Abstract
The occurrence, activity and plasticity of the CAM pathway is described from an introductory viewpoint, framed by the use of the four ‘Phases’ of CAM as comparative indicators of the interplay between environmental constraints and internal molecular and biochemical regulation. Having described a number of ‘rules’ which seem to govern the CAM cycle and apply uniformly to most species, a number of key regulatory points can then be identified. These include temporal separation of carboxylases, based on the circadian expression of key genes and their control by metabolites. The role of a circadian oscillator and interplay between tonoplast and nuclear control are central to maintaining the CAM cycle. Control of reserve carbohydrates is often neglected, but the importance of daily partitioning (for growth and the subsequent night-time CAM activity) and use at night is shown to drive the CAM cycle. Finally, it is shown that the genotypic and phenotypic plasticity in patterns of CAM expression is mediated partly by environmental conditions and molecular signalling, but also by diffusive constraints in succulent tissues. A transformation system is now required to allow these key areas of control to be elucidated.

Key words: CAM, carboxylation, circadian control, metabolite partitioning.

Introduction
Crassulacean acid metabolism (CAM) occurs in approximately 6% of higher plant species (Winter and Smith, 1996) yet suffers from being perceived as a minor photosynthetic pathway, restricted to a small number of highly specialized desert-dwelling plants. Although CAM species are of relatively small economic importance (e.g. vanilla, pineapple and agave) as opposed to C₃ and C₄ crops, this is to lose sight of the many intellectual challenges that CAM poses. The pathway is subject to popular dogma that all CAM plants fix atmospheric CO₂ exclusively at night, whilst the stomata remain closed during the day. Although this may be true for a minority of plants, the flexibility of the pathway, coupled with the diversity of CAM species, ensures a far wider spectrum of response, ranging at the extremes from no net CO₂ uptake (CAM-idling) to atmospheric CO₂ fixation continuously through the 24 h period.

It is the aim of this review to describe the CAM cycle at a level accessible to readers at all degrees of interest in CAM and to highlight the extent of diversity and plasticity of expression. Finally, an attempt is made to explain these plastic responses at the levels of metabolic, molecular and circadian control set in an environmental context.

Taxonomy and diversity: the extent of the problem
CAM in the terrestrial angiosperms is thought to have diversified polyphylogenetically from C₃ ancestors sometime during the Miocene, possibly as a consequence of reduced atmospheric CO₂ concentration (Raven and Spicer, 1996). There is strong evidence that the evolutionary direction has been from C₃–CAM intermediates to full CAM (Pilon-Smits et al., 1996), paralleled by specialization and colonization of new, increasingly arid habitats, i.e. that photosynthetic plasticity has led to speciation (Lüttge, 1996; Kluge et al., 2001).
CAM is found in five taxonomic classes, comprising monocots and dicots, encompassing 33 families and 328 genera (Smith and Winter, 1996), although distribution within the angiosperms will have been adjusted significantly by the suggested revision to higher plant phylogeny (‘deep green’ project, details may be found at the following web site, http://jcps.berkeley.edu/bryolab/greenplantpage.html). These totals incorporate both terrestrial and aquatic angiosperms, together with gymnosperms, including the enigmatic gnetophyte Welwitschia mirabilis. As subtleties in the characterization and determination of CAM develop (Holtum and Winter, 1999; Crayn et al., 2001), together with an increasing number of surveys of groups with a high proportion of CAM representatives (e.g. Orchidaceae, Bromeliaceae and tropical epiphytic ferns), it is likely that the current figure of around 16 000 CAM species will prove to be a considerable underestimate.

Although CAM is unequivocally an adaptation to drought tolerance in terrestrial species, it is manifested in a diverse array of species and life-forms, which makes generalizations about the pathway difficult and dangerous.

What is CAM?

At the simplest level, CAM is a photosynthetic system in which the operation of C₃ (ribulose-1,5-carboxylase: Rubisco) and C₄ (phosphoenolpyruvate carboxylase: PEPC) carboxylases occur within a common cell, with enzyme activity separated temporally. Clearly, CAM requires tight regulation and a number of putative control points will be outlined in this section and discussed in further detail below. Traditionally, diel CAM has been defined within a four-Phase framework as illustrated for well-watered plants of Kalanchoe daigremontiana (Osmond, 1981; Fig. 1A). This scheme is appropriate for a number of species and environmental conditions and will be used as a basis for the generalized overview of CAM provided in Fig. 1A. In this example, the Phases were largely defined on the basis of changes in leaf malate concentration and it is apparent that a clear agreement was not found with patterns of gas exchange. Thus even in a well-characterized species, several parameters should be examined and caution must be exercised in categorizing Phase delineations.

During the night (Phase I), atmospheric and/or respiratory CO₂ is fixed in the cytosol by phosphoenolpyruvate carboxylase (PEPC). The 3C substrate, phosphoenolpyruvate (PEP) is formed by the glycolysis of storage polysaccharide or soluble sugars formed during the previous day (Fig. 2). As such, nocturnal CAM activity imposes a significant carbon demand in addition to growth requirements and it is likely that metabolite flux between the day and night represent a significant control point for the pathway. PEPC is activated by phosphorylation of serine residues, mediated by phosphoenolpyruvate carboxylase kinase (PPCK1: Fig. 2), an enzyme which is recognized as being under circadian control and synthesized de novo each night. Thus, activation of PEPC is regulated at the level of transcription and represents a crucial control point (Nimmo, 2000). The 4C final product, malic acid is stored in the dominant, central vacuole. Malate²⁻ moves passively into the vacuole down an electrochemical gradient through a specific channel, following the active transport of protons via a vacuolar H⁺-ATPase pump (Lüttge, 1987; Smith et al., 1996). During the following day, malate exits the vacuole passively (Lüttge and Smith, 1984). It is feasible that the pronounced tonoplast Phase shifts between an influx and efflux system may represent the central oscillator of observed circadian rhythmicity in CAM (Lüttge, 2000).

Decarboxylation (Phase III) may occur through the single or combined action of three carboxylases: NADP-ME, NAD-ME and PEPC, a feature which is broadly species-dependent (Christopher and Holtum, 1996, 1998).
Decarboxylation generates 3C PEP or pyruvate and CO$_2$ (Fig. 2) at a high internal partial pressure of CO$_2$ ($p$CO$_2$), which is often sufficient to result in stomatal closure. Gluconeogenic recovery of storage carbohydrate imposes a high energetic cost on the pathway, which is supported by increased rates of photosynthetic electron transport during Phase III (Maxwell et al., 1999; de Mattos and Lüttge, 2001) and the rate of decarboxylation is strictly light-dependent. CO$_2$ is fixed via the Calvin cycle with triose phosphate apportioned between growth and the provision of carbon skeletons for Phase I (Fig. 2). In well-watered plants, decarboxylation is flanked by two transitional Phases (Fig. 1A). Phase II at dawn, marks the switch between C$_4$ and C$_3$ carboxylation. PEPC is deactivated in the morning by dephosphorylation, which renders the enzyme sensitive to malate inhibition (Winter, 1982; Nimmo et al., 1984). The co-ordination of carboxylases is essential to the efficient functioning of the CAM pathway if futile cycling of CO$_2$ is to be avoided. Phase II is often characterized by a period of atmospheric CO$_2$ uptake which largely appears to be dominated by PEPC (Fig. 1A; Griffiths et al., 1990). Rubisco remains at a low activation state until PEPC is dephosphorylated (Maxwell et al., 1999). Following the termination of decarboxylation, the stomata may re-open and atmospheric CO$_2$ fixation commences via the C$_3$ pathway (Phase IV), with a major proportion of carbon fixed partitioned for export and growth (Borland et al., 1994; AM Borland and AN Dodd, unpublished observations; Figs 1A, 2).

**Plasticity**

Plasticity in expression of these Phases is a ubiquitous feature of the majority of CAM plants. CAM is intimately linked with the environment and can be perturbed by temperature, light level and water status. Given the predilection of CAM species for arid environments, the probability of disruption and the necessity for photosynthetic plasticity is therefore extremely high.
At the specific level, the four Phases described above provide a convenient framework within which to describe CAM, however, it must be emphasized that this scheme is probably the exception that proves the rule and enormous variation in the patterns of diel CAM photosynthesis are commonplace. An extreme example is found in the atmospheric epiphytic bromeliad Tillandsia usneoides (Spanish moss). As shown in Fig. 3, when grown and measured under constant light, Phase III is extremely truncated and essentially atmospheric CO₂ fixation occurs throughout the 24 h period. Moreover, PEPC sensitivity to malate decreases only extremely slowly during the photoperiod and carbon gain could be mediated by both C₃ and C₄ carboxylases for most of the day (Fig. 3).

With a few notable exceptions (Nobel, 1988; Kluge et al., 1992, 2001) even the most-impossible (sensu Kluge and Brulhart, 1996) CAM species will respond to the environment by adjusting expression of the CAM pathway. As demonstrated in Fig. 1A, under well-watered conditions and constant light Kalanchoe daigremontiana exhibits the classic four Phases of CAM. However, following a short period of drought stress, the pattern of the Phases has been subtly adjusted (Fig. 1B) such that Phase II has been reduced to 1 h and Phase IV CO₂ uptake is lost. Obviously the response can be explained in terms of reducing transpirational water loss under conditions of water deficit, but equally result in significant reductions in carbon gain. Interestingly, mild drought resulted in an increase in the magnitude of CO₂ fixation at night (Fig. 1B) possibly, representing a short-term mechanism to compensate for reduced diurnal supply of carbon. It should be noted that upon rewatering the four-Phase condition is quickly restored, highlighting the photosynthetic flexibility of this archetypal CAM species.

C₃-CAM intermediates possibly exhibit the greatest plasticity in the expression of CAM (Ting, 1985; Kluge et al., 2001). Such species possess an inherent capacity for the induction of CAM depending on environmental conditions and, in particular, water availability. For example, Sedum telephium is a temperate species which grows in shallow soil in conditions whereby water availability fluctuates seasonally. When well-watered, the plants exhibit negligible nocturnal uptake but may refix respiratory CO₂ (Borland and Griffiths, 1990). If droughted, a reduction in relative water content accompanies an increasing contribution of nocturnal uptake as a consequence of an increase in content and activity of a CAM-specific isoform of PEPC over a timescale of days (Smirnoff, 1996; Ting et al., 1996). During the early stages of drought, low levels of malate are synthesized and pCO₂ generated during Phase III may not be sufficient to close stomata fully, although a midday depression of photosynthesis is often apparent, such that atmospheric CO₂ fixation proceeds continuously over 24 h (Borland and Griffiths, 1996). The induction of CAM is considered a stress response which maintains a positive carbon balance (Martin, 1996; Borland and Griffiths, 1996; Herrera et al., 2000; de Mattos and Lütğte, 2001).

The induction and highly plastic expression of CAM in the dicotyledonous tree Clusia minor has been exceptionally well-documented. CAM is rapidly (within one day) and reversibly induced in response to temperature, high light (Haag-Kerwer et al., 1992) and drought stress (Borland et al., 1992; Franco et al., 1992; de Mattos et al., 1999; de Mattos and Lütğte, 2001). It has been demonstrated most elegantly that, when opposite leaves on the same node were subjected to contrasting VPD, CAM was induced only in the leaves exposed to dry air (Schmitt et al., 1988). In marked contrast, the induction of CAM in annual species, such as the well-characterized halophyte Mesembryanthemum crystallinum is protracted and irreversible.

Plasticity in expression of CAM has been extensively explored in surveys of the Crassulaceae (Teeri et al., 1981; Kluge et al., 1991, 1993). At the family level, these data relate to the magnitude of nocturnal CO₂ fixation, which tends to be greater in thicker-leaved, more succulent species (Teeri et al., 1981; Winter et al., 1983) that inhabit arid areas and are evolutionarily more derived (Kluge and

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**Fig. 3.** Diel gas exchange (A), apparent activation state of PEPC (B) provided as the concentration of malate required for 50% inhibition of maximal activity, Kᵢ; (■) and the initial (●) and total activity (○) of Rubisco (B) for the epiphytic CAM bromeliad Tillandsia usneoides. Measurements were made over a diurnal course with a 12 h photoperiod with an incident PFD of 200 μmol photon m⁻² s⁻¹ and day/night temperature of 25 °C and 18 °C, respectively. (Enzyme activities were performed after Maxwell et al., 1999.)
Brulfert, 1996; Kluge et al., 2001). Recent investigations in this laboratory have revealed that thinner-leaved species such as Kalanchoë pinnata are highly plastic in photosynthetic expression and behave like C₃ species, both in terms of the duration of diurnal atmospheric CO₂ uptake and light use efficiency. By contrast, thicker-leaved, more succulent relatives suffer from extreme CO₂-diffusion limitation (Maxwell et al., 1997) and are more rigidly bound to nocturnal CO₂ fixation for the daytime supply of carbon (K Maxwell, unpublished observations).

The CAM cycle displays a series of distinct features that occur at specific points during the 24 h cycle, which is sometimes termed the diel cycle. The diel cycle normally comprises the nocturnal dark-period and the diurnal light-period. However, the specific term ‘diurnal rhythm’ is normally used in reference to the entire diel cycle when the cycle includes a light-period and a dark period. This is distinct from a circadian rhythm, which exhibits 24 h periodicity and is free-running under constant environmental conditions. In the following sections an attempt will be made to explain, firstly, how CAM is regulated and, secondly, how plasticity in expression is modulated by the environment at the molecular and metabolic levels.

**Metabolic ‘rules’ and ‘regulations’ that govern the CAM cycle**

To gain an understanding as to how the plasticity in expression of the CAM pathway allows such a wide range of responses, some basic rules regarding metabolic and molecular control of the CAM cycle must first be examined.

**Temporal separation of carboxylase activity**

The temporal separation of CAM CO₂ uptake into four main Phases, with discrete carboxylase activity, presents a complex system that requires tight metabolic control to prevent futile cycles of carbon. Clear temporal separation of metabolic and transport processes must be maintained, yet this system also incorporates significant flexibility for modulating the supply and consumption of carbon in response to environmental perturbations. The enigma of metabolic control in CAM plants is further compounded by inclusion of all enzymes required for C₃ and C₄ metabolism within a single photosynthetic cell. Table 1 summarizes current thinking with respect to factors which may regulate and thus co-ordinate PEPC and Rubisco activity over the 24 h CAM cycle.

(i) **Regulation of PEPC:** Setting of the CAM Phases and temporal separation of carboxylase activity appears to be regulated by a combination of environmental and endogenous circadian signals. An endogenous rhythm in the rate of dark CO₂ uptake was first reported for...
Bryophyllum fedtschenkoi. 40 years ago (Warren and Wilkins, 1961). Currently, the most highly characterized circadian component of the CAM cycle is the diel change in flux through PEPC, which is mediated by reversible protein phosphorylation. During the day, PEPC is dephosphorylated and strongly inhibited by malate (Table 1; Nimmo et al., 1984, 1986; Grams et al., 1997); at night, the enzyme is phosphorylated and much less sensitive to this inhibition (Table 1; Nimmo, 2000). Regulation of PEPC by reversible phosphorylation tends to restrict C₄-mediated CO₂ uptake to Phase I and early Phase II (Fig. 4) and thus curtails the futile cycling of CO₂ from simultaneous malate synthesis and breakdown during the day when Rubisco dominates carboxylation (Table 1). PEPC phosphorylation status is controlled predominantly by the activity of a specific PEPC-kinase, which is regulated exclusively at the level of transcript abundance (Fig. 4; Table 1; Hartwell et al., 1999; Taybi et al., 2000). As such, PEPC kinase transcript abundance appears to regulate the rate of nocturnal CO₂ uptake tightly (Fig. 4; Table 1).

(ii) Regulation of PEPC kinase: The circadian pattern of PEPC kinase gene expression (Ppck1) exhibits a substantial degree of flexibility, which may explain the plastic nature of CO₂ uptake rates during Phase I and Phase II in response to shifting environmental conditions. Ppck1 expression can be modified by metabolic status leading to the possibility that cytosolic malate can act as a feedback regulator of kinase expression and thus override circadian control of phosphorylation (Borland et al., 1999a). Alternatively, a metabolite upstream of PEPC, such as PEP, could act as a feed-forward activator of kinase expression, as suggested for the induction of Ppck2 by photosynthetic in legume root nodules (Zhang et al., 1995). Furthermore, a protein has recently been discovered in Kalanchoë fedtschenkoi that reversibly inhibits PEPC kinase (Table 1; Nimmo et al., 2001a), which may inhibit the low kinase activity present under conditions where rapid flux through PEPC is not required (Nimmo et al., 2001a).

(iii) Regulation of Rubisco: As outlined above, PEPC is subject to exquisite levels of regulation over the diel course, however, comparable information with respect to Rubisco is just beginning to unfold. Preliminary results have indicated that the activation of Rubisco is exceptionally protracted over the diurnal course, with activity beginning to increase at the onset of decarboxylation to a maximum in Phase IV (Maxwell et al., 1999; Griffiths et al., 2002). Thus activity increases when pCO₂ is high and competition for CO₂ (as cytosolic HCO₃⁻) by PEPC is negligible (Table 1). Evidence from this laboratory suggests that the delayed activation is achieved both through the action of inhibitors during Phase II (Maxwell et al., 1999) and delayed synthesis of Rubisco activase protein (Griffiths et al., 2002). The latter observation indicates that, like PEPC, Rubisco may primarily be regulated at the level of transcription of an effector (Table 1).

Rubisco activase activity is regulated through reductant of the large subunit via ferredoxin-thioredoxin reductase (Zhang and Portis, 1999) and therefore rates of photosynthetic electron transport (Jₑ) influence Rubisco activation (Table 1). Low Jₑ observed under constant saturating PFD during Phase II in the face of high rates of CO₂ assimilation (de Mattos et al., 1999) are probably diagnostic of both continued PEPC activity at dawn and a low Rubisco activation state (Maxwell et al., 1999). Equally, both PEPC and Rubisco are strongly influenced by substrate concentrations. The supply of ribulose-1,5-bisphosphate (RuBP) requires a sufficient Jₑ to regenerate substrate in line with enzymatic demand and is therefore predicted to be limiting during Phase II, when light use is minimal (Table 1).

Circadian and environmental regulation of CAM
Whilst circadian expression patterns of many genes, such as cab (Millar, 1999) are regulated by molecular negative feedback loops termed molecular oscillators, the circadian control of PEPC kinase is likely to be tempered by secondary responses to changes in metabolite concentrations (Nimmo, 2000; Nimmo et al., 2001b). It is possible that tonoplast permeability represents the beat oscillator or ‘master switch’ for circadian regulation of CAM (Lüttge, 2000). The tonoplast of K. daigremontiana mesophyll cells exhibits circadian behaviour of malate retention under constant darkness (Rascher et al., 1998).

**Fig. 4.** Diel CO₂ exchange for juvenile leaves of 6-week-old *M. crystallinum* that was previously watered with 500 mol m⁻³ NaCl for 6 d. Semi-quantitative RT-PCR amplification of PEPC kinase transcripts (Ppck1, GenBank X13660) reveals a tight relationship between rate of Phase I CO₂ uptake and Ppck1 transcript abundance.
Endogenous oscillations in the amount of transcripts for subunit c of the V-ATPase have been found in *M. crystallinum* (Rockel et al., 1997), but it is not known whether the amount or activity of these transporters are subject to circadian regulation. By contrast to malic acid uptake by the vacuole, malic acid efflux is a passive process (Lüttge and Smith, 1984). Regulation of the switch between net malic acid influx, to net malic acid release remains a key constraint to our understanding of the diel co-ordination of the CAM Phases.

The CAM cycle cannot function under a regime of continuous light, and as such is not regulated exclusively by the circadian system. Under continuous light, daytime Rubisco activity persists through the dark period until the onset of the subjective Phase II, when a circadian signal resets the activity of Rubisco activity to match the Phases of the ensuing subjective light period (Buchanan-Bollig et al., 1984; AN Dodd, AM Borland, H Griffiths, unpublished observations). Diel co-ordination of the CAM cycle is, therefore, regulated by a complex interaction between circadian behaviour derived from metabolite feedback loops and environmental cues.

**Control of reserve carbohydrates**

Tight regulation between the amount of reserve carbohydrate retained within leaves, and the carbohydrate exported for growth, ensures that sufficient carbohydrate is retained within the leaves at the end of the light period to furnish the PEP that is required nocturnally to fuel PEP carboxylase during Phase I.

(i) *Nocturnal carbohydrate metabolism*: CAM plants exhibit considerable biochemical diversity in the carbohydrate species which are degraded at night, ranging from cytosolic mono, di- or oligosaccharides to chloroplastic starch (Christopher and Holtum, 1996, 1998). Nocturnal degradation of carbohydrates generates PEP, the 3C substrate for PEPC, and respiratory CO₂ which may be refixed to a greater or lesser extent by PEPC. It has generally been assumed that negligible amounts of carbohydrates are translocated at night and that the growth of CAM plants is fuelled by the selective translocation of soluble sugars formed during Phase IV (Winter, 1985). However, stoichiometric considerations indicate that in some CAM species the net export of carbohydrate occurs by night as well as day (AM Borland, AN Dodd, unpublished observations). Thus, mechanisms which distinguish the carbohydrates required for the generation of PEP, respiratory CO₂ and export at night represent an enigma in terms of metabolic control.

The partitioning of assimilates derived from C₃ photosynthesis and those from decarboxylation of C₄-derived organic acids into discrete pools of carbohydrates during the day has been suggested as a possible mechanism for facilitating control of carbohydrate metabolism in CAM plants (Deleens and Garnier-Dardart, 1977; Borland et al., 1994). This compartmentation could provide a means by which the metabolic pathways that constitute CAM and those that fuel growth, could be regulated independently of one another.

In general, the dark reactions of CAM require elevated rates of carbohydrate degradation in comparison to C₃ counterparts (Keiller et al., 1987). CAM induction in *M. crystallinum* is accompanied by substantial increases (10–20-fold) in the activities of several amylolytic and phosphorolytic starch-degrading enzymes, including α- and β-amylases, starch phosphorylase and glucanotransferase (Paul et al., 1993). CAM induction in *M. crystallinum* is also accompanied by a diel rhythm in activity of starch phosphorylase and various chloroplastic transporters (Paul et al., 1993; Häusler et al., 2000). More recent findings have proposed that a circadian oscillator co-ordinates the timing of carbohydrate degradation with the phosphorylation of PEPC at night. Genes encoding β-amylase and starch phosphorylase show parallel diel expression in *M. crystallinum* and maximal levels of expression precede the up-regulation of PEPC kinase (AN Dodd, AM Borland, H Griffiths, unpublished observations). The patterns of expression are consistent with patterns of enzyme activity/starch degradation, suggesting that circadian regulation of starch degradation could ensure precise control of carbohydrate breakdown which, in turn, determines flux through PEPC.

(ii) *Diurnal carbohydrate metabolism*: Whilst the attention of CAM biochemists has tended to focus on elucidating the multiple levels of control that regulate dark CO₂ uptake, it is apparent that the daytime processes of decarboxylation, gluconeogenesis, C₃ photosynthesis, and carbohydrate partitioning are also subject to co-ordinated control. As in C₄ plants, CAM species may be distinguished by the enzymes which catalyse organic acid decarboxylation, i.e. NAD or NADP malic enzymes or PEP carboxykinase (PEPCK). The induction or enhancement of CAM expression by water deficit has been shown to increase the amount and extractable activities of the relevant decarboxylases in *M. crystallinum* and *Clusia* species (Holtum and Winter, 1982; Borland et al., 1998). However, environmentally induced increases in the decarboxylation rate of organic acids *in vivo* is not always accompanied by a corresponding change in the extractable activity of decarboxylases, as found for malic enzymes in some *Sedum* species (Brulfert et al., 1988; Conti and Smirnoff, 1994) and for PEPCK in pineapple (*J Delahuntly, AM Borland, unpublished observations*). Such findings raise the possibility that: (a) malic acid efflux from the vacuole, rather than decarboxylation, is the rate-limiting step in daytime organic acid breakdown or (b) alterations in the rate of organic acid
breakdown might be achieved by post-translational modification of the decarboxylating enzymes. Cook et al. reported differences in the kinetic properties of NAD-malic enzyme between mitochondria isolated during the day and night from K. fedtschenkoii (Cook et al., 1995). It was suggested that changes in aggregation state of the enzyme, together with differential binding of effectors, contributed to reducing flux through the mitochondrial malic enzyme at night. However, it is not known if these changes are under diurnal or circadian control. The other major decarboxylase in CAM species, PEPCK, has been shown to undergo reversible phosphorylation with the phosphorylated enzyme present at night when, presumably, decarboxylation is curtailed (Walker and Leegood, 1996). There is the intriguing possibility that one protein kinase could phosphorylate both PEPC and PEPCK in vivo and thereby prevent the futile cycling of malate synthesis/degradation.

The carbon skeletons derived from organic acid decarboxylation are used for the synthesis of carbohydrates, a significant proportion of which are retained as reserves to fuel PEPC the following night whilst the remainder may be exported and used for growth. Whilst the dark reactions of CAM have been shown to take precedence over export (Mayoral et al., 1991; Holtum and Osmond, 1995), how CAM plants anticipate the night-time requirements for carbohydrate is unclear. It has been suggested previously that diurnal changes in the concentration of the regulator metabolite Fru-2,6-P₂ may play a key role in partitioning assimilates between storage reserves and export (Fahrendorf et al., 1987). However, manipulation of Fru-2,6-P₂ concentrations in transgenic K. daigremontiana had negligible influence on CAM fluxes associated with the daytime mobilization of malate (Truesdale et al., 1999). It seems more likely that control of assimilate partitioning between reserves for CAM and export will be achieved by a complex diurnal interplay of chloroplastic and vacuolar transporters together with metabolite-mediated fine control of key metabolic enzymes (Keiller et al., 1987; Häusler et al., 2000).

**Integration of CAM carbohydrate fluxes across the diel cycle**

Within the framework of each diel cycle, signal transduction events occur that relay changes in environmental conditions to the plethora of control points which govern plasticity within the four Phases. During the C₃–CAM transition in intermediate species such as *Mesembryanthemum crystallinum*, induction of CAM-specific isoforms of genes that encode enzymes essential for the CAM cycle, such as *Ppc1* (PEPC, Cushman et al., 1989) and *Pgh1* (enolase, Forshoefel et al., 1995) establishes the metabolic template upon which the CAM Phases become imposed. Equally, other C₃–CAM intermediates such as *Clusia minor* exhibit a flexible and indeterminate transition between varying degrees of CAM in response to short and long-term changes in environmental conditions (Borland et al., 1992; Roberts et al., 1997). The shifts in fine and coarse metabolic control that lead to enhancement of CAM occur in response to signal transduction events that are associated with a general transition from a relatively unstressed to a stressed growth environment. For example, the transition from a long to short photoperiod which initiates a C₃–CAM transition in *Kalanchee blossfeldiana* (Brulfert et al., 1988) or the salinity, drought and ontogenetic induction of CAM in *Mesembryanthemum crystallinum* (Winter and Ziegler, 1992). These important, slow, shifts between ‘more C₃’ and ‘more C₄’ modes of carbon fixation belie intriguing optimizations to the CAM Phases on a diel basis, which are governed by issues of carbohydrate supply and demand within the leaf.

It is an inherent feature of CAM that the magnitude of Phase I carboxylation influences the amplitude and duration of the subsequent daytime Phases and vice versa. A number of studies have shown that if CO₂ uptake during the night is high, subsequent daytime atmospheric CO₂ fixation may be reduced, and vice versa (Medina and Delgado, 1976; Fischer and Kluge, 1984; Roberts et al., 1997). The close positive relationship noted between integrated PFD over Phases II–IV and subsequent dark CO₂ uptake (Nobel and Hartsock, 1983) can be attributed to enhanced decarboxylation and daytime accumulation of carbohydrate reserves for CAM under higher PFD. This close coupling between the four Phases of CAM requires a mechanism for (a) ‘anticipating’ the night-time requirements of dark carboxylation and (b) ‘compensating’ any short-fall in dark CO₂ uptake by modulating the amplitude and duration of the subsequent day-time Phases.

Growing evidence suggests that two fundamental levels of control are responsible for coupling the Phases of CAM. Firstly, control by a circadian oscillator, which serves to synchronize and optimize CO₂ flux in anticipation of regular, periodic changes in the environment. Secondly, metabolite control modulates the output from the circadian oscillator to fluctuations in both internal and external CO₂ supply, thereby acting as a compensatory mechanism for adjusting CO₂ uptake over the various Phases of CAM in the face of a rigid endogenous clock cycle. Experimental evidence in support of this dual control has been provided by physiological manipulations of internal CO₂ supply. By preventing the overnight accumulation of malate under N₂ in *K. daigremontiana*, the amplitude and duration of CO₂ uptake by PEPC in normal air the following morning was considerably increased because PEPC kinase mRNA and activity and the phosphorylation status of PEPC were higher than in control conditions and remained so for longer
The experiments demonstrate that whilst a circadian oscillator serves as the ‘on’ switch for PEPC phosphorylation, metabolites, probably cytosolic malate, can modulate the degree of phosphorylation and thus regulate the amount of CO₂ taken up during Phases I and II.

Such metabolic control of PEPC would require an adequate supply of carbohydrates, and in Fig. 5, the effect of carbohydrate depletion following exposure to CO₂-free air for 24 h in *M. crystallinum* is explored. Integrated CO₂ uptake during Phase I (Fig. 5), when compared before (peak A) and after treatment with CO₂-free air (peak B), is reduced in exact stoichiometric proportions to starch reserves and PEP availability (Dodd, 2001). In the ensuing 24 h period, an increase in Phase I CO₂ uptake occurred (peak C), as carbon skeletons for PEP synthesis could now be partitioned from the previous daytime CO₂ fixation, at the likely expense of carbon export. Furthermore, in carbohydrate-depleted leaves of *M. crystallinum*, an increase in PEK kinase (*Ppck1*) transcript abundance was observed towards the end of Phase I and through Phase II, but net CO₂ uptake was curtailed, due to substrate limitation (Dodd, 2001). Thus, whilst the elevated and extended expression of *Ppck1* did not increase flux through PEPC under these circumstances, the results imply that sensing of the leaf carbohydrate pool sizes drives subtle shifts in the relative contributions of the four Phases to leaf carbon gain.

**Conclusions: interplay between CAM plasticity with environmental cues**

The framework of the CAM Phases is not a rigid compartmentation, from either a theoretical or practical point of view, but allows the intricacy and plasticity inherent to the CAM cycle to be described in a comparative manner. The fact that the molecular and biochemical regulation of the CAM circadian cycle can be entrained by metabolites (Borland *et al.*, 1999a, Nimmo, 2000; Nimmo *et al.*, 2001b) is the basis for shifts in the pattern of the CAM cycle in response to environmental cues. Thus, the response to slight drought stress shown in Fig. 1B led to the loss of Phase IV, a reduction in Phase II and an increase in net CO₂ fixation at night. Of course, this is phenotypic plasticity in CAM expression, and shifts in the patterns of CAM also occur genotypically. Thus, more rigid expression of CAM, with minimal Phase II and IV gas exchange, is seen in the most succulent lifeforms such as Agaves, cacti and *Kalanchoë beharensis*, contrasting with the range of expression from *Tillandsia usneoides* (Fig. 3) and though to the rapid induction of CAM in *Clusia* spp (Borland *et al.*, 1992, 1999b; de Mattos *et al.*, 1999).

It has often been noted that the magnitude of Phase I acid accumulation is related to light intensity of the previous day (Nobel and Hartsock, 1983; Borland *et al.*, 1999b), and how sensing and partitioning of carbohydrates may control this response has been described. Additionally, some species, which accumulate large concentrations of citric acid (such as *Clusia minor*: Borland *et al.*, 1992) may also use the daytime degradation of citrate as a means to signal daily light intensity and hence stimulate CAM activity (Roberts *et al.*, 1997; Borland *et al.*, 1999b). Energetic requirements for CAM have recently been elegantly described using chlorophyll fluorescence in the field and laboratory when light intensity is not in excess (e.g. as a diagnostic tool, rather than being dominated by photoinhibitory responses: Maxwell *et al.*, 1999; de Mattos *et al.*, 1999; Griffiths *et al.*, 2002). Thus, the energetics by day (as measured in Fig. 5. Net CO₂ exchange for juvenile leaves of 6-week-old *M. crystallinum* before, during, and after treatment for 24 h with CO₂-free air. See text for CAM-form explanations relating to arrows A, B and C; values below arrows indicate integrated net CO₂ uptake during each Phase I.)
in the electron transport rate, $J_o$, allow ATP supply to match RuBP regeneration early in Phase II and distinguish PEPC- and Rubisco-based carboxylation (Maxwell et al., 1999). At night, the shuttling of a considerable proportion of the malic acid through mitochondria prior to accumulation in the vacuole, allows some to be completely metabolized and meet ATP demands for malate accumulation (Osmond et al., 1988).

Because CAM has evolved convergently in such a diverse array of lifeforms, driven by diffuse limitations in aquatic plants and, perhaps, via succulence itself (as well as drought) in many terrestrial life-forms (Maxwell et al., 1997), alternative uses of this plasticity can be seen in many species. Thus, for Tillandsia usneoides, CO$_2$ uptake can be optimized across a 24 h cycle (Fig. 3) and many C$_3$–CAM intermediates show continuous uptake of CO$_2$. Conceptually, this can be rationalized by the interplay between the operation of stomata and the extremely high carboxylation capacity maintained within CAM tissues (Griffiths et al., 2002). During ‘Phase III’, should the rate and magnitude of CO$_2$ regeneration match Rubisco carboxylation, then internal CO$_2$ will not build up to a concentration which causes stomata to close, and net uptake from the atmosphere will continue (Fig. 3).

Ultimately, whilst K. daigremontiana can be transformed (Truesdale et al., 1999), it is now important to seek to unravel the interplay between environmental cues and molecular and biochemical regulation, particularly for a system not confounded by salt stress (as in Mesembryanthemum). Such approaches would also allow resolution of the interplay between control exerted from the tonoplast and by nuclear gene expression (Lüttge, 2000; Nimmo, 2000), and an understanding of whether so many of the genes regulated by circadian transcription patterns drive, or are driven by, the integrated expression of the CAM cycle.

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References


