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Peer-reviewed author version

Ceusters, Nathalie; Borland, Anne M. & CEUSTERS, Johan (2021) How to resolve the enigma of diurnal malate remobilisation from the vacuole in plants with crassulacean acid metabolism?. In: New Phytologist (print), 229 (6) , p. 3116 -3124.

DOI: 10.1111/nph.17070
Handle: http://hdl.handle.net/1942/34479

How to resolve the enigma of diurnal malate remobilization from the vacuole in plants with crassulacean acid metabolism?

Nathalie Ceusters[1] (0000-0002-8374-0447) , Anne M. Borland[2] (0000-0003-4560-9998) , Johan Ceusters[1, 3]* (0000-0002-5225-9159)

1 Faculty of Engineering Technology, Department of Biosystems, Division of Crop Biotechnics, Campus Geel, KU Leuven, Kleinhoefstraat 4, 2440 Geel, Belgium.

2 School of Natural and Environmental Sciences, Newcastle University, Newcastle upon Tyne NE1 7RU, UK.

3 UHasselt, Centre for Environmental Sciences, Environmental Biology, Campus Diepenbeek, Agoralaan Building D, 3590 Diepenbeek, Belgium

*Corresponding author: Johan Ceusters

e-mail: johan.ceusters@kuleuven.be

telephone +3214 72 13 09

Total word count: 4344

Total number of figures: 2

Total number of tables: 3

Summary

Opening of stomata in plants with crassulacean acid metabolism (CAM) is mainly shifted to the night period when atmospheric CO_2 is fixed by PEPC and stored as malic acid in the vacuole. As such, CAM plants ameliorate transpirational water losses and display substantially higher water use efficiency (WUE) compared to C_3 and C_4 plants. In the past decade significant technical advances have allowed an unprecedented exploration of genomes, transcriptomes, proteomes and metabolomes of CAM plants and efforts are ongoing to engineer the CAM pathway in C_3 plants. Whilst research efforts have traditionally focused on nocturnal carboxylation, less is known regarding the drivers behind diurnal malate remobilization from the vacuole which liberates CO_2 to be fixed by Rubisco behind closed stomata. To shed more light on this process, we provide a stoichiometric analysis to identify potentially rate-limiting steps underpinning diurnal malate mobilization and help direct future research efforts. Within this remit we address three key questions: Q1 Does light-dependent assimilation of CO_2 via Rubisco dictate the rate of malate mobilization? Q2: Do the enzymes responsible for malate decarboxylation limit day-time mobilization from the vacuole? Q3: Does malate efflux from the vacuole set the pace of decarboxylation?

Keywords

Aluminum activated malate transporter – Crassulacean Acid Metabolism – Malate efflux – Malic enzyme – PEPC – PEPCK – Rubisco – Tonoplast dicarboxylate transporter

Introduction

Crassulacean acid metabolism (CAM) is one of the three main metabolic pathways to accommodate CO_2 fixation in the photosynthetic tissues of vascular plants. In addition to tropical field crops such as pineapple and *Agave* CAM is present in a plethora of ornamentals such as *Euphorbia*, *Kalanchoë*, *Yucca*, *Sansevieria*, *Sedum*, various bromeliads, orchids and cacti. In contrast to C_3 and C_4 plants, CAM plants predominantly fix atmospheric CO_2 at night when evapotranspiration rates are low, enabling plants to close stomata for much of the day and thereby optimising water-use efficiency (WUE). This important water-conserving trait has fostered diverse recent efforts to determine the minimal requirements for engineering the CAM pathway into non-CAM crops (CAM Biodesign) in order to improve WUE whilst maintaining high productivity (Borland *et al.*, 2014; Lim *et al.*, 2019). The diel cycle of CAM photosynthesis is commonly divided into four phases that integrate patterns of net CO_2 uptake and changes in the abundance of key metabolites that define carbon supply and demand over the 24 h cycle (Osmond, 1978). When stomata are open at night during Phase I, external CO_2 is converted to HCO_3^- and then sequestered via phospho*enol*pyruvate carboxylase (PEPC) using the 3-carbon substrate phospho*enol*pyruvate (PEP), which is provided from the glycolytic breakdown of carbohydrate accumulated during the previous day (Fig. 1). The final 4-C product, malate, is then pumped in the vacuole, following an inside positive electrical potential difference across the tonoplast generated by a dedicated V-ATPase. During the subsequent light period (Phase III), malate is released from the vacuole and decarboxylated in the cytosol by either malic enzyme (ME) or a combination of malate dehydrogenase (MDH) and PEPcarboxykinase (PEPCK), releasing CO_2 inside the mesophyll cells. Subsequently, CO_2 is refixed by ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) behind closed stomata (Borland *et al.*, 2011). These major CAM phases are punctuated by Phase II at the start of the day and Phase IV at the end of the day (Borland & Taybi, 2004), with open stomata allowing fixation of atmospheric CO_2 by either PEPC and/or Rubisco. The four CAM phases typically show a high degree of plasticity in terms of magnitude and duration, which appears to be critical for optimizing carbon gain and water use under a changing environment (Ceusters *et al.*, 2009, 2010, 2011).

Temporal coordination and co-regulation of both carboxylating enzymes, i.e. PEPC and Rubisco, brings about a unique and flexible carboxylation capacity in CAM plants over the diel cycle (Fig. 1). Circadian and metabolic control of nocturnal carboxylation is mediated via

reversible phosphorylation of PEPC and is well documented. PEPC is activated at night via phosphorylation of a serine residue near the N-terminus of the protein which renders the enzyme more sensitive to PEP and the positive effectors, glucose-6-P and triose-P and less sensitive to the allosteric inhibitor, malate (Hartwell *et al.*, 1999; Borland *et al.*, 1999; Nimmo, 2000). As a first step towards installing CAM into C_3 plants, Kebeish *et al.* (2012) used dark-induced promoters to control the nocturnal overexpression of a PEPC engineered with reduced malate sensitivity in stably transformed Arabidopsis. Whilst these transformed Arabidopsis plants showed enhanced stomatal opening and transpiration rates at night (Kebeish *et al.*, 2012), a key challenge for CAM engineering will be achieving coordinated activation–deactivation of PEPC with Rubisco and malate decarboxylation over the diel cycle.

In contrast to the nocturnal (night-time) carboxylation process, much less is known about the diurnal (day-time) biochemical events in CAM plants and one process in particular, the daytime translocation of malate out of the vacuole, has received relatively little attention to date. Light intensity seems to be a critical factor in orchestrating diurnal malate metabolism and different potential rate limiting steps have been suggested: i) efflux from the vacuole; ii) liberation of CO_2 in the cytosol by decarboxylation or iii) assimilation of the liberated CO_2 via Rubisco in the chloroplast (Luttge, 2004). Driven by its growing eco-agricultural importance, the field of CAM research is now advancing quickly and important technical breakthroughs in the past decade have allowed an unprecedented exploration of CAM genomes, transcriptomes, proteomes and metabolomes (Ming *et al.*, 2015; Yang *et al.*, 2017; Ceusters *et al.*, 2019; Abraham *et al.*, 2020). In combination with rapidly progressing computational modelling approaches (Shameer *et al.*, 2018; Chomthong & Griffiths, 2020) a variety of new tools and genetic resources are now available to shed more light on diurnal malate metabolism in CAM plants. In this research review three important key questions are addressed: Q1 Does light-dependent assimilation of CO_2 via Rubisco dictate the rate of malate mobilization? Q2: Do the enzymes responsible for malate decarboxylation limit day-time mobilization from the vacuole? Q3: Does malate efflux from the vacuole set the pace of decarboxylation? Answers are provided by considering detailed stoichiometric analyses based on the available literature and important directions for future research efforts are highlighted and discussed.

Q1: Does light-dependent assimilation of CO_2 via Rubisco dictate the rate of malate mobilization?

Factors influencing the light-dependent assimilation of CO_2 via Rubisco and its relationship to the onset and subsequent rate of malate mobilization during the photoperiod in CAM plants are still not fully resolved. Rubisco activity in CAM plants appears to be tightly regulated during the light period but differs from C_3 plants in terms of a more protracted activation that is independent of light intensity (Maxwell *et al.*, 1999, 2002; Griffiths *et al.*, 2008). In situations where PEPC activity extends beyond the night period for several hours into the photoperiod, Rubisco in CAM plants remains inactive as indicated by low rates of electron transport and increased non-photochemical quenching which curtails the capacity for light use and dissipation being exceeded (Griffiths *et al.*, 2008). Rubisco activation requires the reversible binding of activator CO_2 and a light-dependent non-enzymatic modification called carbamylation which is stabilized by binding of Mg^{2+} (Lorimer & Miziorko, 1980; Portis, 1992). The stromal enzyme Rubisco activase (RCA) is critically important for mediating the activation and maintenance of Rubisco activity and operates by removing inhibitory sugar phosphates from the catalytic site of Rubisco (Parry *et al.*, 2008). The slow and protracted activation of Rubisco during the day in CAM species appears to be regulated, at least in part, by transcriptional regulation of RCA, which shows an increase in protein abundance as the photoperiod progresses that is independent of light intensity (Maxwell *et al.*, 2002). A low activation status for Rubisco at the start of the photoperiod in CAM plants is predicted to curtail competition between the carboxylases. Davies & Griffiths (2012) demonstrated that this low Rubisco activation state at the start of the photoperiod, was maintained under free running conditions of constant light and temperature in CAM-performing *Mesembryanthemum crystallinum* and coincided with the initiation of malate breakdown and stomatal closure at each subjective dawn. Such observations pose the hypothesis that Rubisco activation status in CAM species is mediated by an interplay of circadian and metabolic cues which might include [malate] and/or pCO_2 (Borland & Taybi, 2004; Davies & Griffiths, 2012).

The elevated [CO_2] that is generated from day-time decarboxylation behind closed stomata has suggested pCO_2 as a key signal for coordinating the rate of CO_2 consumption by Rubisco with malic acid remobilisation from the vacuole during Phase III of CAM (Maxwell *et al.*, 1999; Lüttge, 2002; Borland & Taybi, 2004). During Phase III, pCO_2 can range between 0.08 and 2.50 % in the leaf air spaces which generally exceeds substrate saturation of Rubisco which is estimated around 0.2-0.4% (Maxwell *et al.*, 1998; Lüttge, 2002). Malate decarboxylation is commonly marked by high, non-saturated rates of electron transport, with Rubisco activity, RCA abundance and activation state increasing as Phase III progresses and

malate content diminishes. Quantitative comparisons of measured Rubisco activities (uptake of CO_2) and degradation rates of malate (liberation of CO_2) in the leaf mesophyll cells during Phase III would tend to refute the hypothesis that Rubisco assimilation might represent a possible bottleneck in malate remobilization (Table 1). Furthermore, enhancement of Rubisco activity throughout Phase III points to a feed-forward upregulation of Rubisco activation/RCA in response to the declining pCO_2 as malate content is exhausted (Maxwell *et al.*, 1998).

Sensing of pCO_2 during the shift from Phase III to Phase IV is also indicated at a metabolic level by further increases in the abundance of RCA and activation state of Rubisco towards the end of the photoperiod when cytosolic malate is exhausted and pCO_2 may drop as low as 0.01% (Maxwell *et al.*, 1998). This further enhancement of Rubisco activity in Phase IV occurs at a time when stomata have reopened and diffusional limitations to drawdown of atmospheric CO_2 imposed by closely packed succulent cells in CAM tissues is most likely to limit photon utilization. Clearly, the day-time mobilization of malate from the vacuole and its subsequent decarboxylation alter the catalytic context for Rubisco and RCA in CAM plants by generating a metabolic 'feast and famine' of internal [CO_2] supply (Maxwell *et al.*, 2002; Griffiths *et al.*, 2008). Recent studies have begun to investigate structure/function relationships for both Rubisco and RCA in CAM lineages (Shivhare & Mueller-Cajar, 2017; Hermida-Carrera *et al.*, 2020). Such approaches promise new insights on the molecular evolution of these proteins and their catalytic optimization for the dynamic changes in pCO_2 that are generated as a consequence of the onset and cessation of malate mobilisation during the daytime phases of CAM. Further work is required to establish if the engineering of CAM into C3 plants will require a modification to the kinetic properties of Rubisco and/or its activase in order to optimize diurnal malate mobilization.

Q2: Do the enzymes responsible for malate decarboxylation limit day-time mobilization from the vacuole?

Decarboxylation of malate can proceed either via NAD(P)-Malic enzyme (ME) or phosphoenolpyruvate carboxykinase (PEPCK) dependent on the species considered (Fig. 1). The relative contributions of mitochondrial NAD-ME and chloroplastic/cytosolic NADP-ME to malate decarboxylation in the light have been debated for many years. In 1976, Dittrich postulated that NAD-ME was not the dominating decarboxylase in Crassulaceae, but rather an additional support to NADP-ME activity. However, Dever *et al.* (2015) used transgenic RNAi lines of the obligate CAM species *Kalanchoë fedtschenkoi* to demonstrate that NAD-ME is the key decarboxylase, with a minor contribution from NADP-ME. Subsequent analyses of the

transcriptome (Yang *et al.*, 2017) and proteome (Abraham *et al.*, 2020) of *K. fedtschenkoi* identified diel changes in transcript and protein abundance of *kf*NAD ME1. Protein abundance of *k*fNAD ME1 peaked in the middle of the photoperiod at a time when the rate of malate decarboxylation is maximal, suggesting a level of transcriptional regulation over the daytime breakdown of malate via ME (Abraham *et al.*, 2020). ME liberates CO_2 from malate to accommodate further refixation by Rubisco in the mesophyll via the Calvin-Benson cycle (Fig. 1). The byproduct of this reaction, pyruvate, is consequently converted to PEP at the expense of ATP by cytosolic/plastidic pyruvate orthophosphate dikinase (PPDK) and metabolized and stored during the day either as chloroplastic starch or vacuolar sugars dependent on the species considered (Holtum *et al.*, 2005). PPDK is essential for CAM activity as demonstrated by RNAi knockdown in *K. fedtschenkoi* although the RNAi lines also showed a 30 % decrease in extractable NAD-ME activity relative to wild type (Dever *et al.*, 2015). Furthermore, low PPDK activity was noted in rNAD-ME lines of *K. fedtschenkoi* and was attributed to inactivation of PPDK by phosphorylation in the transgenic plants (Dever *et al.*, 2015). These data indicate post-translational control over malate decarboxylation that is subject to metabolic regulation by CAM-associated metabolites, possibly malate. Diel changes in phosphopeptide abundance of PPDK protein in *K. fedtschenkoi* were reported by Abraham *et al.* (2020) and are consistent with phosphorylation/inactivation of PPDK during the night in this CAM species. Phosphorylation of PPDK in C_4 plants is catalyzed by PPDK regulatory protein (PPDK-RP; Chastain *et al.*, 2011). An ortholog of PPDK-RP in *K. fedtschenkoi* showed increased protein abundance at the end of the photoperiod and for most of the dark period which would be consistent with inactivation of PPDK when malate is accumulating in the vacuole, rather than being processed via decarboxylation (Abraham *et al.*, 2020). Moreover, in the inducible CAM plant *M. crystallinum PPDK-RP* transcript abundance was upregulated when switching to CAM mode (Lim *et al.*, 2019). Further research is required to understand if PPDK and its regulatory protein play pivotal roles in the temporal coordination of decarboxylation with malate efflux from the vacuole during the photoperiod in CAM plants.

PEPCK serves as an oxaloacetate (OAA) decarboxylase and requires the prior operation of malate dehydrogenase (MDH) to convert malate into OAA. With extractable activities 4-110 times higher compared to measured PEPCK activities in a range of CAM plants (Dittrich *et al.*, 1973), MDH is unlikely to impose a limit on the rate of diurnal malate degradation. Also the intrinsic PEPCK activity itself does not seem to be a plausible candidate for restraining diurnal malate mobilization from the vacuole. In 2014 Ceusters *et al.* reported a consistent delay in

organic acid breakdown in the CAM bromeliad *A.* 'Maya', which occurred ~ 4 and 12 h into the photoperiod under either low fluence red or green light in comparison to low fluence blue light. However, no changes in PEPCK (main decarboxylating enzyme in this bromeliad) activity were noticed, indicating that this delay in malate mobilization could not be attributed to insufficient intrinsic decarboxylating capacity but rather to curtailed efflux of malate from the vacuole. These light quality experiments also stress the need to further our understanding of the interaction of different photoreceptors such as phytochromes, cryptochromes and phototropins regarding the temporal coordination of the malic acid cycle in CAM plants. Since PEPCK mediated OAA decarboxylation immediately yields CO_2 and PEP, no additional PPDK activity is needed and only low activity of the latter have indeed been measured in different PEPCK types of CAM plants (Black *et al.*, 1996). Plant PEPCK is cytosolic, as is PEPC, so a reciprocal regulation of both activities is predicted for effective operation of the CAM cycle and to avoid futile cycling of ATP and CO_2. Native PEPCK is phosphorylated in some C_4 leaves and in all CAM leaves studied to date (Walker & Leegood, 1996; Walker *et al.*, 1997). It is hypothesised that phosphorylation might occur at night, rendering the enzyme less active, although a connection between the phosphorylation status of PEPCK and a decreased activity in darkened leaves has only been demonstrated for the enzyme from the C_4 plant Guinea grass (Walker *et al.*, 2002). The kinetic properties of PEPCK in the CAM plant *A. comosus* are subject to modulation by pH and metabolites that include malate, citrate and succinate (Martín *et al.*, 2011), so allosteric regulation of PEPCK-mediated decarboxylation could potentially offer a further layer of control over diel malate mobilization in CAM.

To further investigate whether malate or OAA decarboxylation represent a potential rate limiting processes that could impact malate efflux from the vacuole, we provide a stoichiometric comparison of reported, intrinsic activities of the decarboxylating (related) enzymes with malic acid processing in a range of different CAM species (Table 2). Plants relying on PEPCK mediated decarboxylation such as *Aechmea* 'Maya', *A. comosus*, *Clusia rosea* and *Hoya carnosa* clearly display extractable activities far in excess of those needed to accommodate the observed malate degradation. Values of extractable *in vitro* ME activities in the CAM plants *K. daigremontiana*, *K. pinnata* and *Vanilla planifolia* are more in line with the observed rates of malate breakdown but still seem to have sufficiently high capacity to avoid a rate limiting effect under normal conditions of homeostasis. *In vivo,* however, it is likely that decarboxylating enzyme activity will be modified by cellular conditions such as pH and energy charge and/or by reversible phosphorylation/dephosphorylation. A further literature review of

published values for PPDK activities in different CAM plants show comparable values to those of ME and hence do not seem to confer a rate limiting step in diurnal malate metabolism either (Table 3).

Q3: Does Malate efflux from the vacuole set the pace of decarboxylation?

Malate efflux from the vacuole is still one of the least understood processes in CAM research and different mechanisms of tonoplast transport have been proposed for diurnal malate remobilization. These include passive diffusion, a proton-linked symporter and/or a dedicated malate anion channel (Fig. 2) (Smith *et al.*, 1996). In its undissociated form, $H_2malate^0$ can indeed passively diffuse out of the vacuole but considering the relatively low pK_a of ~3.40, this process is proposed to be relevant only at the lowest vacuolar pH around dawn (Lüttge & Smith, 1984). During the major part of the light period, vacuolar efflux occurs predominantly as $Hmalate^-$ and/or $malate^{2-}$ and a wealth of evidence gathered in the preceding decades indicates an intimate stoichiometry of 2 H^+ : $malate^{2-}$ (or alternatively 1 H^+ : $Hmalate^-$). In addition, a recent integrated diel flux balance analysis (FBA) study by Shameer *et al.* (2018) integrating cytosolic, vacuolar, chloroplastic, peroxisomal and mitochondrial metabolism in a realistic CAM model, also predicts a considerable export of protons out of the vacuole during daytime malate remobilization. These considerations suggest a close relationship between proton and malate efflux out of the vacuole during the major part of Phase III.

Based on investigations in the C_3 model species *Arabidopsis thaliana* the tonoplast dicarboxylate transporter (tDT) has been postulated as a plausible candidate to accommodate diurnal malate remobilization in CAM plants (Emmerlich *et al.*, 2003; Holtum *et al.*, 2005; Borland *et al.*, 2009). More recent work in the CAM species pineapple also showed significant diel cycling of tDT transcripts with peak abundance noted around dawn (Wai *et al.*, 2017). Whilst the exact transport properties of tDT have proved rather elusive, a more recent study by Frei *et al.* (2018) confirmed a specific preference for dianion substrates such as malate, fumarate, citrate and succinate but not α-ketoglutarate, gluconate, sulphate or phosphate. As other related members of the solute carrier family 13 (SLC13) transporters had previously been characterized as sodium carboxylate symporters, it is tempting to propose the existence of tDT mediated co-transport of mal^{2-} and 2 H^+. This would provide both an explanation for concomitant remobilization of malate ions and protons out of the vacuole (Holtum *et al.*, 2005). However, detailed proteolyposome studies by Frei *et al.* (2018) indicated tDT as acting in an

electroneutral manner by employing an antiport mechanism exchanging diverse dicarboxylates such as citrate and malate.

Another potential candidate for accommodating vacuolar malate efflux during the light period is the aluminum activated malate transporter (ALMT). Originally revealed in studies investigating aluminum toxicity, these anion channels have been found to play significant roles in diverse physiological processes such as avoidance of metal toxicity, mineral nutrition, ion balances, turgor regulation, fruit quality and guard cell functioning (Sharma *et al.*, 2016). Originally ALMT's were associated with nocturnal malate accumulation in CAM since initial patch clamp analyses in *K. daigremontiana* were indicative of an inward-rectifying anion-selective channel which imposes unidirectional transport of malate from the cytosol to the vacuole (Hafke *et al.*, 2003). Also in grapes, *VvALMT9* has been found to be constitutively expressed in the mesocarp tissue of berries with a significant upregulation during fruit maturation when malic and tartaric acid accumulate in the vacuoles (De Angeli *et al.*, 2013). However detailed patch clamp experiments by Meyer *et al.* (2011) showed for the first time that AtALMT6 could function either as a malate influx or efflux channel depending on the tonoplast potential, cytosolic malate concentration and the vacuolar pH. Moreover, the particular switch between influx and efflux can occur at physiological membrane potentials, thereby favoring malate export out of the vacuole when vacuolar pH has dropped. Based on these outcomes it can be questioned whether a similar principle could account for both the nocturnal sequestration of malate in the vacuole, which is accommodated by significant acidification and its diurnal remobilization after a certain pH threshold has been exceeded. Recent studies focusing on transcriptional changes over the diel course also seem to highlight a specific role for ALMT concerning CAM functioning (Wai & VanBuren, 2018; Ferrari *et al.*, 2020). These considerations reopen the debate as to whether the malate influx channel in CAM plants can indeed be gated in order to act as an efflux channel at the start of the photoperiod. This interesting hypothesis has already been postulated by Smith *et al.* (1996) but at that time it was believed that the malate channel was strictly inward-rectified thereby only permitting vacuolar malate influx (Iwasaki *et al.*, 1992).

Irrespective of the exact nature of the putative malate transporter, strict coupling is required between the diurnal export of malate and its associated protons out of the vacuole on one hand and the decarboxylation events and cytosolic proton metabolism on the other. Whilst the decarboxylation reactions themselves have been well characterized, the biochemical nature of the most important diurnal proton consuming reactions in the cytosol is still obscure. Recent

FBA CAM models highlight a putative key role for the mitochondrial P_i/H^+ symporter (PiC) to relieve the cytosol from its diurnal "proton pressure" and thus curtail over-acidification (Shameer *et al.*, 2018). In Arabidopsis different isoforms of PiC have been characterized with high abundance in the mitochondrial membrane as predominant carriers to replenish the matrix with inorganic phosphate in order to secure ATP synthesis (Millar & Heazlewood, 2003). Transporters in CAM mitochondria have received much less scrutiny but mitochondrial adenylate and dicarboxylate transporters, belonging to the same mitochondrial carrier protein superfamily (mCP) as PiC, have already been identified in the inducible common ice plant, namely McANT2 and McDCT2 respectively (Kore-eda *et al.*, 2005). Further work in this area is highly encouraged to shed more light on how CAM plants secure cytosolic proton homeostasis during the diurnal period of malate degradation. Due to its relatively small volume, the cytosol is especially prone to over-acidification. In 2011 Ceusters *et al*. showed that a substantial reduction in light availability (from ~3 to 0.5 mol photons m^{-2} d^{-1}) during the photoperiod resulted in chlorenchyma cell death after 8 hours. These observations that specific environmental conditions can bring about uncoupling between malate efflux and its further processing, support our proposed view that the efflux process itself is likely to be dictated by an integration of circadian and metabolic signals which would provide a basis for the synchronization and plasticity of metabolism that is inherent to CAM

Conclusions and future perspectives

Among others, early pioneers in CAM research such as Barry Osmond and Ulrich Lüttge already questioned the enigma of diurnal malate remobilization from the vacuole in CAM plants some decades ago (Osmond, 1978; Lüttge, 2000, 2002).

In the past decade significant technical advances have allowed an unprecedented exploration of CAM genomes, transcriptomes, proteomes and metabolomes (Ming *et al.*, 2015; Yang *et al.*, 2017; Ceusters *et al.*, 2019). Successful implementation of the RNAi approach in the CAM model species *Kalanchoë* by the research group of James Hartwell has provided an experimental means to further unravel the roles of specific enzymes and putative transporters (Dever *et al.*, 2015; Boxall *et al.*, 2020). More recently, John Cushman and colleagues have engineered *Arabidopsis* to over-express several proteins from *M. crystallinum* that accommodate CAM carboxylating and decarboxylating events. Their work showed that malate contents and stomatal conductance patterns indeed can be altered (Lim *et al.*, 2019). However,

several important challenges related to CAM Biodesign (i.e. transfer of the CAM photosynthetic pathway into C_3 or C_4 plants) still need to be addressed. These include mechanisms for achieving appropriate transport, storage and degradation of carbohydrate to fuel PEPC mediated carboxylation as well as appropriate coordination between the diel carboxylation processes. Whilst nocturnal CO_2 fixation (Phase I) is solely mediated by PEPC, the diurnal carboxylation processes are much more complex.

Recently, rapid progress in the advances of implementing CRISPR/Cas9 in CAM plants (Liu *et al.*, 2019), have opened up a novel toolbox to accommodate functional genomics research. However, in order to avoid getting overwhelmed by a plethora of 'omics'data, computational modelling approaches are also becoming increasingly important (Chomthong & Griffiths, 2020). These models are typically based on a bottom up approach and do not require information of the complete transcription-gene regulatory network to provide realistic simulations of plant metabolism (Owen & Griffiths, 2013; Cheung *et al.*, 2014). In 2018 Shameer *et al.* published a two phase CAM model accounting for the diurnal and nocturnal phases and integrating cytosolic, chloroplastic, mitochondrial and peroxisomal metabolism. Efforts are underway to develop a more detailed diel model based on a 2h basis that will capture the dynamics of the four phases of CAM and providean unprecedented level of detail, including the diurnal remobilization of malate.

In conclusion we postulate that the vacuolar efflux of malate itself might be a key candidate for orchestrating the onset and duration/rate of diurnal deacidification in leaves of CAM plants. Controlled release of malate from the vacuole which is influenced by both environmental and circadian cues also offers a mechanistic explanation for the observed plasticity in the magnitude and duration of the daytime Phases. During the early morning Phase II stomates open and malate degradation needs to be curtailed to avoid futile cycling of carbon. Net CO_2 uptake in Phase II can, in some circumstances, occur for several hours dependent on environmental conditions or the species considered (Ceusters *et al.*, 2009, 2010). When malate degradation is initiated due to its controlled release from the vacuole Phase II shifts towards Phase III which is characterized by high intracellular pCO_2 and stomatal closure. Finally, when malate efflux comes to an end, its decarboxylation will slow down, intracellular pCO_2 will drop and stomates will reopen to accommodate direct atmospheric CO_2 uptake in Phase IV. However, more important questions still need to be resolved in the near-future to further unravel the exact interplay of environmental and circadian control on diurnal malate and H^+ remobilization from the vacuole. These include:

- What is the exact identity of the diurnal malate transporter(s)?
- How does vacuolar H^+ efflux occur during daytime?
- What is the contribution of mitochondrial transporters and metabolism to secure diurnal cytosolic proton homeostasis?
- What is the exact role of the different photoreceptors such as phytochromes, cryptochromes and phototropins in mediating environmental control over diurnal efflux of malate?

Acknowledgements

The authors would like to thank Internal Funds KU Leuven for financial support.

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Table 1

Comparison of published values for Rubisco activity (expressed as the amount of incorporated CO_2) with calculated, diurnal malic acid degradation rates in two CAM species. For *Clusia* values are expressed in µmol gFW^{-1} h^{-1} and for *Kalanchoë* values are expressed in mmol m^{-2} h^{-1}.

Plant Species	Malic Acid degradation	Rubisco	References
Clusia rosea	14.0	42	(Borland *et al.*, 1998)
Kalanchoë	12.0	72 – 101	(Maxwell *et al.*, 1999)
daigremontiana	8.64	11 – 16	(Borland & Griffiths, 1997)

Table 2

Comparison of reported, intrinsic activities of the different types of decarboxylating enzymes (ME or PEPCK) with calculated, diurnal malic acid degradation rates, in a range of different CAM plants.

Plant Species	Malic Acid degradation (µmol gFW^{-1} h^{-1})	NAD(P)-ME (µmol CO_2 gFW^{-1} h^{-1})	PEPCK (µmol CO_2 gFW^{-1} h^{-1})	References
Aechmea 'Maya'	3.3	/	33 ± 4	(Ceusters *et al.*, 2014)
Ananas comosus	3.5	6.12 ± 1	151 ± 29	(Christopher & Holtum, 1996) (González-Olmedo *et al.*, 2005)
Clusia rosea	14.0	trace amounts	240	(Borland *et al.*, 1998)
Hoya carnosa	5.7	25 ± 2	126 ± 36	(Christopher & Holtum, 1996) (Martin *et al.*, 2010)
Kalanchoë daigremontiana	6	17 ± 5	ND	(Christopher & Holtum, 1996) (Szymańska & Kruk, 2008)
Kalanchoë pinnata	8.3	20 ± 8	ND	(Christopher & Holtum, 1996) (Jaiswal & Sawhney, 2006)
Vanilla planifolia	5.4	70 ± 17	ND	(Christopher & Holtum, 1996) (Puthur, 2005)

Data of enzymatic activities are means ± standard deviation.
ME – malic enzyme; PEPCK – PEPcarboxykinase; ND – Non detectable

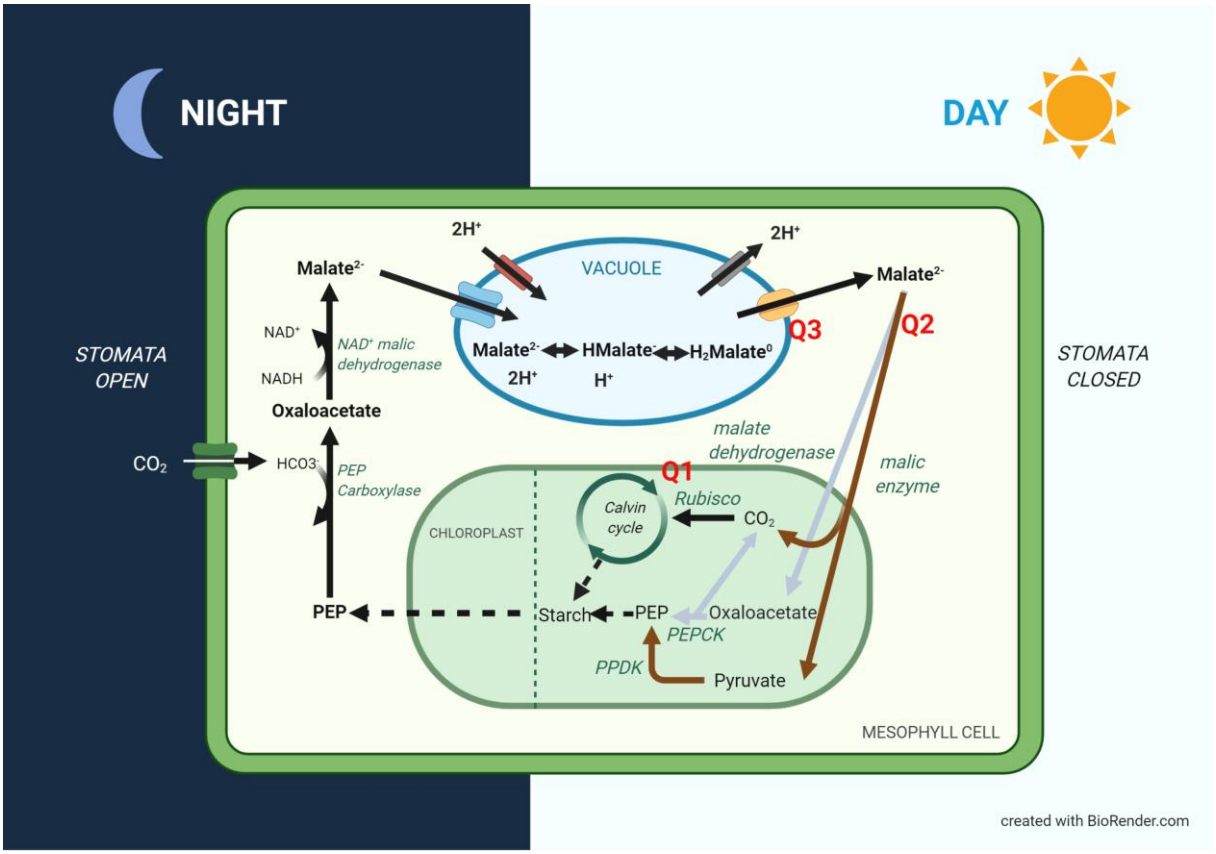
Table 3

Comparison of published values of intrinsic enzyme activity for pyruvate orthophosphate dikinase (PPDK) and NAD(P)-malic enzyme (ME) in different CAM taxa.

Taxa	**PPDK**	**NAD(P)-ME**
	µmol mg^{-1} Chl^{-1} h^{-1}	
Agavaceae	40 -50	43 - 785
Aizoaceae	19	16 - 195
Cactaceae	8 - 200	5 - 920
Crassulaceae	5 - 240	16 - 385
Dracaenaceae	6 - 70	19 - 127

Values based on Black *et al.* (1996) and Kondo *et al.* (2000).

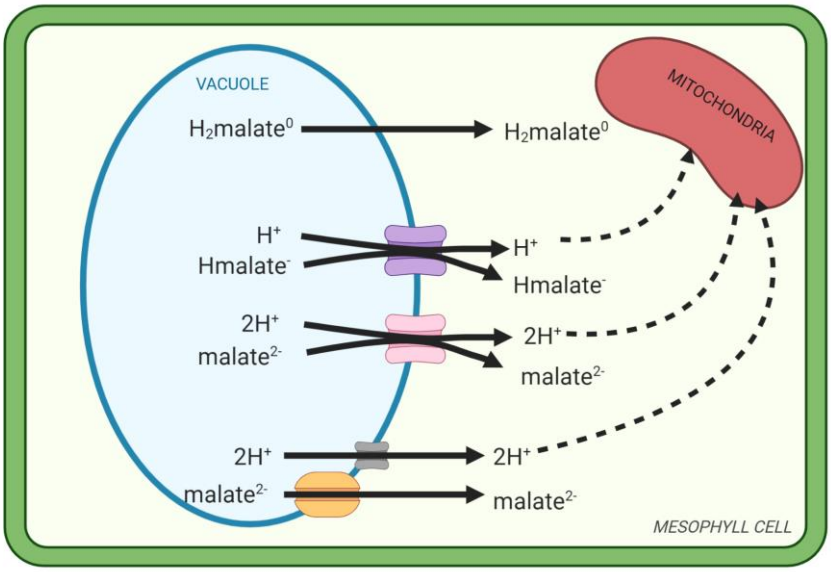
Figure 1



Simplified representation of crassulacean acid metabolism with emphasis on malate metabolism. Dotted arrows represent multiple steps and different species of malate can exist in the vacuole dependent on the pH, i.e. $H_2malate^0$, $Hmalate^-$ and $malate^{2-}$. The mechanism of malate efflux will be discussed in more detail in Figure 2. Three important key questions are addressed in this review i.e. Q1: Does light-dependent assimilation of CO_2 via Rubisco dictate malate mobilization from the vacuole? Q2: Is malate decarboxylation limiting its vacuolar mobilization? Dependent on the species considered, decarboxylation of malate can proceed either via malic enzyme and PPDK (gray arrows) or via malate dehydrogenase and PEPCK (brown arrows). Q3: Does malate efflux set the pace for diurnal malate metabolism?

PEP – phospho*enol*pyruvate; PPDK - cytosolic/plastidic pyruvate orthophosphate dikinase; PEPCK – PEPcarboxykinase

Figure 2



created with BioRender.com

Representation of different hypotheses regarding diurnal malate and proton movements in crassulacean acid metabolism plants namely: passive diffusion of $H_2malate^0$; proton linked symport of $Hmalate^-$ and $malate^{2-}$; and a dedicated malate anion channel for $malate^{2-}$. Whilst $H_2malate^0$ transport is only considered to be meaningful only at the very lowest pH (around dawn) experimental evidence strongly suggests concomitant transport of malate anions (i.e. either $Hmalate^-$ or $malate^{2-}$) and protons out of the vacuole. A putative $H^+/Hmalate^-$ or $2H^+/malate^{2-}$ co-transporter has not yet been identified whilst dedicated malate anion channels have already been characterized such as ALMT and tDT. Recent flux balance analysis models indicate that considerable amounts of protons are consumed by the mitochondria to secure cytosolic proton homeostasis (indicated by dotted arrows).