CAM-Like Traits in C_3 Plants: Biochemistry and Stomatal Behavior

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Contents

Abstract Although it is generally accepted that Crassulacean Acid Metabolism (CAM) originated from C_3 ancestors through a co-option process, this is rarely discussed in terms of specific characteristics and putative mechanisms behind this event. Here we discuss the available data concerning the biochemical and stomatal traits that are present in C_3 plants and could have been enrolled in the CAM cycle. In summary, the biochemical machinery of CAM seems to have originated from a potential stress-driven recruitment of key non-photosynthetic enzymes of the C_3 background which have entrained circadian rhythm. CAM stomatal behavior could be either a direct consequence of an upregulation of the biochemical machinery or it might require additional changes in the signaling/perception pathways controlling stomatal aperture. Considering that CAM has multiple origins, it is likely that each plant group developed it through different combinations of biochemical/stomatal changes, resulting in various degrees of plasticity of this photosynthetic pathway.

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1 Introduction

It has been over 70 years since the term Crassulacean Acid Metabolism (CAM) was used for the first time to indicate the nocturnal acidification observed in species of the genus *Kalanchoë* (Thomas and Beevers 1949; Ranson and Thomas 1960). Ever since, scientists have tried to unravel the mechanisms behind this phenomenon and how it could have appeared along evolution. The emergence of CAM plants multiple times in different taxa and habitats suggests that CAM might have originated by a co-option process in which ancient metabolic pathways were reorganized to generate new functions through modifications in some already-existing key proteins involved in numerous non-photosynthetic processes of C_3 plants (Silvera et al. 2010; Aubry et al. 2011; West-Eberhard et al. 2011; Berry et al. 2013). Apparently, the recruitment of these biochemical elements into the CAM pathway depended on significant increases in the expression of genes involved in both production and transport of C_4-organic acids, as well as alteration in their diel rhythm, coupled with an inversion of stomatal aperture pattern (Taybi et al. 2004; Silvera et al. 2010; Borland et al. 2014). However, a discussion of how elements from C_3 plants could be recruited into CAM is still uncommon. In this chapter we intend to share some insights into the mechanisms that may have led to CAM behavior.

2 General Features of Typical CAM Behavior

The basic C_3 pathway serves as the primary mechanism for the photosynthetic carbon fixation employed by most terrestrial plant species. This mode of CO_2 assimilation, also known as Calvin–Benson cycle, operates with the central participation of RuBP carboxylase-oxygenase (Rubisco, EC 4.1.1.39) as the sole carboxylating enzyme in C_3 plants. Concurrently, in plants performing typical CAM, Rubisco re-fixates the carbon that was previously assimilated by phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) during the nocturnal phase of the CAM cycle (Berry et al. 2013). The atmospheric carbon fixed by PEPC at night is stored in the form of malic acid in the vacuole, also known as Phase I. Then, on the following day, while the stomata remain closed, the malic acid stored during the night is decarboxylated, allowing the CO_2 generated to be photosynthetically reduced in the chloroplasts via Calvin cycle (Phase III), concentrating CO_2 around Rubisco in this phase (Cushman and Bohnert 1999; Dodd et al. 2002; Lüttge 2002, 2004; Keeley and Rundel 2003; Crayn et al. 2004; Silvera et al. 2010; Matiz et al. 2013). Phases II and IV are transitional states between Phases I and III. Phase II occurs at early light period through open stomata, when Rubisco is becoming active while PEPC is being inactivated and CO_2 fixation can happen via both enzymes. In the transition from light period to dark period, the Phase IV occurs, which is characterized by the reopening of the stomata when the storage of

organic acids is already exhausted, allowing atmospheric CO_2 assimilation via Rubisco (Osmond 1978; Dodd et al. 2003; Lüttge 2008; Kluge 2008).

3 Driving Forces of CAM Evolution

It has been proposed that atmospheric CO_2/O_2 ratio reduction in the early Miocene allowed the uprising of CO_2-concentrating mechanisms, such as CAM and C_4 photosynthesis (Ehleringer et al. 1991; Ehleringer and Monson 1993; Raven and Spicer 1996; Winter and Smith 1996; Edwards and Ogburn 2012). Decreasing atmospheric CO_2 had an important impact for terrestrial plants, not only favoring photorespiration by the increasing oxygenase activity of Rubisco (Edwards and Ogburn 2012) but also increasing transpirational cost per unit of carbon fixed (Raven and Spicer 1996; Brodribb and Feild 2010). Thus, by opening stomata during the night and closing them during most of the day, CAM plants achieve a higher water use efficiency than C_3 plants, providing a selective advantage in dry environments (Ehleringer and Monson 1993; Drennan and Nobel 2000; Keeley and Rundel 2003; Winter et al. 2008; Borland et al. 2009, 2014). In fact, terrestrial CAM plants are commonly found in habitats with low water availability, such as deserts and the canopy of tropical forests. Therefore, water deficit and low CO_2 concentrations were important selective driving forces for the emergence of CAM photosynthesis (Keeley and Rundel 2003).

Although the biochemistry of CAM is frequently coupled with nocturnal stomatal opening, sometimes they seem to be independent. *Isoëtes* species, for example, commonly grow in aquatic environments with depleted CO_2 and bicarbonate during the day, due to high photosynthetic activity of the other organisms. As a result, CAM allows the uptake of inorganic carbon (both from water and respiration) at night by storing it as organic acid (Keeley 1985; Ting 1985; Keeley and Rundel 2003). In these taxa the stomata are generally absent or nonfunctional, but all the biochemical machinery of CAM is active. More evidence showing an uncoupling between CAM biochemistry and nocturnal stomatal opening was found in some pseudobulbs and roots of orchids, which are capable of expressing the biochemical reactions of the CAM pathway, but are unable to express typical CAM due to a lack of stomata (Rodrigues et al. 2013). In other words, the biochemistry of CAM may happen despite the absence of stomata or in the case of plants performing CAM cycling and idling, without nocturnal stomatal aperture. In the following sections, it will be discussed how CAM biochemistry could have been selected independently of the nocturnal stomatal opening. Besides, some evidence will be presented about the occurrence of nocturnal stomatal opening without the expression of CAM biochemical machinery. Finally, we are going to address some insights into the potential interactions between the biochemical and stomatal modules operating in the CAM cycle, and their impact on controlling the plasticity of this photosynthetic pathway.

4 Key Biochemical Candidates for Adaptive Selection of CAM-Related Features

Although our current knowledge regarding the evolutionary progression of specific genes selected for CAM expression is somewhat limited, studies of the PEPC gene family have indicated that similar changes to those described for the C_4 pathway might have occurred during the recruitment of non-photosynthetic enzymes from C_3 background into CAM (Silvera et al. 2010). Some important candidates for the evolution of C_4 and CAM photosynthesis appear to involve genes that encode the key enzymes for the carboxylation and decarboxylation processes, such as PEPC, PEPC kinase, malate dehydrogenase (MDH, EC 1.1.1.37), pyruvate orthophosphate dikinase (PPDK, EC 2.7.9.1), and NADH or NADPH-dependent malic enzyme (NADP-ME, EC 1.1.1.40, or NAD-ME, EC 1.1.1.39) (Doubnerová and Ryslavá 2011; Berry et al. 2013).

PEPC activity is thought to be a major factor in limiting the magnitude of the CAM pathway (Taybi et al. 2004); therefore, the essential characteristics of this enzyme should be considered in the context of potential mechanisms involved in determining the evolution and expression plasticity of CAM photosynthesis. In fact, PEPC is a tightly regulated enzyme that is present in the cytosol of all vascular plants and is also broadly distributed in green algae and bacteria. This enzyme represents a crucial regulatory point at a key branch of plant metabolism that confers a highly flexible aspect for synchronizing the carbon partitioning with changing environmental conditions (O'Leary et al. 2011; Shane et al. 2013). The diverse PEPC functions include the regulation of malate production/homeostasis during stomatal conductance modulation, environmental stress responses, and N_2-fixing nodule development in legume roots, among others (Nimmo 2000; Aubry et al. 2011). Furthermore, plant-type PEPCs are particularly relevant for supplying carbon skeletons to the tricarboxylic acid (TCA) cycle, which allows the anaplerotic replenishment of the TCA intermediates redirected for biosynthesis and ammonium assimilation (Gennidakis et al. 2007; Masumoto et al. 2010; Aubry et al. 2011; O'Leary et al. 2011; Shane et al. 2013). O'Leary et al. (2011) considered that, although certainly valid, such a traditional view of the non-photosynthetic PEPC participating only in the replenishment of TCA intermediates oversimplifies the broader contribution of this enzyme to plant metabolism.

In CAM and C_4 plants, PEPC catalyzes the first and pivotal step in CO_2 assimilation which involves the irreversible β-carboxylation of phosphoenolpyruvate (PEP) to yield oxaloacetate (OAA) and inorganic phosphate (Nimmo 2000; Gennidakis et al. 2007). All plant-type PEPCs are regulated by a complex set of posttranslational mechanisms that control their day/night activities, which includes allosteric effectors, phosphorylation, monoubiquitination, and other protein–protein interactions (O'Leary et al. 2011; Shane et al. 2013). Since plant-type PEPCs are allosteric enzymes inhibited by malate and activated by glucose-6-phosphate (Glc-6-P), phosphorylation represents one essential activator of PEPC activity by simultaneously reducing PEPC sensitivity to malate inhibition while enhancing

Glc-6-P activation. In CAM species, therefore, such a posttranslational modification allows this enzyme to overcome feedback inactivation by the end product of nighttime CO_2 fixation (e.g., malate), enabling the abundant nocturnal accumulation of C_4-organic acids required for the proper operation of the CAM cycle (Nimmo 2000; Taybi et al. 2004; Gennidakis et al. 2007; Kluge 2008; Aubry et al. 2011; Berry et al. 2013; Shane et al. 2013).

The PEPC phosphorylation is catalyzed by the presence of a specific Ca^{2+}-independent PEPC protein kinase termed PEPC kinase. In C_3 and C_4 plants, PEPC kinase seems to be activated exclusively by light (Gousset-Dupont et al. 2005; Shenton et al. 2006), while in CAM plants this is a night-specific enzyme whose transcription is mostly under the influence of an internal circadian rhythm (Hartwell et al. 1999, 2002; Nimmo 2000, 2003). Although it is possible that there is a direct connection between the circadian oscillator and the expression of PEPC kinase in CAM plants, through a potential transcription factor directly associated with the endogenous circadian clock, compelling evidence in favor of such a link is still elusive (Nimmo 2000). Another hypothesis concerning the connection between the regulation of PEPC kinase and the circadian clock during the CAM cycle suggests that the circadian rhythm of PEPC kinase expression may be a consequence of fluctuations in the primary metabolism related to the cellular distribution/levels of malate. This hypothesis is based on results showing that the abundance of PEPC kinase transcripts was inversely correlated with cytoplasmic malate concentrations, thus indicating that malate levels could negatively affect PEPC kinase expression and/or its mRNA stability (Borland et al. 1999; Nimmo 2000; Borland and Taybi 2004; Cushman et al. 2008). All together, these evidence indicate that PEPC kinase modulation (by gene expression and/or enzyme activity) might represent one of the strongest candidates required for both the establishment and the maintenance of the core CAM machinery, due to its influence on PEPC expression.

4.1 The Synchronous Modulation of Non-photosynthetic PEPC, MDH, PPDK, and ME by Abiotic Constraints: A "Precondition" for CAM Cycle?

Environmental challenges such as drought, unfavorable temperatures, salinity, and other harsh conditions can considerably hamper the photosynthesis in most plants due to consequences of stress-induced impairment of the photosystems, which, therefore, limit the CO_2 reduction process and can generate oxidative stress (Ashraf and Harris 2013). However, these challenging conditions might have contributed to the adaptive recruitment of specific non-photosynthetic enzymes from the C_3 background into photosynthetic functions in C_4 and CAM plants (Silvera et al. 2010; Berry et al. 2013; Cowling 2013). The selective recruitment of non-photosynthetic genes to a photosynthetic role generally involves modifications

in their default C_3-expression patterns that cause greatly enhanced transcription levels, thus leading to the accumulation of their respective proteins in leaves (Hibberd and Covshoff 2010; Langdale 2011; Williams et al. 2012; Berry et al. 2013). Böcher and Kluge (1978) have already suggested that a pathway of carbon flow similar to CAM could be established in some C_3 plants. In fact, it is generally accepted that CAM evolved through increased expression of C_3 genes involved in both production and transport of organic acids (Taybi et al. 2004).

Some essential components for the CO_2-concentrating process during CAM cycle, such as representatives of the families PEPC, MDH, PPDK, and ME, frequently show increased expression and/or activities in virtually all plants under various types of abiotic constraints (Gonzalez et al. 2003; Aubry et al. 2011; Doubnerová and Ryslavá 2011; Langdale 2011; Cowling 2013). As illustrated in Fig. 1, it is suggested that these enzymes under adverse conditions can form an alternative cycle, which may confer adaptive metabolic adjustments for C_3 plants exposed to challenging environments (Doubnerová and Ryslavá 2011). The coupled activities of PEPC and cytosolic MDH can generate organic acids (such as malate) with important implications in the cellular redox balance (Sriram et al. 2007). Furthermore, the oxidation of malate to pyruvate by ME results in both NAD(P)H production and carbon supply at the involved cellular compartment, which could contribute to a redistribution of the reducing power among different compartments of the cell. Additionally, the combined activities of PEPC, MDH, and ME can form an alternative metabolic flux which provides the ability to respire OAA generated from PEP, instead of relying only on the reaction catalyzed by the cytosolic pyruvate kinase (EC 2.7.1.40) to generate pyruvate. Finally, PPDK activity can regenerate PEP which can be used as substrate for the PEPC reaction. Therefore, this potential alternative cycle formed by PEPC, MDH, ME, and PPDK (Fig. 1) provides and/or redistributes CO_2 and NAD(P)H that can be used by the TCA cycle, antioxidant system, and amino acid metabolism (Doubnerová and

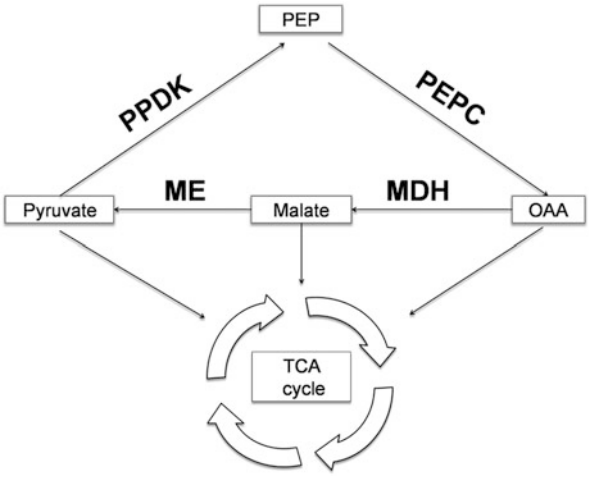

Fig. 1 Scheme of a hypothetical cycle formed by the major key enzymes of CAM in C_3 plants under adverse conditions. PEPC carboxylates PEP, yielding OAA, which undergoes reduction by MDH into malate. Malate is decarboxylated by ME into pyruvate, which, in turn, is converted to PEP by PPDK, closing the cycle. OAA, malate, and pyruvate can be also used to replenish the intermediates of the TCA cycle

Ryslavá 2011; O'Leary et al. 2011; Rodrigues et al. 2014). It is tempting to hypothesize that the recruitment of these metabolic elements used by C_3 plants as a potential strategy to couple with unfavorable conditions, together with the selection of a circadian control of these reactions, might represent important steps for the origin of CAM.

Undoubtedly, a better understanding of the non-photosynthetic roles of these proteins in C_3 species would be useful in predicting the metabolic alterations in a C_3 tissue when components of the CAM pathway are artificially introduced. This is especially relevant when considering that CAM can be interpreted as the most flexible and adaptive photosynthetic pathway and that it has been suggested that economically and ecologically important CAM species should be exploited to support sustainable production in the future(Borland et al. 2011; Cowling 2013). Furthermore, some exciting prospects have been recently envisioned by the scientific community concerning the development of bioenergy feedstocks and food crops engineered with a functional CAM system into C_3 crops (Borland et al. 2014).

5 The Establishment of CAM Stomatal Behavior Could Happen Independently of Biochemistry?

Curiously, stomatal opening during the night does not seem to be exclusive of CAM, as it has already been reported in C_3 and C_4 plants. However, when C_3 and C_4 plants open stomata during nighttime, there is no CO_2 assimilation (Caird et al. 2007), indicating that nighttime stomatal opening in these cases seems to be independent of the enzymatic machinery required for CAM. A recent review pointed out that the possible factors controlling C_3 and C_4 nocturnal stomatal opening may include microclimatic conditions both in soil and in leaves, species-specific variations, and plant and/or leaf age (Zeppel et al. 2013). In the same review, the authors speculate on possible advantages of nocturnal stomatal opening without CO_2 fixation, including embolism removal and nutrient transport (Zeppel et al. 2013). It was also suggested that root temperature may influence nocturnal stomatal conductance in *Vitis vinifera* (Rogiers and Clarke 2013). Interestingly, it was recently discovered that there are specialized stomata in leaves of *Nelumbo nucifera* that open during the night (besides the "normal" ones that open during daytime) and this opening seems to be mainly regulated by darkness (Matthews and Seymour 2013). Undoubtedly, the phenomenon of nocturnal stomatal opening in C_3 and C_4 plants deserves more attention in order to determine its exact consequences for the plant metabolism. Stomatal closure during the day can happen in C_3 plants mainly in response to environmental factors, as will be discussed below.

5.1 Is Stomatal Control in CAM Similar to Its C_3 Counterpart?

As a general assumption, stomata can respond to several environmental factors, such as light, CO_2, drought, pathogens/elicitors, and also endogenous factors, such as circadian rhythm (Klüsener et al. 2002; Chen et al. 2012). Since under drought or pathogen attack both C_3 and CAM plants close their stomata (resulting in CAM idling when CAM biochemistry is present), the differences in stomatal behavior between them are likely to depend on signaling by light, CO_2, or the endogenous clock. For this reason, we will focus on how stomata respond to these factors and the possible changes that may have occurred to yield CAM.

The control of stomata by light, especially in blue wavelength, is already well established for C_3 plants. For instance, AtMYB60 and AtMYB61 are *A. thaliana* transcription factors involved with stomatal control that appear to be regulated by photoreceptors such as cryptochrome and phototropins (Chen et al. 2012). While AtMYB60 is a positive regulator of stomatal aperture and accumulates in the light, AtMYB61 appears to have the opposite function of closing the stomata and accumulates during the dark period (Cominelli et al. 2005; Liang et al. 2005). Additionally, it has been recently shown in *A. thaliana* that the transcription factor ELF3 (*EARLY FLOWERING 3*) is negatively involved in stomatal aperture, while FT (*FLOWERING LOCUS T*) is positively linked to stomatal control (Kinoshita et al. 2011). The same authors suggest that the transcription factor FT either controls an intermediary component in blue-light signaling pathway that mediates stomatal opening or it is the component itself. Interestingly, both ELF3 and FT are also strongly regulated by the circadian clock (Hicks et al. 1996; Covington et al. 2001; Liu et al. 2001; Onai et al. 2004; Hubbard and Webb 2011). In fact, *elf3-201* mutants showed continued open stomata in continuous light with a 50-fold increase in FT expression, while *ft-1* mutants showed continued closed stomata in the same conditions (Kinoshita et al. 2011). Therefore, at least in the C_3 plant *A. thaliana*, light and circadian clock appear to work together to promote the opening of stomata during the day.

CAM plants, however, would require a dampening of stomatal response to light, possibly relying more on circadian rhythms and/or CO_2 levels instead, in order to close stomata during the day. In fact, it was observed that in both *Mesembryanthemum crystallinum* and *Portulacaria afra*, the induction of CAM suppresses the stomatal opening in response to blue light (Lee and Assmann 1992; Tallman et al. 1997). The mechanisms of how this dampening occurs, however, are still unknown. In CAM-induced *M. crystallinum*, an *ELF3* ortholog shows a pattern of expression very similar to that of its C_3 counterpart, accumulating its transcripts during the evening. Therefore, the possible differences between light-regulated stomatal control after induction of CAM do not change expression of ELF3 and, possibly, FT (Boxall et al. 2005). These results indicate that although the central clock remains the same, the output for stomatal aperture in CAM plants may be somehow different from that of *A. thaliana* and other C_3 plants. Mechanisms of

stomatal opening during the night in CAM plants are not known in detail, but it seems there is a strong circadian component since even in continuous light condition stomata of CAM plants continue to open during the subjective night (Wilkins 1984; Lüttge and Beck 1992; Wyka and Lüttge 2003).

5.2 *CO_2 Sensing: The Interaction Between Biochemistry and Stomatal Control*

It has been known that stomata can respond to intercellular CO_2 concentration, but the mechanism underlying this observation still remains largely unknown. It is discussed, for example, whether the guard cells can perceive internal CO_2 directly or whether this gas is perceived by the mesophyll cells (Flexas et al. 2008; Mott et al. 2008; Araújo et al. 2011). In *A. thaliana*, the kinase HT1 (HIGH LEAF TEMPERATURE 1, EC 2.7.11.1) seems to be one of the few components that promotes stomatal aperture and is highly influenced by CO_2 concentrations (Hashimoto et al. 2006). It was also proposed that two carbonic anhydrases (βCA1 and βCA4, EC 4.2.1.1) somehow appear to sense high CO_2 concentrations and promote stomatal closure by inhibiting HT1 activity, indicating that the sensing of CO_2 depends on HCO_3^- generation (Kim et al. 2010). It is important to note that changes in these components affect only CO_2-induced stomatal closure, while stomatal closure in response to the phytohormone abscisic acid (ABA) and blue light remains largely unaffected.

More recently, Merilo et al. (2013) found that OST1 (OPEN STOMATA 1, EC 2.7.11.1), responsible for phosphorylation of SLAC1 (SLOW ANION CHANNEL-ASSOCIATED 1), appears to be essential in CO_2-mediated stomatal closure. SLAC1 was demonstrated to activate Ca^{2+}-dependent slow anion channels and promote stomatal closure (Vahisalu et al. 2008). It is also suggested that there are possibly several points of interaction between the signaling pathways of CO_2, darkness, ozone, drought, and ABA during stomatal closure, including OST1 (Merilo et al. 2013).

Until now, biochemical pathways of stomatal control in charge of sensing intracellular CO_2 concentration were not investigated in plants expressing CAM. Perhaps the expression patterns of HT1 and OST1 orthologs could provide some insight into how CO_2 mediates stomatal behavior in CAM plants; furthermore, since CO_2 could be perceived as HCO_3^-, carbonic anhydrases may also be an important target for research. If there are no changes in these components, then it is probable that malate decarboxylation could generate a sufficiently high internal CO_2 concentration during daytime to induce stomatal closure, while CO_2 assimilation by PEPC during the night may lead to CO_2 concentrations low enough to cause stomata to open during this period (Lüttge 2002; Kluge 2008). Alternatively, stomata of CAM plants could increase their sensibility to CO_2 to follow the organic acid fluctuations.

In a very interesting group of experiments, plants of the CAM species *Kalanchoë daigremontiana* were kept in N_2 during one night, which resulted in a severe

reduction in nocturnal malate accumulation during Phase I (Borland and Griffiths 1997; Borland et al. 1999). The results showed that on the following day CO_2 assimilation values were higher and lasted longer during Phase II. The authors suggested that this effect could be due to higher PEPC activity as a consequence of lower malate content (as malate inhibits PEPC) and activation by PEPC kinase. However, these plants still showed a Phase III and not much difference was detected in Phase IV. Further work in the same species under continuous light showed that the circadian rhythm of CO_2 uptake and stomatal conductance was not heavily affected by nocturnal malate depletion (Wyka et al. 2004).

Von Caemmerer and Griffiths (2009) tested stomatal CO_2 responses in both *K. daigremontiana* and *K. pinnata* by manipulating CO_2 availability during different moments in the CAM cycle and also by depleting intracellular malate accumulated during the night. Interestingly, they found that stomata did not open during phase III, even when combining a lowering of internal CO_2 (reduction in malate accumulation in the previous night) and atmospheric CO_2. They suggest that there must be a signal other than CO_2 that causes stomata to close during phase III. The developmental changes in expression of CAM in *Peperomia scandens*, a plant capable of going from CAM cycling to typical CAM, showed that the stomatal behavior changed regardless of alterations in the amount of organic acids accumulated during the night (Holthe et al. 1987), suggesting that in this species it was not an upregulation of biochemical machinery that caused the changes in stomatal behavior.

Recently, Owen and Griffiths (2013) developed a model to predict CAM behavior based on *K. daigremontiana*, showing that metabolic control may be a major factor in determining the CAM phases. It was also shown that, at least theoretically, it is possible to extinguish phase III with a severe downregulation of malate decarboxylation. Although this model was built mainly over stomatal control by metabolic factors, this leads to the hypothesis that a simple upregulation of CAM biochemistry could generate CO_2 variations high enough to result in CAM stomatal behavior. Accordingly, Kluge (1968) already demonstrated that phase III is shortened under high light due to more rapid consumption of nocturnally stored malate, resulting in earlier stomatal opening for phase IV than in low light.

Gathering all these observations, it is still not clear whether the stomatal behavior of CAM plants could simply be a consequence of the biochemical machinery (generation of CO_2 variations large enough to supplant other stimuli) or whether it would require changes in other control mechanisms (abolishment of opening in response to light, inversion of circadian rhythms, increased sensitivity to CO_2, etc.). More likely, both factors contribute differently in each species, conferring different degrees of plasticity. A biochemistry-driven stomatal control could probably result in a more rapid and plastic expression of CAM, allowing a species to be capable of going from C_3 to CAM and back in response mainly to the environment. Examples of this plasticity are rare so far, as it was only confirmed that species such as *Calandrinia polyandra*, *Clusia pratensis*, and *Clusia minor* are capable of such event (Lüttge 2008; Winter et al. 2008; Winter and Holtum 2014). On the other hand, species such as *M. crystallinum* are not capable of returning to a

C_3 state once CAM has been established (Winter and Holtum 2007; Winter et al. 2008), perhaps due to permanent changes in stomatal control. Undoubtedly, even irreversible CAM plants show some degree of metabolic control over stomatal aperture that confers some plasticity regarding the strength of CAM.

6 Conclusions and Perspectives

The discussion presented in this review, although still speculative to some extent, raises some interesting questions that deserve further attention in future research. It is still not known whether the stomata of CAM plants function, in terms of perception and response to signals, are similar to those of C_3 plants. We believe that permanent changes in stomatal behavior would lead to a less plastic CAM. The understanding of circadian clock elements and their functions is definitely vital for the comprehension of how crucial enzymes such as PEPC, MDH, ME, and PPDK started to show diverse patterns of activity along the day/night cycle. A key point seems to rest on understanding the upstream controllers of PEPC kinase expression and activity.

A very interesting subject of study is the so-called C_3-CAM facultative plants. Winter et al. (2008) demonstrated that the switch from C_3 to CAM can occur in response to the environment as well as to ontogeny, in a degree that varies with the plant species: some species are heavily affected by the environment, while others rely mainly on ontogeny, with numerous behaviors between these extremes. These massive changes in metabolism could answer some questions as to how CAM stomatal behavior is achieved: is it simply through upregulation of the biochemical machinery or through changes in perception of signals related to stomatal control? Does the biochemical machinery consist of specific isoenzymes for CAM or does it originate from the same isoenzymes present in the C_3 mode?

The answers to those questions would certainly lead to important targets to work on engineering CAM into C_3 crops, allowing these plants to grow in semiarid habitats and, therefore, increase agricultural production (Borland et al. 2014).

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