30 min. After cooling, extracts were each incubated with 5 U of amyloglucosidase and 0.5 U of α -amylase (Sigma-Aldrich) to digest starch at 40°C overnight. After centrifugation at $12\,000\text{ g}$ for 10 min, the supernatant was assayed for glucose equivalents as described previously (Dubois *et al.*, 1956).

Metabolite analyses

Methanol extracts were desalted on columns comprising 0.5 ml of Dowex AG50W X4-200 (anion exchanger) layered over 0.5 ml of Amberlite IRA-67 (cation exchanger). Desalted extracts were dried down, re-suspended in 1 ml of H_2O , and stored at -20°C . The amounts of sugar alcohols (cyclitols) and specific neutral sugars (sucrose, fructose, and glucose) in desalted extracts were analysed using high-pressure liquid chromatography. Sugars were separated on a CarboPac PA-100 column run isocratically using 150 mM NaOH at a flow rate of 1 ml min^{-1} and detected via pulsed amperometry (ED40 Electrochemical detector, Dionex). Statistically significant differences between the wild type and mutant at comparable stages of salinity were assessed using *t*-tests.

Sodium determination

The sodium content of leaves and roots was determined by flame photometry. Dried plant tissue was combusted overnight in a muffle furnace at 550°C . The ashed samples were placed into crucibles, moistened with a few drops of distilled water, and 2 ml of concentrated HCl was added. Samples were placed in a steam bath and allowed to dry. A further 2 ml of concentrated HCl was added and the samples were left in the bath for 1 h. Then, a further 10 ml of warm 25% HCl was added and the contents were transferred into 100 ml graduated flasks through filter paper (Whatman No. 1). The crucibles were washed several times with warm 1% HCl. Contents in the flasks were allowed to cool and were diluted to 100 ml. The sodium was then determined with the flame photometer by using Na standards in the range $0.8\text{--}4.0\text{ }\mu\text{g ml}^{-1}$.

Measurement of root respiration

Root respiration was measured polarographically using a Hansatech Clark type oxygen electrode (Williams and Farrar, 1990; Grantz *et al.*, 2003). Freshly excised roots were placed in the

electrode chamber containing an air-saturated nutrient solution of exactly the same composition as that in which the plants were grown. Root respiration was measured over 5 min and was expressed relative to the blotted fresh weight of root in the vessel. Temperature was maintained at 20°C via water circulation around the reaction vessel. The electrode was calibrated using air-saturated nutrient solution and an oxygen-free nutrient solution that was obtained by adding a few crystals of sodium hydrosulphite ($\text{Na}_2\text{S}_2\text{O}_4$; dithionite) to the reaction vessel.

Assay of nitrate reductase activity

The activity of nitrate reductase *in vivo* was measured according to Deane-Drummond (1982). Fresh plant material (leaf PL5 and root tissue) was sampled during the middle of the photoperiod. Approximately 0.75 g of sliced leaf tissue or 0.25 g of root tissue were placed in foil-covered vials with 10 ml of buffer (pH 8) that contained $50\text{ mol m}^{-3}\text{ KH}_2\text{PO}_4$, $100\text{ mol m}^{-3}\text{ KNO}_3$, and 1% (v/v) propanol. The buffer was bubbled with N_2 gas for 30 min before use to produce anaerobic conditions and prevent competition with oxygen for endogenously generated reductant during the assay. The plant material was vacuum infiltrated with buffer for 3 min. After removal of the time zero sample (1 ml), the vials were stoppered and incubated at 27°C for 30 min. A further 1 ml of sample was taken from the vial at the end of the incubation period. Each sample (1 ml) was mixed with 1 ml of sulphanilamide (10 g in 1.0 l of 3 M HCl) and 1 ml of $2\text{ mol m}^{-3}\text{ N}$ -naphthylethylenediamine dichloride (NED), and left to stand at room temperature for 1 h. Absorbance was read at 540 nm against a standard curve prepared with 0–0.05 mM NaNO_2 .

A model for leaf 24 h carbon balance

A carbon budget approach (Borland, 1996; Borland and Dodd, 2002) was used to provide an integrated view of how leaves of wild-type and mutant plants of *M. crystallinum* adjusted carbohydrate partitioning between contrasting sinks during acclimation to salinity. The input of carbon from C_3 photosynthesis was calculated by integrating net CO_2 uptake over the 12 h photoperiod. The input of carbon from C_4 metabolism was calculated from the net breakdown of malate over the 12 h photoperiod with 1 mol malate=4 mol C. The net flux of carbon into the starch pool was calculated from the net accumulation of starch over the photoperiod with 1 mol hexose=6 mol C. Net flux of carbon into the sugar pool was calculated from the net accumulation of soluble sugars over the 12 h photoperiod with 1 mol hexose=6 mol C. Daytime export was calculated as the sum of carbon in excess of that accumulated in starch and soluble sugars during the day. The net flux of carbohydrate into CAM at night was calculated from the PEP needed to accommodate the measured overnight accumulation of malic acid, assuming that 1 mol hexose produces 2 mol PEP and 1 mol PEP=3 mol C. The net flux into respiratory CO_2 was calculated from the amount of net CO_2 efflux at night (as in control plants) or, in the case of salt-treated plants, from the overnight accumulation of malic acid minus net CO_2 uptake at night (i.e. respiratory recycling). Nocturnal export was calculated as the amount of carbon depleted at night that could not be accounted for by the requirement to produce the 3-C substrate (PEP) for nocturnal carboxylation or the generation of respiratory CO_2 . Units in the model are $\text{mmol C m}^{-2}\text{ d}^{-1}$.

Results

CAM expression in the wild type and mutant

In wild-type plants, net dark CO_2 uptake was apparent after 6 d of salinity, and nocturnal net CO_2 uptake increased further over the time period of the salinity treatment (i.e.

14 d; Fig. 1). In contrast, the mutant showed no net uptake of CO_2 at night over the entire 14 d of salinity, indicating CAM deficiency. The wild-type plants showed higher rates of net CO_2 uptake during the day (on average 20% higher) compared with the mutant at comparable stages of salinity. After 14 d of exposure to salinity, 24 h net CO_2 uptake in the wild-type plants was reduced to 36% of that of well-watered controls, but in the mutant was reduced to only 10% of that of well-watered controls.

Changes in leaf titratable acidity measured at the start (dusk) and end (dawn) of the dark period were used as an additional measure of the magnitude of CAM expression between the wild type and mutant (Fig. 2a, b). Overnight accumulation of acidity was noted in the wild type after 4 d of salinity, with the organic acids probably derived from the re-fixation of respiratory CO_2 because there was no net uptake of CO_2 at this stage of the salinity treatment (Fig. 1). Overnight accumulation of acidity in the wild type increased steadily over the course of the salinity treatment and even control plants accumulated some acids after 14 d. At all stages of salinity, the amount of acid accumulated overnight in the wild type was significantly higher than that in the mutant (Fig. 2a, b). For the mutant, overnight accumulation of acids was noted after 14 d of salinity, and this must have been derived from re-fixation of respiratory CO_2 . On

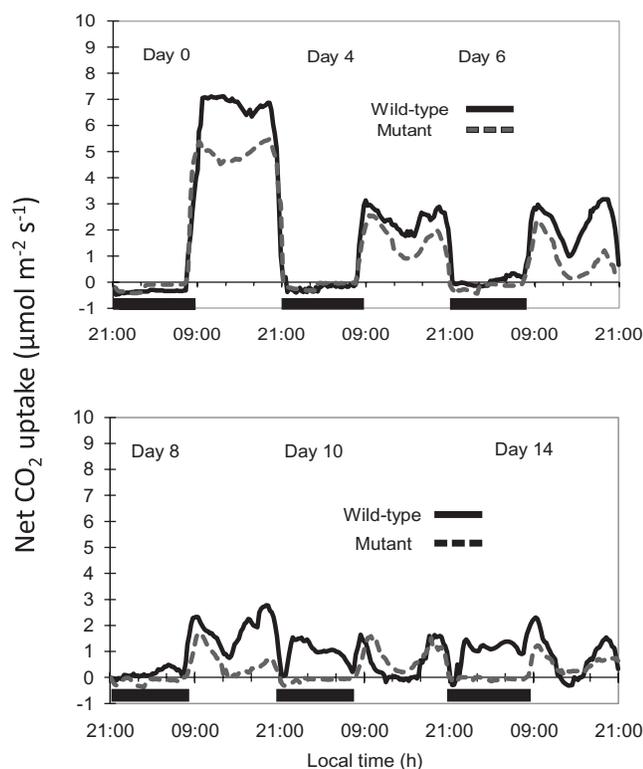


Fig. 1. Day/night profiles of net CO_2 exchange for leaves of wild-type (solid line) and CAM-deficient mutant (dashed line) plants of *Mesembryanthemum crystallinum* grown under control conditions (day 0) and after 4, 6, 8, 10, and 14 d of treatment with 300 mM NaCl in hydroponics. The solid bars on the x-axes represent the periods of darkness and each gas exchange profile is for a representative leaf from three replicate determinations.

the basis of nocturnal acid accumulation, CAM activity in the mutant was $\sim 40\%$ of that in the wild type after 14 d exposure to salinity.

Changes in carbohydrate and cyclitol contents imposed by salinity

The imposition of salinity enhanced day/night changes in starch content in leaves of the wild type over the 14 d treatment (Fig. 2c). In contrast, well-watered plants showed an overall increase in leaf starch content over the 14 d time period. Very low (but detectable) concentrations of starch were found in leaves of the mutant (Fig. 2d). The roots of the mutant were also depleted in starch compared with the wild type (Fig. 2e, f). There were no significant changes in carbohydrate contents of roots over a 24 h period (data not shown), so all data for roots show carbohydrate contents measured at the end of the day.

Leaves of the mutant contained significantly higher concentrations of soluble sugars compared with the wild type, particularly glucose and fructose (Fig. 3). Concentrations of glucose, fructose, and sucrose increased over the course of the day in the mutant at all stages of salinity, indicating that in the absence of starch, the mutant made use of soluble sugars to maintain 24 h net carbon balance. In the roots, the mutant showed higher concentrations of glucose and fructose compared with the wild type at day 0 and after 4 d of salinity (Fig. 4). At other stages of salinity, concentrations of root soluble sugars were similar in the wild type and mutant, and overall concentrations of all soluble sugars declined over the duration of the salinity treatment in both the wild type and mutant (Fig. 4).

Roots and particularly shoots accumulated substantial amounts of Na over the 14 d salinity treatment (Fig. 5a, b). Wild-type leaves showed significantly higher Na contents than mutant leaves ($P < 0.05$), but Na contents of roots of the wild type and mutant were similar. Imposition of salinity resulted in comparable increases in cyclitols in the leaves of both the wild type and mutant (Fig. 5c), with a 5-fold increase in leaf cyclitol content noted over the 14 d treatment period. There was no significant day–night change in leaf cyclitol content (data not shown). The roots of well-watered mutants contained significantly higher concentrations of cyclitols compared with the wild type and, after 4 d of salinity, the cyclitol content of the mutant roots was 5-fold higher than that of wild-type roots (Fig. 5d). However, with increasing salinity stress, root cyclitol content declined in the mutant and, by day 14 of salinity, cyclitol contents were similar in the roots of the mutant and wild type.

Dry matter production, nitrate reductase activity, and root respiration

Shoot dry matter content (which excluded the weight of NaCl) of wild-type plants was significantly higher ($P < 0.05$) than that of the mutant under well-watered and saline conditions, whereas root dry matter content was similar in the wild type and mutant under watered and saline conditions

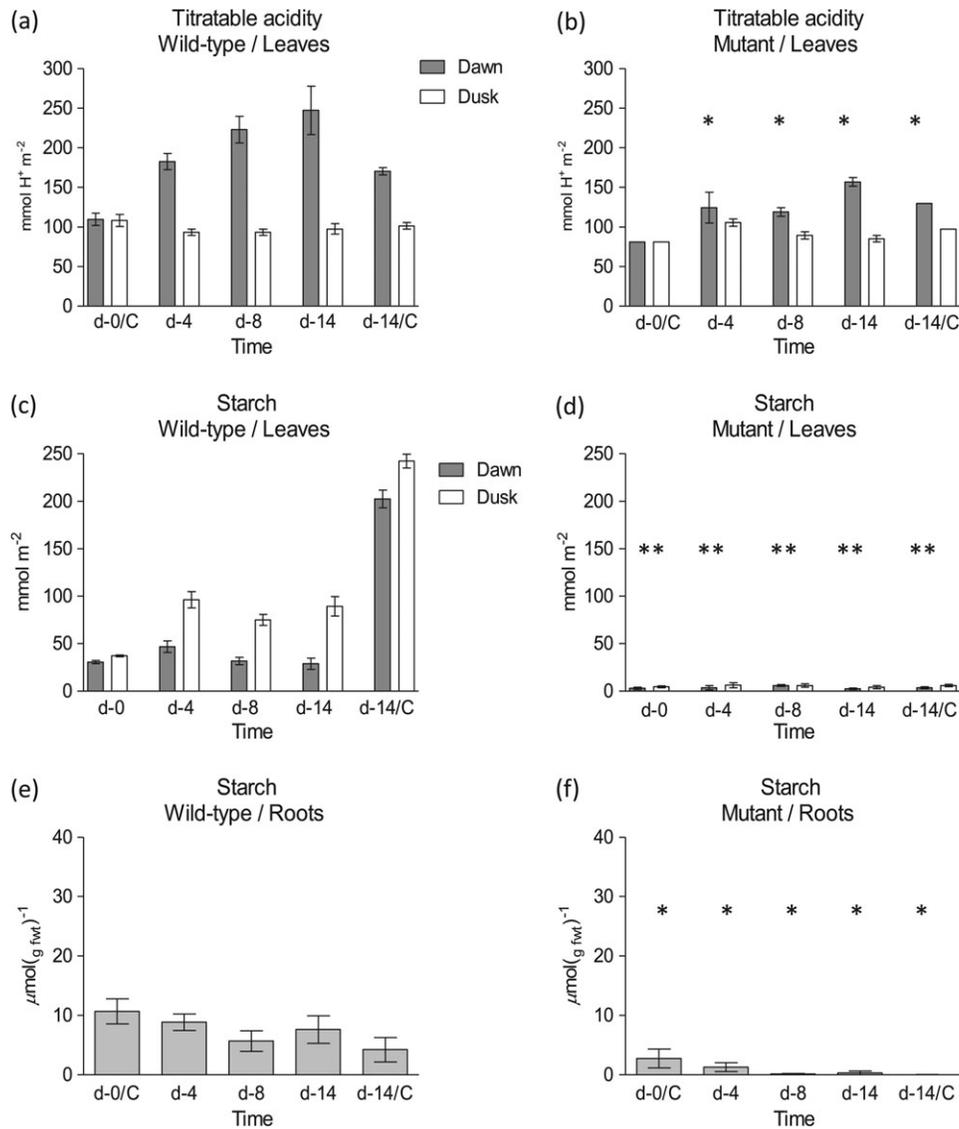


Fig. 2. Leaf titratable acidities (a, b) and starch content in leaves (c, d) and roots (e, f) of wild-type and CAM-deficient mutant plants of *Mesembryanthemum crystallinum* under control conditions (day 0/C and day 14/C) and following the imposition of 300 mM NaCl for 4, 8, and 14 d. Measurements on leaves were made at the start (dawn) and end (dusk) of the photoperiod and for roots at the end of the photoperiod. Each bar is the mean of four replicates \pm SE. Asterisks indicate statistical differences at $P < 0.05$ between the wild type and mutant at comparable stages of salinity treatment as determined by t -tests.

(Fig. 6a, b). Exposure to salinity for 14 d reduced root growth compared with 14 d control plants in both the wild type and mutant, whereas shoot dry matter content (excluding the weight of NaCl) of salt-treated plants was either similar to or slightly higher than that of control plants. The highest rates of nitrate reductase (NRA; measured on a dry weight basis) in well-watered plants were found in the roots of wild-type and mutant lines (Fig. 6c, d). Salinity resulted in a significant reduction in NRA activity in both leaves and roots. NRA was significantly ($P < 0.01$) higher in leaves of wild-type plants compared with those of the mutant under well-watered and saline conditions, whereas the reverse was true of root NRA activity, which was highest in the mutant under watered and saline conditions.

Rates of root respiration were significantly higher in the mutant compared with the wild type under well-watered

and saline conditions (Fig. 6e). Overall, the rate of root respiration declined under saline conditions in both the wild type and mutants.

Integration of leaf 24 h carbon balance

The net 24 h carbon budgets presented in Fig. 7 provide a quantitative illustration of the sources (C_3 or C_4) of carbon in the leaf, how this carbon was partitioned between starch and soluble sugars, and how much of each fraction was used to generate the substrate for CAM, respiratory CO_2 , or cyclitol synthesis. Export was calculated as the sum of carbon in excess of that accumulated in starch and soluble sugars during the day, plus carbon degraded at night that is not accounted for by the requirement to produce the 3-C substrate (PEP) for nocturnal carboxylation or the generation of

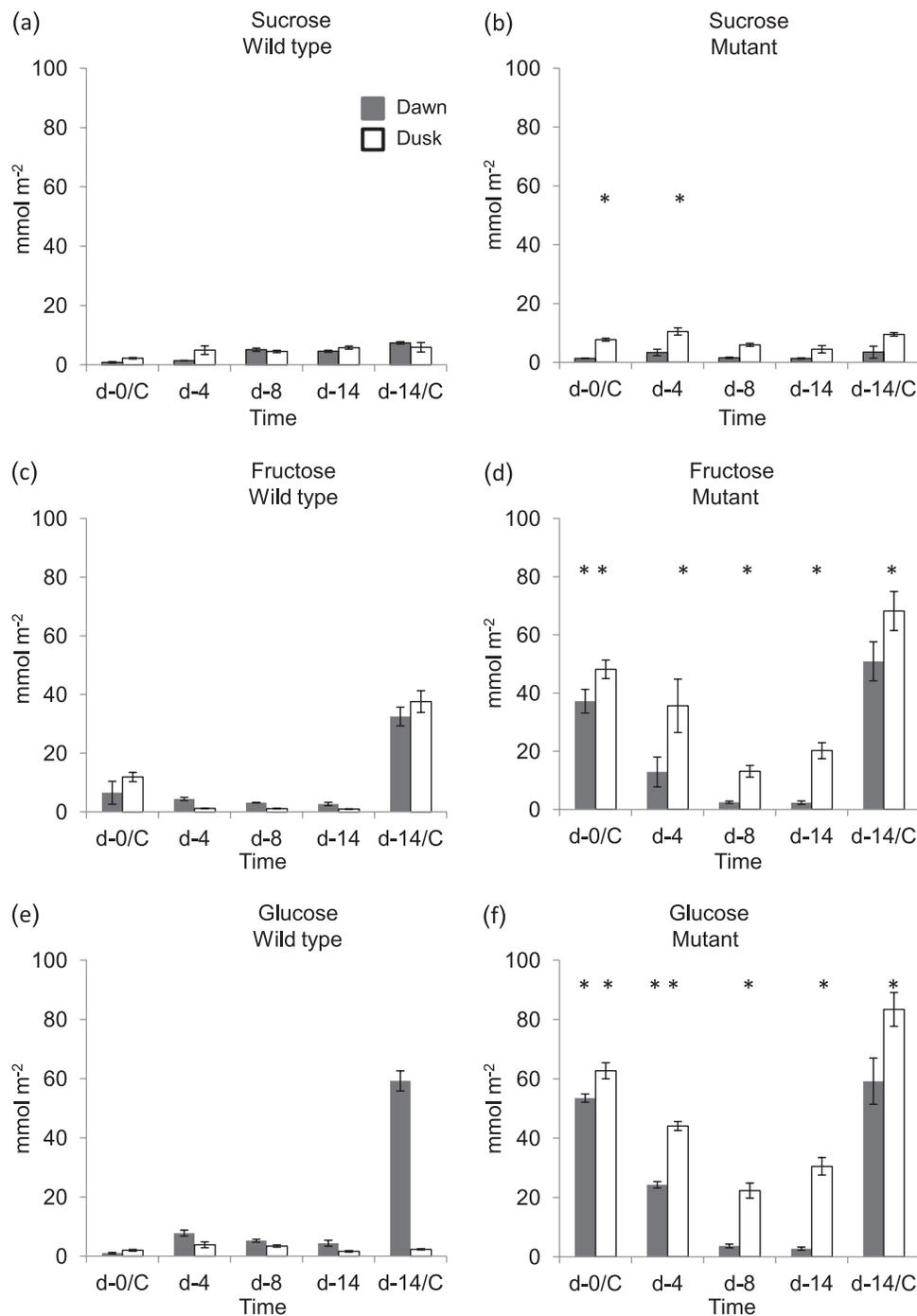


Fig. 3. Sucrose (a, b), fructose (c, d), and glucose (e, f) contents in leaves of wild-type and CAM-deficient mutant plants of *Mesembryanthemum crystallinum* under control conditions (day 0/C and day 14/C) and following the imposition of 300 mM NaCl for 4, 8, and 14 d. Measurements were made at the start (dawn) and end (dusk) of the photoperiod, and each bar is the mean of four replicates \pm SE. Asterisks indicate statistical differences at $P < 0.05$ between the wild type and mutant at comparable stages of salinity treatment as determined by *t*-tests.

respiratory CO_2 . Under control conditions, wild-type plants took up $\sim 20\%$ more carbon compared with the mutants, with $\sim 25\%$ of this carbon stored as starch; the remaining 75% entered the soluble sugar pool, with some 40% of these soluble sugars exported over the course of the day. At night, the amount of sugars exported from control leaves was >2 -fold higher than those exported during the day. In the starch-deficient mutants, all carbon entered the soluble sugar pool,

with daytime export $\sim 70\%$ of that noted in control plants. At night, export from leaves of control mutants was $\sim 80\%$ of that calculated for the wild type.

After 14 d exposure to salinity, in the wild-type plants starch became the major sink for daytime carbon whereas a small proportion of carbon was partitioned towards the accumulation of cyclitols. At night, 67% of the starch degraded was used for the synthesis of substrate for CAM

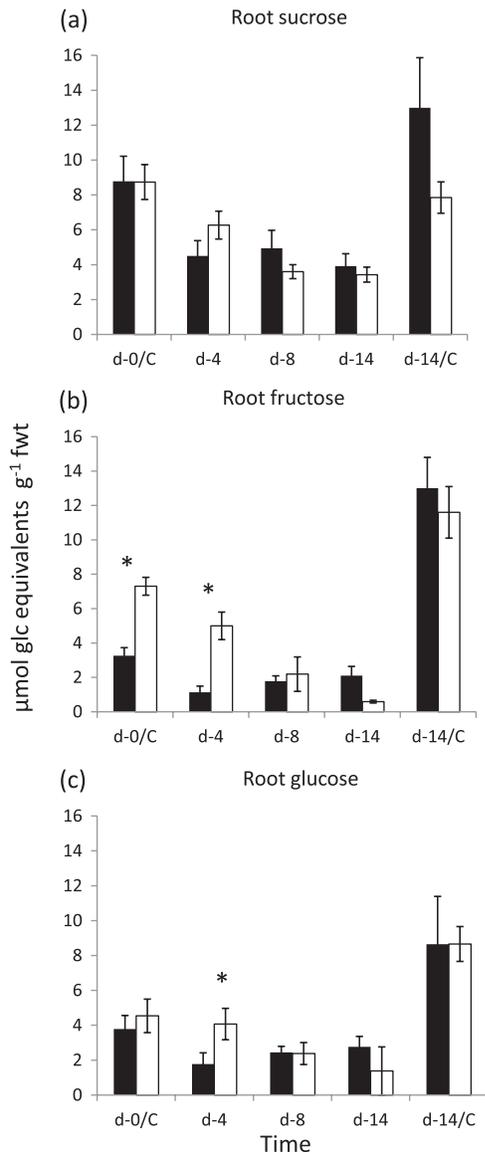


Fig. 4. Contents of sucrose (a), fructose (b), and glucose (c) in roots sampled at the end of the photoperiod of wild-type (solid bars) and CAM-deficient mutant (open bars) plants of *Mesembryanthemum crystallinum* under control conditions (day 0/C and day 14/C) and following the imposition of 300 mM NaCl for 4, 8, and 14 d. Each bar is the mean of four replicates \pm SEM. Asterisks indicate statistical differences at $P < 0.05$ between the wild type and mutant at comparable stages of salinity treatment as determined by t -tests.

whereas the remainder of starch degradation products entered the soluble sugar pool. Approximately 10% of these sugars were respired, with this CO_2 recaptured by PEPC. Induction of CAM and acclimation to salinity in wild-type plants reduced net export by $>60\%$ compared with controls, and all net export occurred at night. In the salted starch-deficient mutants, some 65% of soluble sugars were degraded at night to provide PEP and a further 22% of sugars were respired with the CO_2 re-fixed by PEPC. Induction of CAM and acclimation to salinity in the mutants reduced net export by $>90\%$ compared with non-salt-treated mutants. Overall,

the amount of sugars exported from leaves of salt-treated mutants was only 15% of that noted in salt-treated wild type.

Discussion

Starch deficiency limits CAM via direct and indirect effects on substrate availability

The photosynthetic metabolism of CAM plants has an absolute requirement for reserve carbohydrates that provide carbon for the synthesis of PEP during the night. These storage carbohydrates might be starch or soluble sugars, depending on the species (Christopher and Holtum, 1996). CAM induction in *M. crystallinum* is usually associated with enhanced degradation of transitory starch (Keiller *et al.*, 1987; Paul *et al.*, 1993; Dodd *et al.*, 2003). In the present study, as noted elsewhere (Borland and Dodd, 2002), there was a close stoichiometry between the amount of starch degraded during the night to provide carbon skeletons for PEP synthesis and nocturnal acid accumulation in the wild type. Although leaves of the mutant had barely detectable amounts of starch, soluble sugar content was higher than that in the wild type. A previous study indicated that the CAM-deficient mutant of *M. crystallinum* is deficient in plastidic PGM (Cushman *et al.*, 2008), and increased sugar content has also been observed in leaves of plastidic PGM mutants of *Arabidopsis* and *Nicotiana sylvestris* (Caspar *et al.*, 1985; Hanson and McHale, 1988). In the CAM-deficient mutant of *M. crystallinum*, exposure to salinity for 2 weeks induced significant overnight accumulation of acids ($\sim 40\%$ of that noted in the wild type), and indicated that soluble sugar breakdown can provide PEP for PEPC-mediated carboxylation in *M. crystallinum*. This is in agreement with observations that feeding exogenous sugars to detached leaves of the CAM-deficient mutant of *M. crystallinum* restored overnight accumulation of malic acid (Cushman *et al.*, 2008) and indicates the flexibility of the CAM pathway for utilizing different carbohydrate sources to generate substrate for nocturnal acidification.

Starch deficiency also limited CAM in the PGM mutants of *M. crystallinum* by curtailing nocturnal stomatal opening and uptake of atmospheric CO_2 at night. In C_3 plants, the endogenous opening of stomata at night is dependent on the breakdown of starch to provide an osmoticum to balance K^+ within the guard cells (Lascève *et al.*, 1997; Easlon and Richards, 2009) and it would appear from the present work that starch plays a similar role in the guard cells of CAM plants at night.

Cyclitols as sinks for carbohydrate during acclimation to salinity

Cyclitol accumulation is believed to play a vital role in salinity acclimation in *M. crystallinum* by maintaining osmotic adjustment between the vacuole (rich in NaCl) and cytoplasm (low in NaCl) and by alleviating oxidative burden (Popp and Smirnov, 1995; Borland *et al.*, 2006; Sunagawa *et al.*, 2010). In *M. crystallinum*, the *de novo*

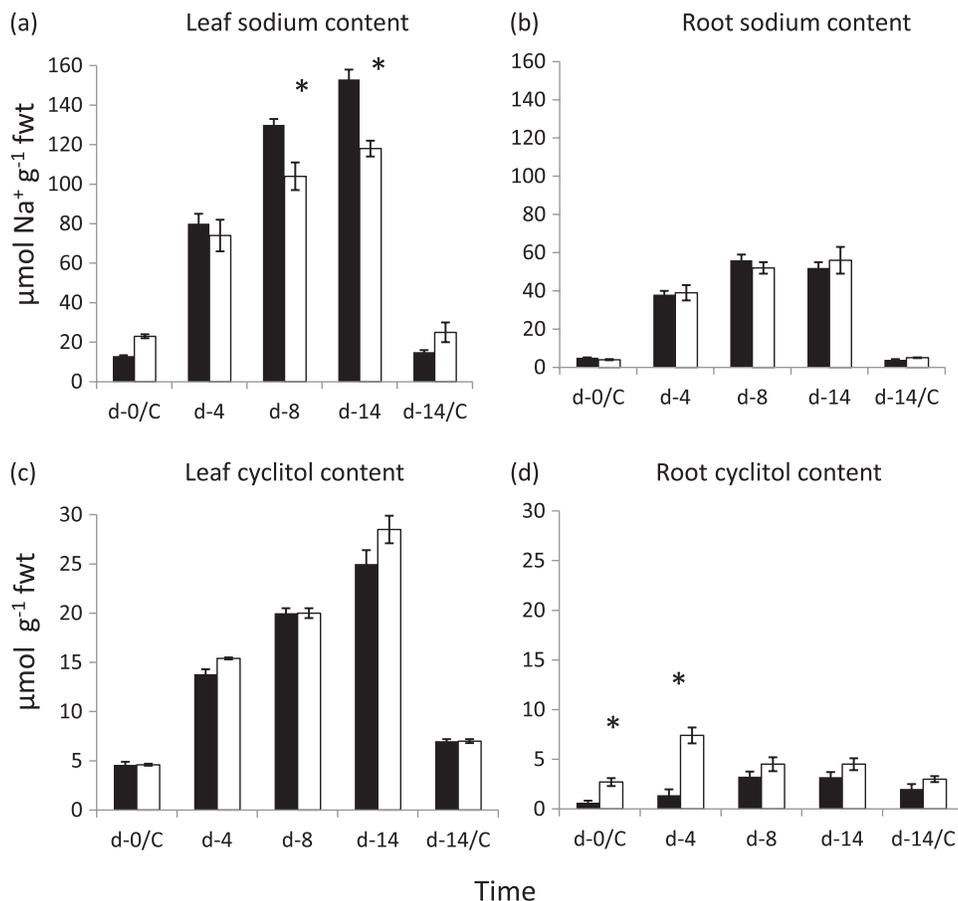


Fig. 5. Contents of sodium (a, b) and cyclitols (c, d) in leaves and roots of wild-type (solid bars) and CAM-deficient mutant (open bars) plants of *Mesembryanthemum crystallinum* under control conditions (day 0/C and day 14/C) and following the imposition of 300 mM NaCl for 4, 8, and 14 d. Each bar is the mean of four replicates \pm SE. Asterisks indicate statistical differences at $P < 0.05$ between the wild type and mutant at comparable stages of salinity treatment as determined by *t*-tests.

synthesis of inositol (as a precursor to ononitol and pinitol) proceeds from glucose 6-phosphate through two steps catalysed by myo-inositol-1-phosphate synthase (INPS) and inositol monophosphatase (IMP; Nelson *et al.*, 1998). Wild-type and mutant *M. crystallinum* accumulated similar amounts of cyclitols under salinity, indicating that starch mobilization is not required for the synthesis of these compatible solutes, which are thus likely to be derived from primary photosynthate and/or processing of the soluble sugar pool. That the mutant accumulated amounts of cyclitols comparable with the wild type yet showed a 60% reduction in CAM expression might imply that cyclitols were a stronger sink for carbohydrates than CAM. However, cyclitol accumulation accounted for only 2–5% of 24 h carbon gain under salinity, indicating that cyclitols represent a low-cost C-based osmotic adjustment whereas CAM required the nocturnal mobilization of >70% of primary carbohydrate that accumulated over the previous day. Differences in the kinetics of transcriptional activation of INPS and IMP and key CAM-related genes (e.g. PEPC) suggest that the genetic machinery that underpins salinity acclimation in *M. crystallinum* ensures an early commitment of carbohydrate to cyclitols that continues throughout the period of salt stress. The longer term induction of the

enzymatic machinery of CAM (which happens 1–2 d after increases in INPS and IMP proteins; Adams *et al.*, 1992; Nelson *et al.*, 1999) prevents competition with cyclitol synthesis for carbohydrate during the early stages of salinity and ultimately ensures sustained net carbon gain under saline conditions as long as carbohydrate reserves are maintained.

Previous studies have reported that salinity elicits an up-regulation of both the INPS and IMP proteins in leaves in *M. crystallinum*, but a down-regulation of the INPS protein in the roots (Nelson *et al.*, 1998). Import of myo-inositol to the roots probably serves to indicate photosynthetic competence under salinity and also is thought to act as a leaf-to-root signal that promotes sodium uptake by the root via Na^+ /myo-inositol symporters and subsequent transport to the leaf via the xylem (Nelson *et al.*, 1999). Given that cyclitols are believed to regulate Na uptake as a function of photosynthetic capacity, it is notable in the present work that the amounts of cyclitols accumulated in leaves (or roots) was not related to the amount of net CO_2 uptake which, under salinity, was 50% lower in the mutant compared with the wild type. While the Na content was marginally lower in leaves of the mutant (~20%) compared with the wild type, it seems likely that this reflects the reduced rate of transpiration (data not shown) and lowered

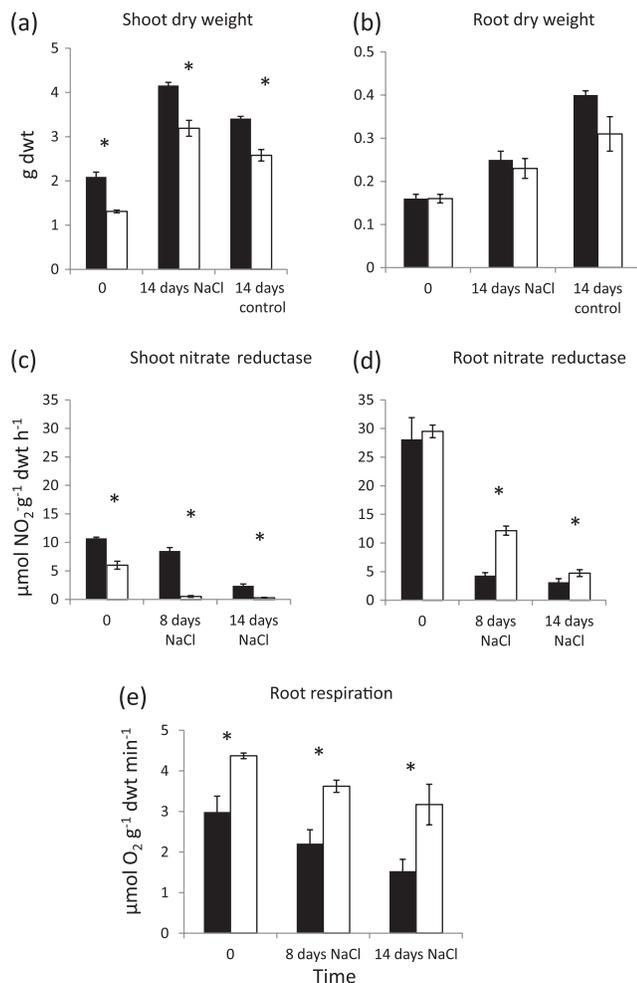


Fig. 6. Growth of leaves (a) and roots (b), nitrate reductase activity of leaves (c) and roots (d), and root respiration (e) in wild-type (solid bars) and CAM-deficient mutant (open bars) plants of *Mesembryanthemum crystallinum* under control conditions (0) and following the imposition of 300 mM NaCl for 8 d and 14 d. Each bar is the mean of four replicates \pm SE. Asterisks indicate statistical differences at $P < 0.05$ between the wild type and mutant at comparable stages of salinity treatment as determined by *t*-tests.

xylem transport in the mutant compared with the wild type. Thus, it would appear that starch deficiency can potentially uncouple photosynthetic carbon balance from Na accumulation in the halophytic species *M. crystallinum*.

Respiration as a sink for carbohydrate

The acid that accumulated overnight in leaves of the salt-treated CAM-deficient mutant must have been derived from the re-fixation of respiratory CO_2 at night, given that there was no nocturnal net uptake of atmospheric CO_2 . Reduced nocturnal stomatal conductance in the mutant is likely to have curtailed leakage of respiratory CO_2 from the leaf, thereby encouraging re-fixation by PEPC. Enhanced nocturnal re-fixation in the mutant also implies higher rates of leaf dark respiration compared with the wild type. Rates of

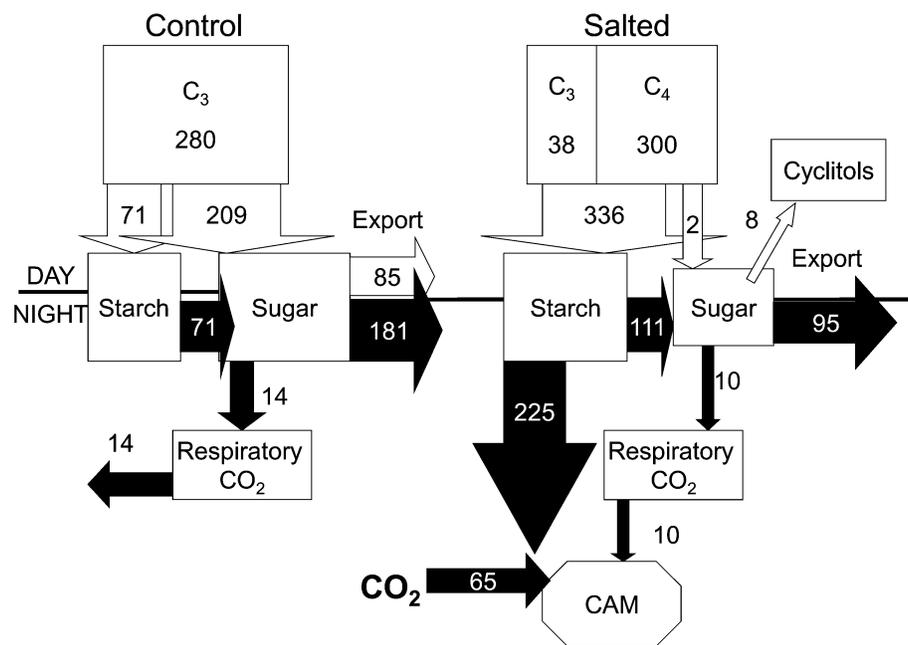
leaf dark respiration in CAM species can be estimated from calculations of the amount of acid that accumulates in excess of net dark CO_2 uptake. Thus, despite the fact that photosynthetic inputs of carbon into the leaf were significantly lower in mutant compared with wild-type *M. crystallinum*, leaves of the salt-treated mutant were estimated to show a 2- to 3-fold higher rate of dark respiration than that in the wild type. Direct measurements of respiration in the roots of *M. crystallinum* indicated that the mutant also had significantly higher rates of root respiration than the wild type, particularly under salinity, with mutant roots showing 2-fold higher rates of O_2 uptake compared with the wild type. That respiration was a stronger sink for carbohydrate in the *M. crystallinum* mutant might be a consequence of a more ready supply of soluble sugars as respiratory substrate, as was suggested for starchless mutants of *Arabidopsis*, which also showed elevated rates of dark respiration compared with the wild type (Sun *et al.*, 2002).

Integration of leaf carbon balance reveals sink hierarchy in *M. crystallinum* under saline conditions

The inability to make starch significantly reduced the leaf biomass of the mutant *M. crystallinum*. This can be attributed to several interacting factors. Lower rates of net CO_2 assimilation were noted in the mutant, which could be due to Pi limitation for ATP synthesis and ribulose 1,5-bisphosphate (RuBP) regeneration due to the inability to use triose-phosphate/glucose-6-phosphate for starch synthesis (Lytovchenko *et al.*, 2002). Lower rates of leaf nitrate reductase activity in the mutant and the inability to break down starch for growth processes at night would also limit leaf biomass in the mutant *M. crystallinum*, as also shown for PGM mutants of *Arabidopsis* (Schulze *et al.*, 1991). The higher rates of root respiration and root nitrate reductase in the mutant are indicative of an enhanced import of soluble sugars to the roots compared with the wild type, indicating that mutant roots were stronger sinks for carbohydrate. This was also implied by the elevated concentrations of root cyclitols in the mutant during the early stages of salinity. Despite this elevated metabolic activity, root biomass and root Na content were similar in the wild type and mutant under well-watered and salt-treated conditions, implying that starch deficiency and reduced CAM activity were accompanied by higher metabolic costs for maintaining the uptake of Na, Cl, and nutrients in the mutant.

Comparing the leaf carbon budgets for the wild type and the mutant of *M. crystallinum* indicated that after 2 weeks of exposure to salinity stress, CAM was a stronger sink for carbohydrate compared with C-based osmotic adjustment, leaf growth, and export. Some 70% of net carbon balance in the wild type was accounted for by the generation of PEP and respiratory CO_2 for nocturnal carboxylation, whereas in the CAM-deficient mutant 86% of net carbon was committed to generating substrates for CAM. Thus, CAM was a relatively stronger sink for carbohydrate in the mutant compared with the wild type, with the consequence that after 2 weeks of salinity, net export of sugars from

(a) Wild type



(b) Mutant

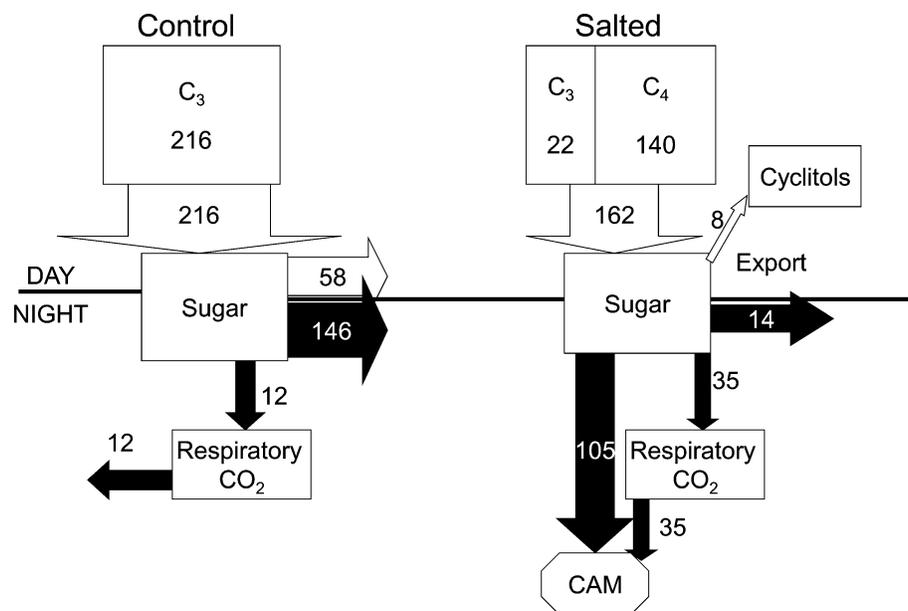


Fig. 7. The 24 h net carbon budgets for leaves of wild-type and CAM-deficient mutants of *M. crystallinum* showing partitioning of carbon skeletons derived from C₃ and C₄ carboxylation between different carbohydrate pools, export, dark respiration, and CAM in control plants and after exposure to 300 mM NaCl for 14 d. The source of carbon in the leaf during the day is C₃ (daytime photosynthesis) or C₄ (from breakdown of malate over a 12 h photoperiod with 1 mol malate=4 mol C). Export includes growth/maintenance, and fluxes shown in the white (open) arrows are daytime processes, while those in black (solid) arrows are night-time processes. All numbers presented on the budgets are mmol C m⁻² d⁻¹.

leaves of the CAM-deficient mutant was <15% of that in the wild type.

It appears likely that the substantial allocation of carbohydrate to CAM curtailed export of sugars to the roots of both the wild type and mutant. Reduced export of sugars to the roots under salinity was implied by a steady

decline in the rate of root respiration and root nitrate reductase activity in both the wild type and mutant, whereas the decline in root carbohydrate content implies remobilization of reserves to support acclimation and maintenance under salinity. Consequently, the root:shoot ratio decreased under salinity and suggests that the carbohydrate demands

of CAM in the photosynthetic shoot take precedence over root growth and metabolism under water-limited conditions.

In both salt-treated wild-type and CAM-deficient mutant plants, the elimination of daytime carbohydrate export from leaves of the salt-treated wild type and mutant ensured the retention of significant carbohydrate stores for the nocturnal generation of PEP. In the wild type, this appears to have been achieved by: (i) the daytime allocation of 99% of carbon to starch at the expense of the (more readily exportable) soluble sugar pool; and (ii) restriction of starch degradation to the night period, a phenomenon which might be a function of circadian control (Borland and Taybi, 2004; Ceusters *et al.*, 2010; Graf and Smith, 2010). In the mutant, where all carbohydrate was allocated to the soluble sugar pool, the elimination of daytime export in the CAM-performing plants suggests a key role for the vacuole in regulating supply and demand for carbohydrate over the diel CAM cycle. Vacuolar sugar transporters have been highlighted previously as key candidates for regulating carbon flow during the diel CAM cycle in soluble sugar-accumulating CAM species such as pineapple and *Agave* (Antony and Borland, 2008; Antony *et al.*, 2008; Borland *et al.*, 2009). The vacuole seems to have taken on a similar role for regulating the expression of CAM in the starch-/CAM-deficient mutant of *M. crystallinum*. This finding supports the idea that the biochemistry and transport processes that exist in all plant species for the turnover of starch and soluble sugars can easily be recruited to achieve the coordinated supply and demand for carbohydrate that sustains the 24 h operation of CAM.

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