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Review

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The photosynthetic plasticity of crassulacean acid metabolism: an evolutionary innovation for sustainable productivity in a changing world

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Summary

The photosynthetic specialization of crassulacean acid metabolism (CAM) has evolved many times in response to selective pressures imposed by water limitation. Integration of circadian and metabolite control over nocturnal C_4 and daytime C_3 carboxylation processes in CAM plants provides plasticity for optimizing carbon gain and water use by extending or curtailing the period of net CO_2 uptake over any 24-h period. Photosynthetic plasticity underpins the ecological diversity of CAM species and contributes to the potential for high biomass production in water-limited habitats. Perceived evolutionary constraints on the dynamic range of CO_2 acquisition strategies in CAM species can be reconciled with functional anatomical requirements and the metabolic costs of maintaining the enzymatic machinery required for C_3 and C_4 carboxylation processes. Succulence is highlighted as a key trait for maximizing biomass productivity in water-limited habitats by serving to buffer water availability, by maximizing the magnitude of nocturnal CO_2 uptake and by extending the duration of C_4 carboxylation beyond the night period. Examples are discussed where an understanding of the diverse metabolic and ecological manifestations of CAM can be exploited for the sustainable productivity of economically and ecologically important species.

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I. Introduction

Crassulacean acid metabolism (CAM) is a striking example of convergent evolution that substantially improves plant water use efficiency by enabling partial or predominant uptake of CO_2 at night; at least 343 genera in 35 plant families (i.e. *c.* 6% of higher plants) are known to engage in this photosynthetic specialization (Smith & Winter, 1996; Silvera *et al.*, 2010). In essence, CAM is expressed on a background of Rubisco-mediated CO_2 fixation via the engagement of phosphoenolpyruvate carboxylase (PEPC) for nocturnal uptake of CO_2. The malic acid accumulated overnight is subsequently broken down to release CO_2 that is fixed by Rubisco during the following day behind closed stomata, thereby conserving water and resulting in water use efficiencies that exceed those of C_4 and C_3 plants by at least three and sixfold, respectively (Nobel, 1996). Over the 24-h CAM cycle, the main periods of stomatal opening (night) and stomatal closure (middle part of the day) are punctuated by periods of variable duration where stomata open for direct uptake of CO_2 at the start and end of the day.

The temporal separation of C_3 and C_4 carboxylation processes that defines CAM provides plasticity for optimizing carbon gain and water use in response to changing environmental conditions by extending or curtailing the period of net CO_2 uptake over a 24-h period (Dodd *et al.*, 2002). At an ecological level, it has been proposed that such photosynthetic plasticity opens up new opportunities for subsequent diversification and speciation, as evidenced by the exploitation by CAM species of hot and arid climates (e.g. semi-deserts), semi-arid regions with seasonal water availability (e.g. Mediterranean climates), or microclimates characterized by intermittent water availability (e.g. tropical epiphytic habitats; Silvera *et al.*, 2005, 2010). It has also been advocated that the magnitude of biomass productivity of agronomically important CAM species (e.g. pineapple (*Ananas comosus*) and *Agave*) owes much to the plasticity in deploying both C_3 and C_4 carboxylation to enhance the magnitude and duration of net uptake of CO_2 over a 24-h cycle in resource-limited environments (Nobel, 1996; Borland *et al.*, 2009; Holtum *et al.*, 2011).

In the light of climatic data that show an increase in the frequency of drought over the past century and with climate models predicting further impacts of drought as well as substantial variation in its global occurrence (IPCC, 2007; Portmanna *et al.*, 2009), CAM plants might be predicted to play increasingly important roles in terms of carbon sequestration and sustainable biomass production in a changing world (Borland *et al.*, 2009; Davies *et al.*, 2011). The aim of the current review is to provide an overview of the metabolic basis of plasticity in the deployment of C_3 and C_4 carboxylation processes in CAM plants and to assess evidence for selective and evolutionary constraints on this photosynthetic plasticity. The mechanistic basis of such constraints will be considered and links between photosynthetic plasticity and productivity will also be discussed. Examples will be explored where knowledge of the underpinning costs and benefits of photosynthetic plasticity can be exploited for the sustainable productivity of economically and ecologically important CAM species.

II. Defining and describing the plasticity of CAM

The magnitude of CAM expression is determined by species, by the stage of leaf/plant development and by environmental conditions. A number of terms have been used to define modes of CAM expression (Fig. 1). 'CAM cycling' is used to describe the re-fixation of internal

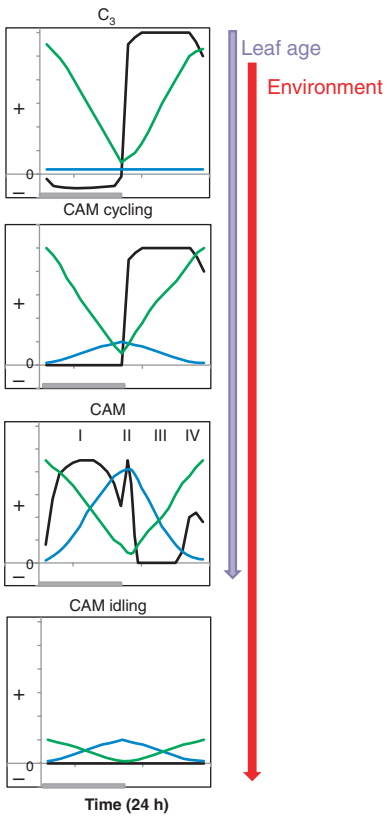

Fig. 1 Modes of crassulacean acid metabolism (CAM) expression illustrated as a continuum of CO_2 acquisition strategies that are determined by leaf age and prevailing environmental conditions (commonly water limitation). Different levels of CAM are indicated by day : night changes in net CO_2 uptake (black line), malic acid (blue line) and carbohydrate (green line) contents. The solid grey bars below the *x*-axes indicate the periods of darkness. 'CAM cycling' describes refixation of respiratory CO_2 at night to provide malic acid, and CAM is shown to consist of four phases of gas exchange, with most net CO_2 uptake at night. 'CAM idling' involves refixation of respiratory CO_2 at night to form malic acid and decarboxylation of malic acid during the day to recover carbohydrate.

respiratory CO_2 at night via PEPC with the resultant malic acid broken down during the day to release CO_2 for Rubisco while stomata remain open. 'CAM' indicates net uptake of CO_2 at night via PEPC with substrate (phosphoenolpyruvate (PEP)) provided by nocturnal degradation of carbohydrates with malic acid accumulated overnight. The main periods of stomatal opening (night) and stomatal closure (middle part of the day) may be punctuated by periods of variable duration where stomata open for direct uptake of CO_2 at the start and end of the day, giving rise to four 'typical' phases of leaf gas exchange in the CAM mode (Osmond, 1978). 'CAM idling' describes the situation where stomata remain closed over the 24-h day : night cycle and there is no net CO_2 uptake but carbon (C) skeletons are recycled through the synthesis of malic acid at night from recapture of respiratory CO_2 and subsequent decarboxylation of this malic acid the following day to recover carbohydrate.

The CAM modes illustrated in Fig. 1 should not be viewed as distinct photosynthetic phenotypes, but rather as a continuum of CO_2 acquisition strategies that are determined by the evolutionary history of a given species and the prevailing environmental conditions (Silvera *et al.*, 2010). CAM cycling may be viewed as the first stage in the shift from C_3 to CAM that occurs in some species as leaves age, or in response to water limitation in species known to exhibit facultative CAM (Cushman & Borland, 2002). However, some species, including many epiphytic orchids and bromeliads and some temperate succulents, such as several of the *Sedum* species, do not progress beyond the CAM cycling stage (Borland & Griffiths, 1990; Martin, 1996).

In general, the mode/magnitude of CAM expression is governed by the stage of leaf development in those species viewed as constitutive CAM and by the environment in those species viewed as facultative CAM. For example, constitutive CAM species such as *Kalanchoë daigremontiana* and *Opuntia ficus-indica* which exhibit CAM photosynthesis from early in development can increase the amount of net dark CO_2 uptake in response to environmental changes such as the imposition of drought, but the magnitude of this facultative component of CAM tends to be curtailed as leaves age (Griffiths *et al.*, 2008; Winter *et al.*, 2008). By contrast, truly facultative CAM plants such as the well-studied *Mesembryanthemum cystallinum* and many species within the tropical tree genus *Clusia* may operate entirely in the C_3 mode when unstressed or in the CAM mode when subjected to drought or salinity (Lüttge, 2006; Winter & Holtum, 2007; Winter *et al.*, 2008). Many CAM species can potentially progress to a state of CAM idling under periods of extended drought, with reports that the resultant recycling of C skeletons behind closed stomata can maintain photosynthetic competence and a positive C balance at the expense of growth for up to 5 yr without water in the case of some desert cacti (Nobel, 1988).

III. Metabolic basis of photosynthetic plasticity

1. Four phases of carbon supply and demand underpin the deployment of C_3 and C_4 carboxylation

Encompassed within the continuum of CO_2 acquisition strategies provided by CAM (Fig. 1) is the metabolic potential to continually adjust and optimize C gain under changing environmental conditions by modulating the proportions of CO_2 taken up by PEPC and Rubisco over the 24-h light : dark cycle. The metabolic processes that underpin plasticity in deployment of C_3 and C_4 carboxylation can be described in four phases (Osmond, 1978). Phase I describes the nocturnal opening of stomata when evapotranspiration rates are low and atmospheric plus respiratory CO_2 is converted to HCO_3^- via carbonic anhydrase. Bicarbonate is then fixed by PEPC and the 3-C substrate PEP is formed from the glycolytic breakdown of starch or soluble sugars, depending on species. Carbohydrate supply is thus a key determinant of the magnitude of dark CO_2 uptake in CAM plants (Dodd *et al.*, 2002). The final 4-C product resulting from nocturnal carboxylation is malate, which is transported into the vacuole, where it is accumulated as malic acid as a result of the high concentration of H^+ generated by the vacuolar H^+-ATPase and/or H^+-PPiase (Bartholomew *et al.*, 1996; Tsiantis *et al.*, 1996). CO_2 uptake and malate accumulation continue for most of the dark period, such that the concentration of vacuolar malic acid can reach > 200 mM by dawn. Vacuolar storage capacity is a further determinant of the magnitude of dark CO_2 uptake in CAM plants (Winter & Tenhunen, 1982; Nelson *et al.*, 2005).

PEPC is activated at night by a dedicated protein kinase phosphoenolpyruvate carboxylase kinase (PPCK), the transcription of which is regulated by the circadian clock (Hartwell *et al.*, 1996, 1999). PPCK phosphorylates PEPC, rendering it insensitive to inhibition by malate (Carter *et al.*, 1991). At the start of the day, phase II is recognized by a surge in the rate of net CO_2 uptake which can be mediated via both PEPC and Rubisco. The degradation of PPCK results in the dephosphorylation of PEPC in phase II, rendering the enzyme more sensitive to inhibition by malate. During phase III, malate exits the vacuole and is decarboxylated through the single or combined action of three enzymes (depending on plant species): NADP malic enzyme (NADP-ME), NAD-ME and phosphoenolpyruvate carboxykinase (PEPCK). In addition to the 3-C product PEP or pyruvate, CO_2 is released at a high internal partial pressure (pCO_2) and is fixed by Rubisco. This is accompanied by stomatal closure and transpirational water loss is curtailed. The high pCO_2 generated via decarboxylation in phase III may limit photorespiration for much of this phase and, while the recovery of carbohydrate via gluconeogenesis in phase III imposes a high energetic cost on the CAM

pathway, the production of substrate for subsequent nocturnal carboxylation and partitioning for growth is ensured. During phase IV, when malate reserves have been exhausted, the stomata may re-open and atmospheric CO_2 may be fixed directly by Rubisco. The carbohydrates that will provide substrates for the nocturnal reactions are retained either in the chloroplast as starch or as sucrose/hexoses within the vacuole, depending on species (Christopher & Holtum, 1996).

2. Synchronizing the supply of and demand for carbon over the 24-h CAM cycle

The four phases of C supply and demand described in the previous section extend the dynamic range of CO_2 acquisition strategies in CAM plants by enabling some species to restrict net CO_2 uptake to the night (e.g. many desert cacti), some species to extend net CO_2 uptake beyond the night but continue phase II for several hours after dawn (e.g. tropical trees of the genus *Clusia*; Borland *et al.*, 1993; Winter *et al.*, 2009), and some species to show continuous net uptake of CO_2 for 24 h, albeit with reduced, but positive, net uptake over the middle part of the day (Borland & Griffiths, 1996; Borland *et al.*, 1998; Lüttge, 2006, 2008). Such plasticity requires robust synchronization of C_3 and C_4 carboxylation processes in order to curtail futile cycling over the 24-h CAM cycle (Borland & Taybi, 2004).

Circadian control of the transcript abundance of PPCK has been proposed to play a cardinal role in setting the phases of CAM by controlling the activation status of PEPC (Nimmo, 2000). In principle, clock control of the degradation of PPCK late in phase I should enable an anticipation of dawn and ensure a rapid inactivation of PEPC at the start of the day in phase II, thereby avoiding futile cycling of malate synthesis and decarboxylation. However, in some CAM species the deactivation of PEPC at the start of the day has been shown to be extremely protracted, such that net CO_2 uptake and malic acid accumulation can continue for several hours during phase II (Borland *et al.*, 1993; Roberts *et al.*, 1997; Haslam *et al.*, 2002; Winter *et al.*, 2009). Experimentally curtailing nocturnal CO_2 uptake and malic acid accumulation by enclosing plants in an anaerobic atmosphere of N_2 gas overnight increased the magnitude and duration of PEPC-mediated phase II net CO_2 uptake in both a constitutive and a facultative CAM species (Fig. 2; Borland & Griffiths, 1997). Thus, PEPC activation status can be modified by leaf metabolic status, so that PPCK transcripts can persist while cytosolic malate is maintained below a threshold concentration (Borland *et al.*, 1999). Such a mechanism provides an explanation for reports that the duration/magnitude of phase II in diverse CAM species is related to the storage capacity of the vacuole for malic acid (Winter & Tenhunen, 1982). High vacuolar storage capacity would be predicted to maintain a low cytosolic [malate]

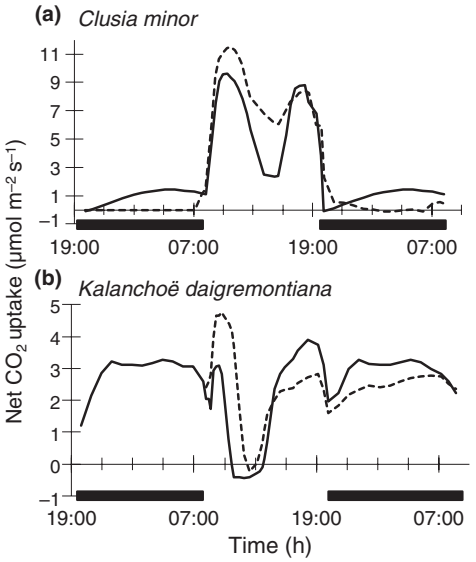

Fig. 2 Enhanced phase II uptake of CO_2 as a consequence of curtailing nocturnal malic acid accumulation in leaves of the facultative crassulacean acid metabolism (CAM) species *Clusia minor* (a) or the constitutive CAM species *Kalanchoë daigremontiana* (b). Leaves were enclosed in an anaerobic atmosphere of N_2 overnight to curtail dark CO_2 uptake. Solid lines, control; dashed lines, $+N_2$. The solid bars on the *x*-axes represent the periods of darkness (redrawn from Borland & Griffiths, 1997, with kind permission from Springer Science+Business Media B.V.).

and thereby curtail feedback inhibition of PEPC-mediated CO_2 uptake at the start of the day. Extending PEPC activation into the photoperiod can compensate plants for any nocturnal shortfall in net CO_2 uptake (Fig. 2) and can make a significant contribution to 24-h C gain, as noted for several species of *Clusia* in the field (Borland *et al.*, 1993; Roberts *et al.*, 1997; Winter *et al.*, 2009). There is also evidence that Rubisco activation status is modulated reciprocally with that of PEPC to curtail competition for CO_2, such that Rubisco activation status is low in phase II and peaks only a few hours before the end of phase IV (Maxwell *et al.*, 1999; Griffiths *et al.*, 2002), implicating the clock in some measure of control over C_3 carboxylation.

The day : night turnover of carbohydrate also determines the magnitude of C_4 carboxylation by providing substrate for PEPC-mediated CO_2 uptake at night. Maintaining growth in the face of dynamic shifts in the deployment of C_3 and C_4 carboxylation over the CAM cycle requires a means of curtailing competition between, on the one hand, the provisioning of substrates for growth and export and, on the other hand, the retention of carbohydrate to provide PEP for C_4 carboxylation (Borland & Dodd, 2002). Evidence is accumulating to suggest that pervasive clock control over carbohydrate partitioning and growth in CAM plants ensures a threshold *potential* for PEPC-mediated CO_2 uptake, irrespective of environmental conditions. Unlike most C_3 plants where growth occurs predominantly at night, in CAM species leaf expansion growth is maximal

in the middle of the day, a phenomenon that persists under constant environmental conditions, indicating circadian control of growth (Gouws *et al.*, 2005; Walter & Schurr, 2005). Moreover, seasonal consistency in the provision of storage reserves for nocturnal CO_2 uptake in a CAM bromeliad grown under contrasting day length, photon flux density (PFD) and temperature regimes (Ceusters *et al.*, 2010) is consistent with the proposed clock control of carbohydrate partitioning over the CAM cycle. Thus, circadian uncoupling of growth (during the day) from degradation of carbohydrates (at night) to provide substrates for PEPC would curtail competition for C skeletons over the 24-h CAM cycle and maintain photosynthetic plasticity. Robust circadian control of transcript abundance of genes implicated in carbohydrate turnover and chloroplast transport of C metabolites has been reported to accompany CAM induction in the facultative species *Mesembryanthemum crystallinum* (Haüsler *et al.*, 2000; Dodd *et al.*, 2003; Koreeda *et al.*, 2005; Cushman *et al.*, 2008). Partitioning of isotopically distinct, C_3- or C_4-derived classes of C pools is also likely to be critical for plasticity in the deployment of PEPC and Rubisco-mediated CO_2 fixation (Borland & Dodd, 2002; Ceusters *et al.*, 2008a).

IV. Ecological and evolutionary significance of photosynthetic plasticity

Field-scale assessments of the ecological and phylogenetic significance of the various photosynthetic manifestations of CAM within communities may be accomplished by analysis of the C isotope composition ($\delta^{13}C$) of photosynthetic tissues. The technique is based on the principle of differential enzyme-mediated discrimination against $^{13}CO_2$ that is exhibited by PEPC and Rubisco. In a study on the constitutive CAM plant *Kalanchoe daigremontiana*, in plants only allowed to assimilate CO_2 at night the $\delta^{13}C$ of leaf organic material was −10.6‰, while plants only allowed to assimilate CO_2 during the light period showed a $\delta^{13}C$ of 25.5‰ (Nalborczyk *et al.*, 1975). The relative proportions of C_3 and C_4 carboxylation processes in CAM species will thus be expected to be integrated by the distribution of $\delta^{13}C$ between these values (Griffiths, 1992; Borland & Griffiths, 1996). These assumptions were refined by Winter & Holtum (2002), who elegantly demonstrated how $\delta^{13}C$ provides a quantifiable index of the proportions of CO_2 fixed via C_3 and C_4 carboxylases over the CAM cycle.

Given the inherent plasticity of CAM for modulating the relative proportions of direct C_3- and C_4-mediated CO_2 uptake and given that whole tissue $\delta^{13}C$ is also affected by diffusional limitations, biochemistry and the isotopic signature of source air, ecological surveys of potential CAM activity within plant assemblages might be predicted to reveal a continuum of isotopic signatures ranging from −10 to −26‰. Instead, numerous studies have revealed a bimodal distribution of $\delta^{13}C$ in various families known to contain both C_3 and CAM species (Fig. 3; Winter & Holtum, 2002; Pierce *et al.*, 2002; Crayn *et al.*, 2004; Holtum *et al.*, 2004; Silvera *et al.*, 2005). Using the relationship between the percentage of nocturnal CO_2 uptake and the $\delta^{13}C$ of newly assimilated C, Winter & Holtum (2002) calculated that the cluster of carbon isotopic signatures around the range of −12 to −16‰ were indicative of CAM species that obtained *c.* 60–75% of their C through PEPC-mediated nocturnal CO_2 uptake (i.e. the 'CAM' mode illustrated in Fig. 1). However, low-level CAM species or C_3–CAM intermediates have $\delta^{13}C$ values that can overlap with those of C_3 species because the majority of CO_2 is fixed by the C_3 pathway. Resolving the relative frequency of CAM species within the first cluster of isotopic signatures (i.e. those within the range −24 to −32‰) requires assessment of the overnight accumulation of titratable acidity as a marker for

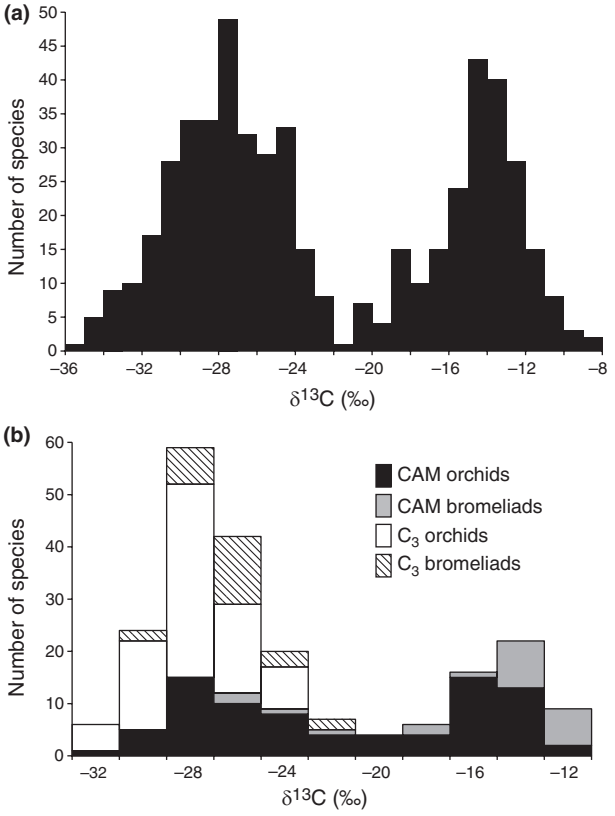

Fig. 3 Bimodal distribution of photosynthetic modes as indicated by (a) the $\delta^{13}C$ of over 500 species from the Asclepiadaceae, Bromeliaceae, Crassulaceae, Clusiaceae, Cucurbitaceae, Didereaceae, Euphorbiaceae, Polypodiaceae and Vittariaceae, families known to contain both C_3 and crassulacean acid metabolism (CAM) (redrawn from Winter & Holtum, 2002; Copyright 2002, reproduced with permission of American Society of Plant Biologists), and (b) frequency of leaf $\delta^{13}C$ values for 165 orchid species and 50 bromeliad species with the presence or absence of CAM identified by day : night measurements of titratable acidity. Values were abstracted from Pierce *et al.* (2002) and Silvera *et al.* (2005).

CAM. Including this information on frequency distributions of isotopic values for 165 orchid species and 50 species of the Bromeliaceae revealed a bimodal distribution of CAM across the entire isotopic range of studied species, with low-level CAM species, with values of *c.* −24 to −28‰, predicted to obtain 5–20% of their C at night (Fig. 3b; Pierce *et al.*, 2002; Silvera *et al.*, 2005).

The bimodal distribution of CAM species across the entire isotopic range implies that strongly or weakly expressed CAM is favoured over intermediate metabolism. Such observations suggest that the degree of photosynthetic plasticity is constrained by environmental selection pressures such that species relying predominantly on strong or weak CAM are favoured based on available ecological niches (Winter & Holtum, 2002). C isotope ratios also reflect water use efficiency (gH_2O transpired g^{-1} above-plus below-ground dry biomass accumulated) as dark CO_2 uptake is associated with lower transpiration rates than C_3 photosynthesis in the light (Winter *et al.*, 2005). Thus, strong CAM species, that is, those that obtain *c.* 65% or more of their C at night, are likely to inhabit severely water-limited habitats. Taxa within this category include the Cactaceae and Agavaceae and other highly succulent taxa. The high vacuolar storage capacity in succulent CAM species will enhance PEPC activity and nocturnal malic acid accumulation and may also enhance water storage, thereby reducing vulnerability to water stress.

The cluster of species with 'weak' CAM probably reflects a more diverse range of niches. Permanently low CAM (or 'CAM cycling'; Fig. 1) is proposed to aid survival during periods of intermittent water shortage when refixation of respired CO_2 at night can supplement net C gain and, in some cases, improve instantaneous water use efficiency (Martin, 1996; Cushman & Borland, 2002). Species with 'weak' CAM can be found growing in shallow soils in temperate regions or as epiphytes in humid tropical forests (e.g. orchid species and bromeliads). In the context of evolution, it has been proposed that weak CAM could serve as a genetic reservoir that facilitates the adaptive radiation of CAM into more arid niches or microclimates, as in the case of epiphytes (Crayn *et al.*, 2004; Silvera *et al.*, 2005, 2009).

The other metabolic scenario for CAM species exhibiting C_3-like C isotopic ratios is for plants to engage predominantly in C_3 photosynthesis but to switch to strong CAM for short periods of time, usually in response to periods of water limitation. This strategy appears to be particularly prevalent in tropical trees of the genus *Clusia*, which show remarkable plasticity for switching between C_3 photosynthesis and CAM in response to changes in environmental conditions (Holtum *et al.*, 2004; Lüttge, 2006). Species such as *Clusia pratensis*, *Clusia cylindrica* and *Clusia minor* are capable of inducing strong (up to 50% of CO_2 fixed at night) and fully reversible CAM but show C isotope ratios typically in the 'weak' CAM range (Borland *et al.*, 1992; Holtum *et al.*, 2004; Winter *et al.*, 2009). In this context, it is important to appreciate that leaf $\delta^{13}C$ is dominated by structural material which comprises at least 50% of leaf dry weight (Borland *et al.*, 1994). Thus, if leaves flush during periods of adequate rainfall, the deposition of structural material will be dominated by a C_3 signal which may obscure the later contributions from C_4 carboxylation, even though these can be substantial. *Clusia* species with this photosynthetic strategy of facultative CAM occupy a diversity of habitats, including those that experience strongly seasonal changes in water availability, and many species exhibit a range of life forms that include trees, shrubs, hemi-epiphytes and epiphytes (Lüttge, 2006).

V. Mechanistic basis of constraints on photosynthetic plasticity

The relative paucity of CAM species with intermediate metabolism suggests the existence of mechanistic constraints to the assimilation of equal amounts of CO_2 through Rubisco and PEPC for sustained periods of time. CAM plants show evolutionary convergence in terms of particular anatomical and metabolic traits and it is feasible that certain of these traits constrain the range of photosynthetic plasticity that is possible.

1. Anatomical determinants of photosynthetic plasticity

Leaf and/or stem succulence is a functional anatomical trait in CAM plants, resulting from the presence of large vacuoles which are needed to store organic acids accumulated during the night but which can also act as water reservoirs. Previous studies have reported positive relationships between succulence and the magnitude of CAM in a taxonomically diverse range of CAM lineages (Nelson *et al.*, 2005; Nelson & Sage, 2008). It has been suggested that succulence is an anatomical trait that might have predisposed ancestral CAM taxa towards the evolution of this photosynthetic specialization in water-limited habitats (Sage, 2002). However, succulence presents a trade-off between functional requirements for C_4 carboxylation and direct C_3-mediated uptake of atmospheric CO_2 by impacting on leaf mesophyll conductance (Maxwell *et al.*, 1997). A higher degree of succulence means less intercellular air space (IAS) between mesophyll cells and a reduction in the length of mesophyll exposed to intercellular air spaces (L_{mes}/area; Smith & Heuer, 1981; Maxwell *et al.*, 1997; Nelson *et al.*, 2005; Nelson & Sage, 2008), traits that reduce internal conductance to CO_2. Reduced internal CO_2 conductance could provide higher photosynthetic efficiency to 'strong CAM' plants which rely heavily on dark CO_2 uptake (phase I) because less CO_2 efflux at night maximizes net nocturnal carbon gain by minimizing the loss of C previously fixed

during the day (Griffiths, 1992). By contrast, reduced internal CO_2 conductance may confer reduced photosynthetic efficiency in 'weak CAM' plants which rely heavily on daytime/late afternoon (phase IV) atmospheric uptake of CO_2, because diffusion through mesophyll limits C availability for Rubisco (Evans & von Caemmerer, 1996; Maxwell *et al.*, 1997; Nelson & Sage, 2008). Thus, the bimodal distribution of weak and strong CAM plants that is indicated by $\delta^{13}C$ may reflect the presence of a compromise between the optimal anatomy for high PEPC activity and the internal structure ideal for C_3 photosynthesis (Silvera *et al.*, 2005). Leaf anatomical measurements gathered for a phylogenetically diverse group of CAM species have indicated that photosynthetic divergence between weak and strong CAM is mediated by the per cent IAS and L_{mes}/area, which collectively present a functional threshold for predominantly Rubisco or predominantly PEPC-mediated net CO_2 uptake (Nelson & Sage, 2008).

The concept of a functional anatomical threshold for the dominance of C_3 or C_4 carboxylation is somewhat confounded by the existence of species such as those belonging to the genus *Clusia*, which show a more comprehensive and dynamic range of carboxylation patterns. Some *Clusia* species are fully C_3 (e.g. *Clusia multiflora*) and others (e.g. *C. minor* and *C. pratensis*) are capable of reversibly switching between C_3 photosynthesis and strong CAM, while other species often considered as strong CAM (e.g. *Clusia rosea* and *Clusia quadrangula*) may exhibit an almost continuous range of CO_2 uptake strategies, as indicated by highly variable $\delta^{13}C$ within individual species (Fig. 4a; Borland *et al.*, 1992; Holtum *et al.*, 2004; Vargas-Soto *et al.*, 2009). The capacity for CAM (per cent dark CO_2 uptake) within eight species of *Clusia* showed an inverse relationship with the anatomical characteristic of L_{mes}/area (Fig. 4b), as found for other CAM species (Nelson and Sage 2008). However, the values of this anatomical parameter within *Clusia* lie much closer to those of C_3 species, rather than CAM species, even though the CAM *Clusia* species included in this study fixed > 70% of CO_2 at night (A. Barrera Zambrano, unpublished observation). A relatively well-aerated mesophyll would help to optimize C_3-mediated CO_2 fixation in *Clusia* species, but adequate vacuolar storage capacity would be required for significant nocturnal C_4 carboxylation.

The leaves of *Clusia* possess clearly differentiated palisade and spongy mesophyll tissues along with an adaxial layer of water storage parenchyma, and it is feasible that a differentiated leaf anatomy presents more options for accommodating a greater range of C_3 and C_4 carboxylations compared with the largely undifferentiated leaf anatomy

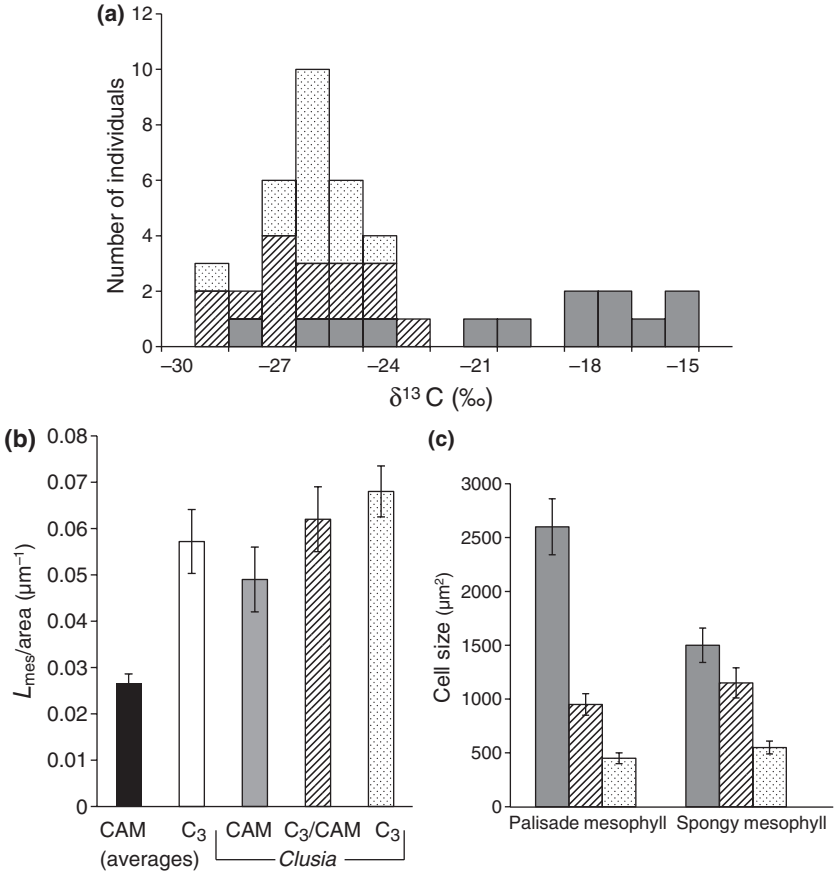

Fig. 4 Photosynthetic plasticity within individual species of *Clusia* that show different capacities for crassulacean acid metabolism (CAM), as indicated by frequency of leaf $\delta^{13}C$ (a). Data were abstracted from Borland *et al.* (2002), Holtum *et al.* (2004) and Vargas-Soto *et al.* (2009). *Clusia rosea* (CAM), grey bars; *C. minor* (C_3/CAM), hatched bars; *C. multiflora* (C_3), dotted bars. (b, c) Leaf anatomical characteristics underpinning this plasticity were examined for eight species of *Clusia* with different modes of photosynthesis and showed (b) variations in the length of mesophyll exposed to intercellular air space (L_{mes}/area) in comparison to average values obtained for different taxonomic lineages of C_3 and CAM species (data abstracted from Nelson *et al.*, 2005) and (c) variations in cell size of palisade mesophyll and spongy mesophyll (CAM, grey bars; C_3/CAM, hatched bars; C_3, dotted bars).

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that is typical of most CAM species (Nelson and Sage, 2005). Measurements of cell sizes in different leaf tissues of eight species of *Clusia* have indicated that the palisade mesophyll cells of strong CAM *Clusia* species are significantly (*c.* 2.5–5-fold) larger than those in facultative CAM or C_3 *Clusia* species (Fig. 4c). Palisade mesophyll depth also showed a significant correlation with CAM strength in the same eight species of *Clusia* and immunolocalization studies indicated that palisade mesophyll cells of *C. rosea* (CAM) contained threefold higher levels of both Rubisco and PEPC proteins compared with the spongy mesophyll cells (A. Barrera Zambrano and E. Olmos, unpublished observations). Enhanced development of palisade mesophyll tissue is commonly found in thicker-leaved C_3 species and is believed to act as a strategy for improving the harvesting of light, thereby helping to offset the increased investment in biomass in thicker leaves (Smith & Hughes, 2009). In CAM *Clusias*, the same strategy of investment in palisade mesophyll cells, together with a relatively high L_{mes}/area, would enhance the capacity for C_3 carboxylation while the enlarged palisade mesophyll cells would also offer potential for significant C_4 carboxylation by serving to accommodate more PEPC protein and large vacuoles to store organic acids overnight. In principle, such anatomical features could accommodate the dynamic range of photosynthetic manifestations that distinguish *Clusia* from many other CAM lineages.

2. Metabolic constraints to photosynthetic plasticity

The metabolic enzymes and transporters necessary for CAM are assumed to be present in the chloroplast-containing cells of all higher plant species. However, CAM species, in comparison to C_3, generally show increased activity and abundance of PEPC protein together with elevated activities of decarboxylases (i.e. PEPCK and $NADP^+$-/NAD^+-ME) and enzymes of the glycolytic and gluconeogenic pathways in order to support the synthesis of the substantial reciprocating pools of carbohydrates that underpin the CAM cycle (Holtum & Winter, 1982; Paul *et al.*, 1993; Borland *et al.*, 1998). The substantial daily reciprocal transfer of C between acids and carbohydrates that underpins CAM also requires extensive and regulated transport of metabolites across membranes bounding the vacuoles, chloroplasts and mitochondria (Holtum *et al.*, 2005).

Analyses of expressed sequence tags and mRNA expression profiling of C_3 and CAM-performing *M. crystallinum* have indicated that salt-induced CAM is accompanied by significant increases in transcript abundance of genes implicated in C_4 acid carboxylation/decarboxylation, glycolysis/gluconeogenesis, carbohydrate biosynthesis/degradation, protein degradation, transcriptional activation, signalling, and transport facilitation (Kore-eda *et al.*, 2004; Cushman *et al.*, 2008). By contrast, CAM induction in *M. crystallinum* was accompanied by a decrease in transcript abundance of genes encoding light-harvesting and photosystem complexes and C_3 photosynthetic enzymes, including Rubisco large and small subunits (Cushman *et al.*, 2008). However, changes in steady-state transcript abundance provide only a partial indication of the potential relative importance of particular enzymes and transporters, because of possible post-translational modifications and *in vivo* regulation of enzyme/transporter activity. This point is illustrated by the metabolite-mediated increase in the contribution of C_4 carboxylation to 24-h C gain (Fig. 2) that did not require any change in the abundance of PEPC or Rubisco proteins (Borland & Griffiths, 1997). Currently, it is not known if increased investment of resources in the protein machinery required for C_4 carboxylation is achieved at the expense of proteins required for C_3 photosynthesis, but such a trade-off scenario could provide a mechanistic underpinning to the bimodal distribution of weak vs strong CAM species.

Another way to address the question of mechanistic limitations to plasticity is to examine molecular processes in 'weak CAM' species which have the potential to engage in 'strong CAM', but only for limited periods of time. *Clusia minor* exhibits such photosynthetic plasticity, having $\delta^{13}C$ in the C_3 weak-CAM range (Fig. 4a), but is capable of accumulating over 1.5 M titratable protons overnight (the highest reported for any CAM species) when CAM is up-regulated in the wet–dry season transition in the tropics (Borland *et al.*, 1992). It has been reported that *C. minor* fares worse than the sympatric constitutive C_3 species *C. multiflora* under periods of extended drought in the field (Lüttge, 1999), implying that photosynthetic plasticity constrains long-term acclimation to severe water limitation. Under laboratory conditions, *C. minor* can increase the per cent of nocturnal uptake from 20% under well-watered conditions to *c.* 75% after 7 d without water (Borland *et al.*, 1998). However, extended drought (up to 12 wk) led to accelerated leaf loss in *C. minor* and the leaves that were retained showed a marked decline in photosynthetic competence compared with leaves of the C_3 *C. multiflora* (Shorrock, 2009).

Using suppressive subtractive hybridization to enrich for genes that show modified abundance under drought has revealed striking differences between the C_3 and facultative CAM *Clusia* species in the major categories of genes expressed under short-term (7 d) drought (Fig. 5). Drought induced more and a greater diversity of genes for metabolism in *C. minor*, compared with the C_3 species, with over 40% of these genes implicated in carbohydrate metabolism, energy processing and membrane transport (Shorrock, 2009). Many of the genes implicated in CAM showed enhanced abundance under drought in *C. minor*, while in the C_3 *C. multiflora*, many of these genes showed a decrease in abundance under drought (Shorrock, 2009). Drought induced more and a greater diversity of genes

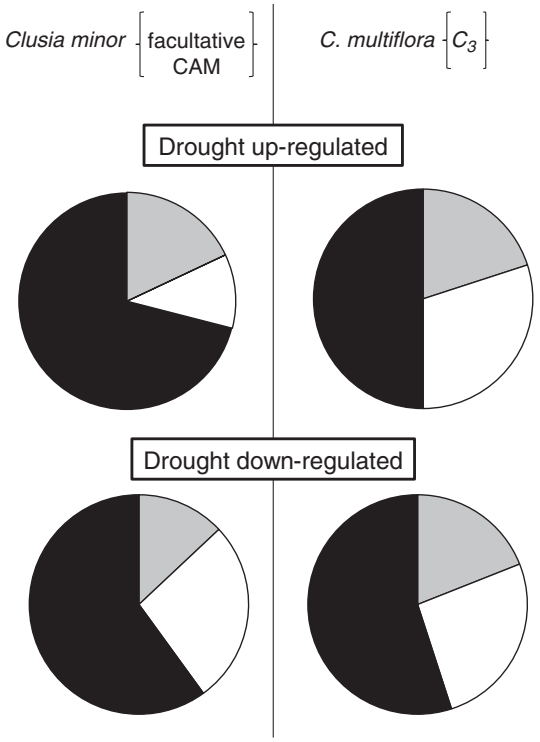

Fig. 5 Drought-induced changes in major categories of genes in leaves of the facultative crassulacean acid metabolism (CAM) species *Clusia minor* and the constitutive C_3 species *Clusia multiflora*. RNA was extracted at two time-points in a 24-h cycle after 2, 4 and 7 d without water and suppressive subtractive hybridization was used to generate four libraries of expressed sequence tags that showed increased or decreased abundance with drought in the two species, with 1000 clones sequenced from each library. Black, metabolism; white, genetic information processing; grey, cell processes and environmental information processing.

within the 'genetic information processing' category in the C_3 species compared with the facultative CAM species (Fig. 5). In *C. multiflora*, genes implicated in protein processing such as chaperones, thioredoxins and proteosome components showed increased abundance under drought while proteases dominated the genes under this category in *C. minor* (Shorrock, 2009).

Changes in transcript abundance can give only a partial indication of the relative importance of particular proteins in contrasting strategies for drought acclimation in the photosynthetically divergent *Clusia* species. However, the data suggest that a protein maintenance strategy, as implied for the C_3 *Clusia* species, would be more conservative in terms of resource use in comparison to the facultative CAM species where up-regulation of genes for primary metabolism would probably involve more costly *de novo* protein synthesis. Leaf loss under extended drought may constitute a drought avoidance strategy for the photosynthetically plastic *C. minor*, which permits conservation of resources as well as rapid regrowth of new leaves (with a predominantly C_3-like $\delta^{13}C$) when water is more readily available. Such a strategy provides another possible mechanistic scenario for the apparent paucity of CAM species that show intermediate metabolism for extended periods of time.

VI. Exploiting the photosynthetic plasticity of CAM for sustainable productivity

Understanding the ecological and mechanistic bases of photosynthetic plasticity in CAM plants has practical applications in terms of optimizing the selection and sustainable cultivation of species for agronomy, horticulture and habitat conservation. Three examples are discussed below.

1. Photosynthetic plasticity under low light: implications for cultivation of ornamentals

The preponderance of CAM species in exposed habitats reflects the energetic costs of the pathway and presents a challenge for sustainable cultivation of economically important ornamental species (e.g. orchids and bromeliads) in northern temperate latitudes. In energetic terms, CAM imposes an additional metabolic cost of *c.* 10% compared with the standard C_3 pathway (Winter & Smith, 1996). These costs arise from the requirements of transporting malic acid into the vacuole at night and from converting the C_3 residue resulting from malate decarboxylation during the daytime back to the level of storage carbohydrate via gluconeogenesis. Limiting light conditions are probably atypical for most CAM species in their natural habitats where daily integrated PFD often exceeds 20 mol photons m^{-2} d^{-1} (Borland *et al.*, 1992). The moderate light conditions (*c.* 8 mol photons m^{-2} d^{-1}) encountered in glasshouses during the brightest months in northern Europe can decline to < 2 mol photon m^{-2} d^{-1} in autumn and winter and can present problems for CAM ornamentals unless one resorts to supplementary lighting. The incidence of necrotic lesions on the foliage of many glasshouse-grown *Aechmea* (Bromeliaceae) and some *Phalaenopsis* (Orchidaceae) cultivars has been attributed to constraints on the operation of CAM under low light (De Proft *et al.*, 2007; J. Ceusters, unpublished observation). Light-limited decarboxylation of malic acid has been linked with localized cell death, as a result of an over-acidification of the cytosol in *Aechmea* under deep shade conditions or during transport (Ceusters *et al.*, 2008b, 2011). However, studies under contrasting light treatments have illustrated how metabolic and photosynthetic plasticity in the deployment of the four phases of CAM can facilitate acclimation to conditions of deep shade in *Aechmea*, preventing necrotic lesions and optimizing biomass productivity throughout the year with minimal requirements for supplementary lighting (Ceusters *et al.*, 2010, 2011).

Extending the short autumn/winter photoperiod experienced in northern Europe with low illumination of 30 μmol m^{-2} s^{-1} was sufficient to maintain the CAM

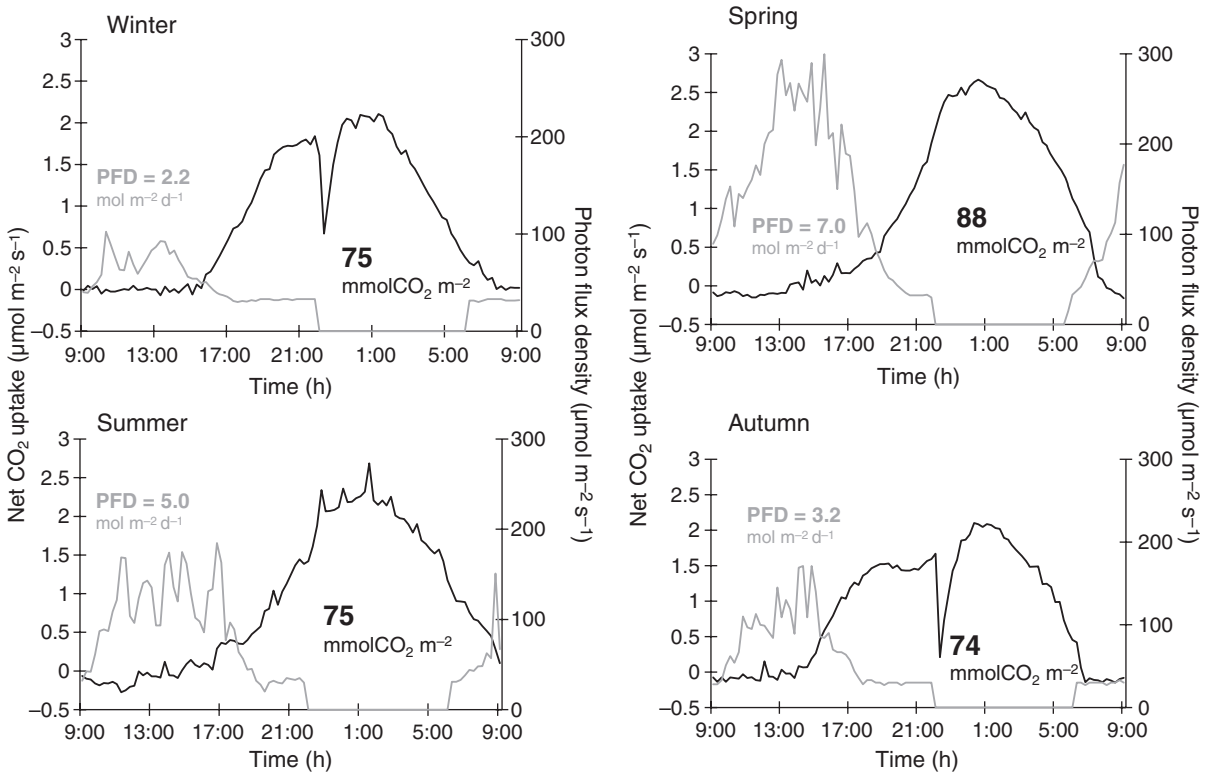

Fig. 6 Seasonal changes in light regime as indicated by integrated photon flux density (grey line) and influence on plasticity in deployment of day- and night-mediated net CO_2 uptake (black line) by the ornamental bromeliad *Aechmea 'maya'* to maintain leaf carbon balance, which is indicated by the values shown for integrated 24-h CO_2 uptake. The figure was redrawn from Ceusters *et al.* (2010), by permission of Oxford University Press.

cycle, prevent cell death and maintain 24-h C gain throughout the year in *Aechmea 'maya'* grown under natural illumination despite large seasonal discrepancies in integrated PFD (Fig. 6; Ceusters *et al.*, 2010). Photosynthetic plasticity in deployment of the C_3 and C_4 carboxylases was evident, with the temporary dip in net CO_2 uptake noted at the end of the artificially extended photoperiod in autumn and winter indicative of a greater contribution from Rubisco to 24-h C gain during the darker months (Fig. 6; Ceusters *et al.*, 2010). Use of a mass balance modelling approach to dissect out the metabolic components of photosynthetic plasticity indicated that constraints on the amount of carbohydrate exported during the day maintained a consistent pool of transient carbohydrate reserves (Ceusters *et al.*, 2010). This gave remarkable seasonal consistency in the amount of storage reserves available at night, thereby optimizing biomass gain throughout the year. The photosynthetic plasticity of CAM allows successful acclimation of *Aechmea* to natural conditions of shade (Skillman & Winter, 1997) as well as to light-limiting conditions under artificially extended photoperiods in glasshouses. These photosynthetic attributes have important practical applications for the sustainable horticultural productivity of other economically important CAM ornamentals in northern latitudes.

2. The photosynthetic plasticity of *Clusia*: applications for reforestation and habitat restoration

The noted ability of many *Clusia* species as early colonizers on a range of semi-arid, nutrient-poor soils has led to their use as nurse plants, allowing the establishment of diverse vegetation islands on marginal land. The CAM species *Clusia hilariana* is an important nurse plant in the coastal restingas of Brazil, an effect attributed in part to the stratification provided by the height and architecture of *Clusia* in these semi-arid habitats, which enhances the activity of seed dispersers such as birds and bats (Dias & Scarano, 2007). *Clusia* also provides a large contribution to biomass stock in nutrient-poor restingas, thus impacting positively on ecosystem services such as C sequestration, biomass productivity and nutrient cycling, which in turn influence recruitment processes and biodiversity (Dias *et al.*, 2006).

The facultative CAM species *C. minor* often grows in disturbed areas and savanna borders and is an important component of the shrubland stage in cloud forest formation. By contrast, the C_3 *C. multiflora* is important in forest gaps and also in both mature and successional forest (Cáceres & Cuenca, 2006). It seems likely that these contrasting niches can be attributed, at least in part, to the contrasting photosynthetic physiologies of these species, and thus knowledge

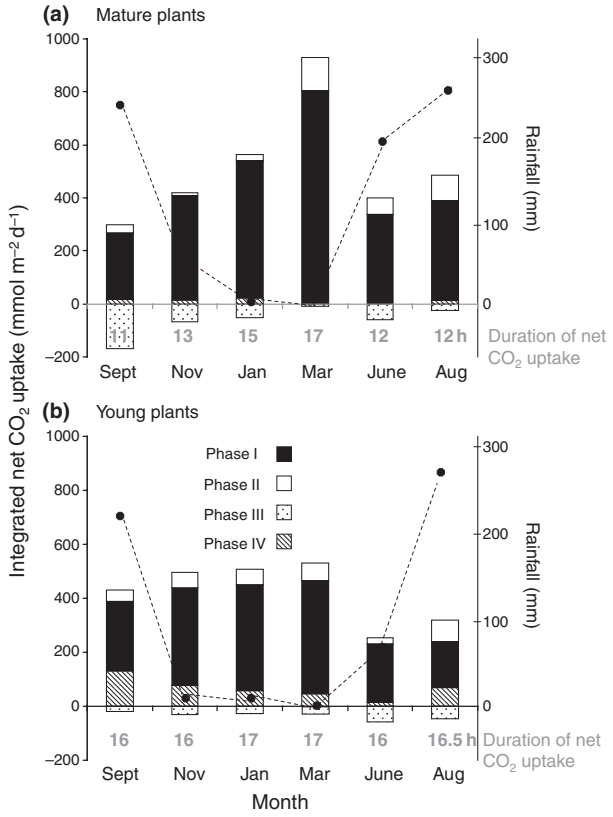

Fig. 7 Seasonal changes in the contributions of the four phases of crassulacean acid metabolism (CAM) to net CO_2 uptake over 24-h periods for (a) mature (5-yr-old) and (b) young (2-yr-old) plants of *Agave tequilana* grown in west-central Mexico in a warm subtropical environment with monthly rainfall patterns shown by the dashed lines. Data were re-calculated from 24-h gas exchange profiles obtained by Pimiento-Barrios *et al.* (2001, 2006).

of the range of photosynthetic manifestations that can be exhibited by different *Clusia* species could be exploited for successful restoration of degraded habitats.

Clusia rosea, often considered a strongly constitutive CAM species, exhibits a facultative component of CAM (Winter *et al.*, 2009) and displays a wide range of $\delta^{13}C$ in nature (Fig. 4a) which implies extensive photosynthetic plasticity in the field. The relevance of photosynthetic plasticity for optimizing the C balance of 3-yr-old shrubs of *C. rosea* during wet and dry field conditions in Panama was assessed via seasonal measurements of whole-canopy CO_2 exchange (Winter *et al.*, 2009). Under well-watered conditions, nocturnally fixed CO_2 contributed *c.* 50% to 24-h C gain on sunny days but the contribution decreased to zero following overcast days. However, compensation by daytime net CO_2 uptake on overcast days meant that 24-h C gain was largely conserved across the range of daily photon flux densities experienced in the field (Winter *et al.*, 2009). The compensation of C balance in low CAM-performing *C. rosea* was largely attributable to enhanced and extended phase II, a situation analogous to that observed in the laboratory-based manipulations of leaf malate content (Fig. 2), indicating the key role of malate as a C balance gauge (Borland & Griffiths, 1997; Borland *et al.*, 1999). The response of *C. rosea* to drought was similarly buffered and reversible increases in nocturnal C gain were shown to offset drought-induced reductions of CO_2 fixation in the light (Winter *et al.*, 2009). Thus, flexible deployment of Rubisco and PEPC over the day : night cycle can maintain the C gain of *C. rosea* throughout wet and dry conditions. The photosynthetic plasticity of *C. rosea* is currently being exploited in a reforestation project in Panama on degraded lands where soil erosion is a problem and conservation of soil water is important (K. Winter, pers. comm.).

3. Plasticity and productivity: *Agave* for high biomass production on marginal land

Succulent CAM species of the genus *Agave* have been cultivated for centuries as sources of alcohol and fibres from rain-fed semi-arid lands. Certain species have been reported to display annual above-ground productivities that are comparable to those of the most water-use efficient C_3 or C_4 crops but with only 20% of the water required for cultivation (Borland *et al.*, 2009). Such characteristics have stimulated interest in the potential of *Agave* as a sustainable source of bioenergy feedstock that will not compete with food and fodder production, while offering the potential for C sequestration on marginal and degraded land (Davies *et al.*, 2011; Holtum *et al.*, 2011). The $\delta^{13}C$ values of *Agave* species are typically in the 'strong CAM' range (i.e. > 70% of C gained at night). However, it has been proposed that atmospheric CO_2 fixed directly by Rubisco for several hours at the end of the day (i.e. phase IV) contributes a substantial proportion of the C skeletons required for growth in high-yielding CAM species (Bartholomew & Kadzimin, 1977; Winter, 1985; Borland *et al.*, 1994). Establishing the extent of direct Rubisco-mediated uptake of atmospheric CO_2 in the field is an important factor to consider in terms of selecting appropriate cultivars/species of *Agave* for geographical regions with contrasting rainfall patterns, PFD and maximum and minimum temperatures.

The magnitude of daily C gain and plasticity in the deployment of C_3 and C_4 carboxylation was shown to depend on plant age in *Agave tequilana* grown under rain-fed field conditions (Fig. 7, data recalculated from Pimiento-Barrios *et al.*, 2001, 2006). Phase I net CO_2 uptake dominated C gain throughout the year, and maximum rates of instantaneous net CO_2 uptake in mature plants (*c.* 25 μmol CO_2 m^{-2} s^{-1}) were *c.* 40% higher than those in young *A. tequilana* (*c.* 16 μmol CO_2 m^{-2} s^{-1}), with the highest rates measured at night (phase I) for mature plants but during the phase IV–phase I transition in young plants (Pimiento-Barrios *et al.*, 2001, 2006).

Maximal daily C gain was highest in the dry season (while moderate day and night temperatures prevailed) for both young and mature plants when the daily duration of net CO_2 uptake was at its maximum (i.e. 17 h d^{-1}; Fig. 7).

The data indicate the importance of extending the period of net C gain beyond the night to achieve maximal C gain (Fig. 7). However, this was achieved by deployment of different carboxylases in the young and mature plants (Fig. 8). Phase IV (Rubisco) made a greater contribution to net C gain in the young plants but phase II showed a better correlation with maximal C gain in the mature plants (Fig. 8). These results can be explained by the increase in leaf succulence (decrease in internal conductance) in older *Agave* plants which is likely to curtail CO_2 availability for Rubisco in phase IV. Phase II will be less affected by low internal conductance to CO_2 if PEPC remains active, given this enzyme's high affinity for CO_2 (HCO_3^-; Maxwell *et al.*, 1997). Leaf succulence would appear to represent a key trait for optimizing C gain in *Agave* by serving not only to buffer water availability when rainfall is low but also to provide a high vacuolar storage capacity for malic acid which maximizes nocturnal PEPC capacity and the potential for extending PEPC activation for several hours into the day. Extending the duration of phase II has a further positive influence on 24-h C gain by delaying the onset of phase III decarboxylation until the warmest, brightest part of the day (Borland & Griffiths, 1996). This is likely to improve the efficiency of refixation of CO_2 by Rubisco and minimize the net efflux of CO_2 during phase III, another key factor in maximizing 24-h carbon gain in mature plants of *A. tequilana* (R^2 = 0.603; Fig. 8).

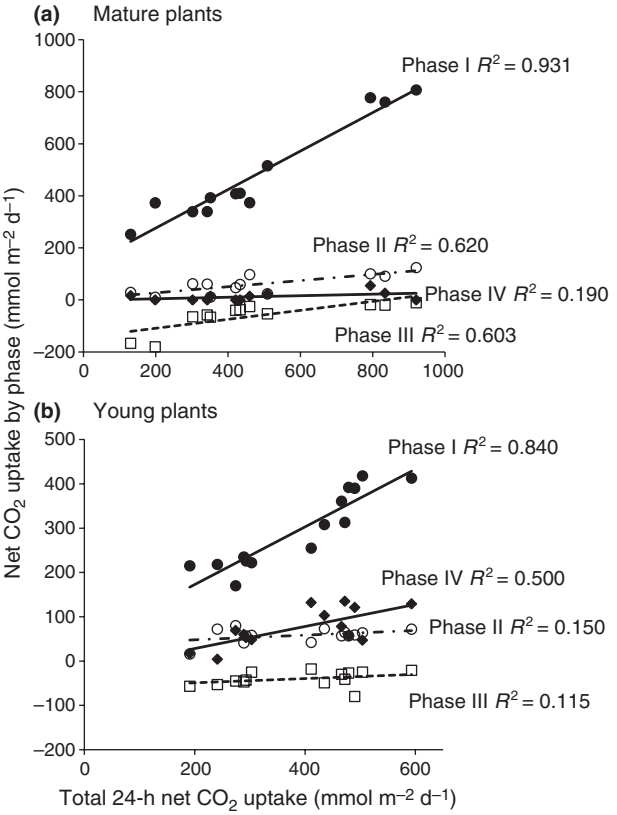

Fig. 8 The significance of individual phases of crassulacean acid metabolism (CAM) in contributing to daily net CO_2 uptake by leaves of (a) mature (5-yr-old) and (b) young (2–3-yr-old) plants of *Agave tequilana* grown in west-central Mexico. Data were re-calculated from 24-h gas exchange profiles obtained by Pimiento-Barrios *et al.* (2001, 2006).

VII. Concluding remarks

Numerous examples have been described above and elsewhere in which reciprocal interplay between C_3 and C_4 carboxylation processes sustains a positive C balance in CAM species under varying environmental conditions. The integration of circadian and metabolite control over carboxylation processes and carbohydrate partitioning provides the means for optimizing the C gain and water use of CAM plants that typically inhabit variable and potentially limiting environments. The functional anatomical requirements for a large vacuolar storage capacity in CAM plants appears to constrain the degree of plasticity in engaging both C_3 and C_4 carboxylases for direct uptake of atmospheric CO_2 and may, in part, account for evolutionary selection of either 'weak' or 'strong' CAM species. However, succulence can facilitate photosynthetic plasticity by maintaining a low cytosolic [malate] and thus extend PEPC-mediated net CO_2 uptake for several hours into the photoperiod, thereby optimizing C gain under potentially limiting environmental conditions. By providing a reservoir for soluble photoassimilates, high vacuolar storage capacity may also curtail feedback inhibition of photosynthesis under elevated [CO_2]. Succulence appears to be a key trait for maximizing the productivity of agronomically important CAM species under semi-arid conditions and probably underpins the reported incremental increases in net CO_2 uptake and biomass production under long-term exposure to elevated atmospheric concentrations of CO_2 (Drennan & Nobel, 2000; Garcia-Moya *et al.*, 2011).

The polyphyletic origins of CAM across 35 taxonomically diverse families suggest that the ecological convergence of this photosynthetic specialization in water-limited environments is underpinned by metabolic diversity for dealing with extreme environments. An understanding of the diverse metabolic and ecological manifestations of CAM will be accelerated by the integration of physiological, biochemical and molecular processes using a systems approach. Such knowledge should be exploited to inform the adoption and improvement of economically and ecologically relevant CAM species for sustainable agronomy and horticulture and for maintaining ecosystem services in a changing world.

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