REVIEW PAPER

Exploiting the potential of plants with crassulacean acid metabolism for bioenergy production on marginal lands

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Abstract

Crassulacean acid metabolism (CAM) is a photosynthetic adaptation that facilitates the uptake of CO2 at night and thereby optimizes the water-use efficiency of carbon assimilation in plants growing in arid habitats. A number of CAM species have been exploited agronomically in marginal habitats, displaying annual above-ground productivities comparable with those of the most water-use efficient C3 or C4 crops but with only 20% of the water required for cultivation. Such attributes highlight the potential of CAM plants for carbon sequestration and as feed stocks for bioenergy production on marginal and degraded lands. This review highlights the metabolic and morphological features of CAM that contribute towards high biomass production in water-limited environments. The temporal separation of carboxylation processes that underpins CAM provides flexibility for modulating carbon gain over the day and night, and poses fundamental questions in terms of circadian control of metabolism, growth, and productivity. The advantages conferred by a high water-storage capacitance, which translate into an ability to buffer fluctuations in environmental water availability, must be traded against diffusive (stomatal plus internal) constraints imposed by succulent CAM tissues on CO2 supply to the cellular sites of carbon assimilation. The practicalities for maximizing CAM biomass and carbon sequestration need to be informed by underlying molecular, physiological, and ecological processes. Recent progress in developing genetic models for CAM are outlined and discussed in light of the need to achieve a systems-level understanding that spans the molecular controls over the pathway through to the agronomic performance of CAM and provision of ecosystem services on marginal lands.

Key words: Biomass, CAM, carbon sequestration, circadian control, marginal lands, productivity.

Introduction

The photosynthetic specialization of crassulacean acid metabolism (CAM) permits the net uptake of CO2 at night and thereby dramatically improves the water-use efficiency (WUE) of carbon assimilation in plants growing in arid habitats. CAM is estimated to be expressed in ~7% of vascular plant species (Winter and Smith, 1996a), many of which dominate the plant biomass of arid, marginal regions of the world. Typically, the water use-efficiency of CAM plants, expressed as CO2 fixed per unit water lost, may be 3-fold higher than that of C4 plants and at least 6-fold higher than for C3 species. Examples of cultivated CAM species include Ananas comosus (pineapple), Opuntia ficus-indica, Agave sisalana, and A. tequilana, all of which can achieve near-maximal productivity over areas in which precipitation is inadequate or evapotranspiration so great that rainfall is insufficient for the cultivation of many C3 and C4 crops. Of these examples, pineapple (A. comosus: Fig. 1A) is cultivated over 60° of latitude and produces up to 86 Mg fruit ha⁻¹; the international trade value of the fresh produce, not including processed fruit, was recorded as US$1.9 billion year⁻¹ in 2003 (FAOSTAT, 2005). The high yielding potential of Opuntia achieved notoriety in the 1900s when
an O. stricta monoculture grew to occupy >25 million hectares in central eastern Australia and produced a total biomass of ~1.5 billion Mg in ~80 years (Osmond et al., 2008). Today, Opuntia species are part of natural and agronomic ecosystems in many parts of the world, with commercial cultivation (primarily for fodder and forage) occupying over 1 million hectares and annual dry biomass productivity for O. ficus-indica of 47–50 Mg ha$^{-1}$ year$^{-1}$ (Nobel, 1996; where 1 ha = 10 000 m$^2$). The current worldwide cultivation of Agave is >500 000 ha (Nobel et al., 2002), mostly for fibre (primarily sisal: Fig. 1B) and fodder, but also for the production of alcohol, either in the form of tequila (produced from the double distillation of fermented sugars from the stems and attached leaf bases of A. tequilana), or as mezcal (a singly distilled beverage extracted from ~10 other species).

The potential for Agave as an economically viable source of bioethanol with a zero-waste platform has recently been highlighted in Mexico as well as for the eroded lands of the Great Karoo in South East Africa where the climate and soil are not suitable for the cultivation of other crops (Boguslavsky et al., 2007; Burger, 2008). The high annual productivity of A. tequilana (50 Mg dry biomass ha$^{-1}$ year$^{-1}$ on semi-arid land) and high total sugar content (27–38%) in leaves/stems/fruits (cf. sugar cane 15–22%) have led to reports that distilled ethanol yields of 14 000 l ha$^{-1}$ can be obtained from some cultivars, with predictions of a further 33 650 l ethanol ha$^{-1}$ from cellulose digestion (Burger 2008). It is recognized that economic and environmental sustainability in the cultivation of dedicated bioenergy crops will require greater emphasis on the application and diversification of low-input agriculture on marginal land. This article will highlight the key attributes of CAM that contribute towards high biomass and bioenergy production in marginal habitats. Areas of current and future research are outlined for elucidating the causes and consequences of CAM and for providing a knowledge base that might inform and improve the potential of CAM plants for carbon sequestration and bioenergy production on marginal and degraded lands.

Biochemistry and regulation of CAM

CAM, like C$_4$, employs the enzyme phosphoenolpyruvate carboxylase (PEPC) for the capture of atmospheric and respiratory CO$_2$, thereby providing a means of ‘turbo charging’ Rubisco-mediated C$_3$ photosynthesis and reducing photorespiration for much of the photoperiod. However, whilst the C$_4$ pathway functions through spatial separation of PEPC and Rubisco between the mesophyll and bundle sheath cells, the complete CAM pathway occurs in each photosynthetic mesophyll cell and relies on strict temporal regulation of C$_4$ and C$_3$ carboxylation processes. CAM plants open their stomata and perform PEPC-mediated CO$_2$ uptake in the dark to form malic acid, which is subsequently broken down to release CO$_2$ that is fixed by Rubisco during the following day behind closed stomata. The closure of stomata in the light period and concomitant almost complete cessation of transpiration from the shoot surface underpins the high WUE of CAM plants. In addition, the temporal separation of metabolism in CAM provides plasticity for optimizing carbon gain in response to changing environmental conditions via adjustments in both the magnitude and relative proportions of direct C$_3$- and
C₄-mediated CO₂ uptake. Whilst the enzymatic machinery required for these carboxylation and decarboxylation reactions is present in all higher plants, evolution of the pathway required a change in the regulation of key enzymes and transporters in order to sustain the temporal separation of the two carboxylation processes that are central to CAM.

**CAM biochemistry**

The 24 h day/night cycle is the only meaningful unit of time within which to consider the biochemical processes of CAM which may be delineated into four phases (Osmond, 1978). Starting from the end of the photoperiod, the CAM cycle proceeds with phase I and the metabolic steps are illustrated in Fig. 2. In the cytosol, PEPC uses phosphoenolpyruvate (PEP) and HCO₃⁻ (HCO₃⁻ resulting from the action of carbonic anhydrase on CO₂) to generate oxaloacetate, which is rapidly converted to malate by malate dehydrogenase. Malate is transported into the vacuole through a voltage-gated, inward-rectifying anion channel (Hafke et al., 2003). The current best candidate for the molecular identity of this channel protein is the CAM orthologue of the Arabidopsis thaliana protein ALMT9 (aluminium-activated malate transporter 9: Kovermann et al., 2007), although the ALMT family of proteins has yet to be characterized in a CAM species. Inside the vacuole, malate accumulates as malic acid due to the high concentration of H⁺ generated by the vacuolar H⁺-ATPase and/or H⁺-PPiase (Bartholomew et al., 1996; Tsiantis et al., 1996). Indeed, it is the electrical component of the H⁺ electrochemical difference established by these two H⁺ pumps that maintains the inside-positive potential needed to drive the influx of malate– anions across the tonoplast through the vacuolar malate channel (Hafke et al., 2003). CO₂ uptake and malate accumulation continue for most of the dark period, such that the concentration of vacuolar malic acid can reach upwards of ~200 mM by dawn. PEPC is activated during the dark period due to phosphorylation by a dedicated PEPC kinase (PPCK), which mediates a 10-fold increase in the Kᵢ of PEPC for its feedback inhibitor, malate (Nimmo et al., 1984, 1987 Carter et al., 1991). The post-translational activation of PEPC in the dark period is hypothesized to draw down the internal partial pressure of CO₂ inside the leaf, and it is further hypothesized that this signals stomatal opening in the dark, thus sustaining the supply of CO₂ to PEPC.

In the few hours prior to dawn, PPCK is degraded and PEPC is dephosphorylated, rendering the enzyme 10 times more sensitive to inhibition by malate. The malate-mediated shutdown of PEPC at the start of the photoperiod may be considered a critical step for curtailing futile cycling at the start of the photoperiod in CAM plants (Borland et al., 1999). The export of malate from the vacuole to the cytosol (the site of PEPC activity) has long been considered a passive process, coupled in some way to the stoichiometric efflux of 2 H⁺ per malate (Smith et al., 1996), but the molecular identity of this transport system has also not been identified. An intriguing possibility is that the CAM orthologue of the tonoplast dicarboxylate transporter identified in A. thaliana (Emmerlich et al., 2003; Hurth et al., 2005) could mediate malate efflux from the vacuole at dawn. Rubisco activation, mediated via Rubisco activase, is believed to commence at the start of the photoperiod, and for a brief period CO₂ may be fixed by both PEPC and Rubisco, a period referred to as phase II of CAM (Fig. 3). However, it is noteworthy that a study on Kalanchoë daigremontiana found that the Rubisco activation state was low in phase II and increased gradually throughout the photoperiod, peaking some 3–4 h before the end of the photoperiod (Griffiths et al., 2002). This correlated with a peak in transcript and protein abundance of Rubisco activase, which occurred around the middle of the light period, in contrast to the peak of Rubisco activase transcript abundance that occurs shortly after dawn in C₃ species (Griffiths et al., 2002). Additionally, in Kalanchoë, Rubisco has kinetic properties which are intermediate

![Fig. 2. The pathway of crassulacean acid metabolism in a mesophyll cell of an NADP-ME (malic enzyme) species such as Mesembryanthemum crystallinum. Filled arrows represent dark reactions whilst open arrows represent light reactions. The dashed line running across the centre separates dark at the top from light at the bottom. The black-filled boxes on the left of the diagram represent the leaf epidermis, with the gap representing a stomatal pore. The enzymes that catalyse the reactions are as follows: (1) nocturnal starch breakdown possibly via chloroplastic starch phosphorylase and the export of G6P via the glucose 6-phosphate:phosphate translocator (GPT) followed by glycolytic conversion of G6P to PEP which is then used by phosphoenolpyruvate carboxylase (PEPC) as a substrate for CO₂ fixation; (2) carbonic anhydrase; (3) PEPC; (4) malate dehydrogenase; (5) voltage-gated malate channel, possibly a tonoplast membrane-targeted aluminium-activated malate transporter; (6) vacuolar H⁺ ATPase; (7) unknown protein proposed to mediate malate efflux, possibly the tonoplast dicarboxylate transporter; (8) NADP-ME; (9) ribulose bisphosphate carboxylase oxygenase; (10) unknown pyruvate transporter on the inner envelope membrane of the chloroplast; (11) pyruvate orthophosphate dikinase; (12) phosphoenolpyruvate:phosphate translocator; (13) gluconeogenesis; (14) GPT; (15) starch synthesis beginning with ADP-glucose pyrophosphorylase.](image-url)
between C₃ and C₄, with a reduced specificity (compared with C₃) but lower Kₘ for CO₂ (compared with C₄), perhaps reflecting the daily switch in carboxylase dominance that occurs over the CAM cycle (Griffiths et al., 2008).

The decarboxylation of malate (phase III of CAM) may be catalysed by: mitochondrial NAD⁺-ME (malic enzyme), cytosolic NADP⁺-ME, chloroplastic NADP⁺-ME, or cytosolic PEPCK, depending on the plant species (Dittrich et al., 1973; Dittrich, 1976; Holtum et al., 2005; the decarboxylation of malate via ME is shown in Fig. 2). In some CAM species, there is correlative evidence that malate is decarboxylated by more than one of these enzymatic routes due to high activities of more than one decarboxylase (Dittrich et al., 1973). Decarboxylation by ME generates pyruvate. In order for this pyruvate to be recycled through gluconeogenesis to starch, it must be converted to PEP by pyruvate, orthophosphate dikinase (PPDK; Fig. 2). PPDK has long been assumed to be a chloroplastic enzyme, and this was shown to be the case in Mesembryanthemum crystallinum (Kondo et al., 1998). In cytosolic NADP-ME and mitochondrial NAD-ME CAM species, PPDK in the chloroplast requires a pyruvate transporter on the chloroplast inner envelope membrane (Kore-eda et al., 1996). The molecular identity of this chloroplast membrane pyruvate transporter remains to be elucidated. However, in contrast to M. crystallinum, other CAM species, including K. daigremontiana and K. pinnata, have isoforms of PPDK localized to both the cytosol and chloroplast (Kondo et al., 2000, 2001). These observations are consistent with data reported for the C₃ species A. thaliana, in which a single PPDK gene was found to have two promoters that produced two different transcripts, one of which was translated into a chloroplastic PPDK, whilst the other transcript produced a cytosolic isoform of PPDK (Parsley and Hibberd, 2006). The regulation and localization of ME isoforms, PEPCK, and PPDK have received very limited attention in CAM species, and the function of the PPDK-regulatory protein (Chastain et al., 2008)—a homologue of which has been identified in M. crystallinum—remains to be established in terms of CAM operation.

The high internal concentration of CO₂ generated in the intercellular spaces by malate decarboxylation in phase III while stomata are closed effectively saturates the carboxylase activity and suppresses the oxygenase function of Rubisco, even though internal O₂ concentrations are also elevated at this time. In well-watered CAM plants, stomata may open once the supply of malate is exhausted and internal CO₂ concentrations drop. Direct fixation of atmospheric CO₂ by Rubisco can then proceed for the remainder of the light period, a period known as phase IV (Fig. 3; Osmond, 1978). The magnitude and duration of each phase of the CAM cycle is highly plastic and varies (i) between species; (ii) in response to the environment; and (iii) with leaf development (Winter et al., 2008). As illustrated for Kalanchoë fedtschenkoi (Fig. 3), the magnitude and duration of phases II–IV is inversely related to the magnitude and duration of CO₂ taken up at night (phase I). The older leaf pair 5 showed most net CO₂ uptake at night, and this was reflected in a diminished phase II and IV and extended phase III (the latter probably due to the time taken to decarboxylate the larger pool of malate present in leaf pair 5 compared with leaf pair 4). These different gas exchange patterns can also be attributed to the contrasting degree of succulence and commitment to CAM seen in young and old leaves of K. fedtschenkoi, which is equivalent to the difference between mature leaves of K. daigremontiana and K. pinnata (Griffiths et al., 2008; von Caemmerer and Griffiths, 2009).

Energetically, the CAM cycle incurs an additional cost when compared with the standard C₃ mode of carbon fixation. This arises from two sources: first, the cost of transporting malic acid into the vacuole at night, driven by one or both of the tonoplast H⁺ pumps; and secondly, the cost of converting the C₃ residue resulting from malate decarboxylation during the daytime back to the level of storage carbohydrate via gluconeogenesis, this being needed to provide the substrate for PEPC during the following night-time. In aggregate, these processes probably represent an additional metabolic cost of ~10% compared with the standard C₃ pathway (Winter and Smith, 1996b), which is relatively minor when considering that CAM plants typically grow in high-light environments in which photon supply is not normally limiting for growth. In compensation, however, CAM plants benefit during phase III from the suppression of photorespiration, which in C₃ plants can increase the cost of net CO₂ fixation by a minimum of 25%. Even under optimal temperature conditions, therefore, the energetic cost of net CO₂ fixation is significantly lower in CAM plants than in C₃ plants (Nobel, 1996; Winter and Smith, 1996b). This advantage increases with higher ambient temperatures, at which photorespiration increases steeply in C₃ plants, which is relevant when considering the temperature regimes characteristic of semi-arid habitats at tropical and subtropical latitudes.

Temporal control of CAM

The complete 24 h cycle of CAM occurs within individual leaf mesophyll cells, and strict temporal regulation of the various metabolic and transport components of the pathway is required to avoid the futile cycling that would result from uncontrolled, simultaneous CO₂ fixation and malate decarboxylation. The coordination and optimization of CO₂ fixation and subsequent metabolism during CAM is controlled by the endogenous circadian clock (Hartwell, 2005a). In particular, the leaves of CAM species perform a persistent circadian rhythm of CO₂ fixation at constant, permissive temperatures in continuous light, normal air, and continuous darkness under CO₂-free air (Wilkins, 1992; Hartwell, 2005a,b). Effective circadian control is probably critical to the optimal functioning of the CAM pathway, and has been covered in detail elsewhere (Borland and Taybi, 2004; Hartwell, 2005a,b). However, it is important to highlight some key features here, as circadian control may be a major yield constraint in highly productive CAM
species such as *Agave* and *Opuntia*, and thus have relevance in the selection of cultivars as feed stocks for bioenergy production.

The control of carbon flux through PEPC in CAM species is known to be mediated via circadian control of the synthesis and degradation of the dedicated regulatory kinase PPCK (Nimmo *et al.*, 1984; Carter *et al.*, 1991; Hartwell *et al.*, 1996, 1999, 2002). The nature of the underlying circadian oscillator that provides the temporal signals to optimize the CAM cycle as a whole, however, remains rather more elusive. Orthologues for many of the *A. thaliana* central clock genes have been cloned and characterized from the facultative CAM plant, *M. crystallinum* (Boxall *et al.*, 2005). This work has demonstrated that CAM species possess a multigene loop oscillator very similar to that in *A. thaliana* (Hotta *et al.*, 2007; McClung, 2008), supporting the hypothesis that the convergent evolution of CAM exploited an existing C₃ oscillator for the coordination and optimization of C₃ and C₄ carboxylation processes. Moreover, phase advancing the *M. crystallinum* multigene loop oscillator using a temperature pulse in the late dark period resulted in a highly correlated phase advance of circadian-regulated processes associated with CAM, including PPCK gene transcript abundance and malate oscillations (Hartwell, 2005; SE Boxall, JM Foster, and J Hartwell, unpublished data). However, the most convincing data linking the multigene loop oscillator in the nucleus to the circadian regulation of CAM comes from transgenic lines of *K. fedtschenkoi* overexpressing the central circadian clock gene, *TIMING OF CAB EXPRESSION1* (*TOC1*). In these *TOC1*-overexpressor lines, the circadian rhythm of CO₂ fixation collapses rapidly to arrhythmia following the onset of constant conditions (Hartwell, 2005; C Dall’omo, SE Boxall, JM Foster and J Hartwell,
unpublished data). This is consistent with the phenotype of output rhythms in lines of A. thaliana that strongly over-express TOC1 (Mas et al., 2003), and thus suggests that the multigene loop oscillator does coordinate CAM. The K. fedtschenkoi TOC1-overexpressor lines grow less rapidly and have smaller leaves, indicating that circadian control of CAM is critical to the growth and development of the plant (C Dal’ıomo and J Hartwell, unpublished). The detailed characterization of the phenotype of these K. fedtschenkoi TOC1-overexpressor lines will be the subject of a future publication.

In the light of the growth penalties associated with incorrect circadian control of CAM in TOC1-overexpressor lines, it is clear that detailed understanding of circadian control will be a key element in dissecting yield constraints in potential CAM biofuel species such as Agave and Opuntia. The importance of circadian control to growth and reproductive success has previously been demonstrated in A. thaliana, where complete arrhythmia of the central circadian oscillator was accompanied by an ~50% decrease in net carbon gain from photosynthesis (Dodd et al., 2005). Furthermore, altered circadian control has recently been shown to regulate growth vigour in allotetraploids and F1 hybrids of Arabidopsis (Ni et al., 2009). It is generally considered that CAM evolved via genome duplication or polyploidy, followed by evolution of the CAM-specific role for the extra redundant copies of relevant genes such as PPC and PPCK (Cushman and Bohnert, 1999). It is intriguing to speculate that the clock-dependent up-regulation of output genes may predispose a newly formed allopolyploid to the evolution of the CAM pathway under certain environmental conditions. Incorrect timing of biological activity in arid habitats tends to have more rapid negative consequences simply by virtue of the greater environmental extremes (e.g. temperature) that occur in such habitats. Thus, it can be argued that accurate circadian control tends to be even more important in arid-zone species. It remains to be established if the clock in CAM species has an even more critical role in reproductive success than that in C3 species. Certainly, it seems likely that the clock is central to the photoperiodic induction of CAM and onset of flowering in Kalanchoe blossfeldiana (Taybi et al., 2002), and may be associated with the onset of the dry season (Kluge and Brulfert, 1996).

**Carbohydrate economy and growth processes**

Whilst the circadian clock plays a cardinal role in establishing and maintaining the characteristic phases of CAM, the day/night turnover of carbohydrate is a key component in determining the magnitude of CAM expression. The nocturnal uptake of CO2 is sustained by degradation of carbohydrate reserves to PEP as substrate for PEPC-mediated carboxylation (Fig. 2). Up to 20% of leaf dry biomass can be allocated to carbohydrates for CAM, and these reserves may be accumulated in the form of soluble, low molecular weight sugars in the vacuole or as insoluble, polymeric glucan (starch) in the chloroplast (Kenyon et al., 1985; Christopher and Holtum, 1998, 1999). The typically high content of non-structural carbohydrates in CAM leaves can be viewed as a desirable trait for bioethanol production (Smith, 2008). Moreover, despite the requirement to bank reserves for dark CO2 uptake, in many CAM species (e.g. pineapple, Agave, Opuntia) the potential for high biomass productivity is not compromised. CAM allows metabolic flexibility in the use of different carbohydrate sources for nocturnal substrate provision, as demonstrated by the restoration of nocturnal acidification by feeding glucose or sucrose to leaves of a starch-deficient mutant of M. crystallinum (Cushman et al., 2008a). Clearly, the partitioning of carbohydrate for dark carboxylation must be modulated in line with the requirements of other competing sinks that include dark respiration, export, and growth (Borland and Dodd, 2002).

The phasing of leaf expansion growth over the diel CAM cycle has important implications for the mechanisms that regulate assimilate partitioning over a 24 h period. Using high-resolution digital image sequence processing to examine diel patterns of leaf growth, Gouws et al. (2005) showed that leaf growth accelerated at night and decelerated during the day in C3-performing M. crystallinum. In contrast, leaves of the CAM species Kalanchoe beharensis and cladodes of Opuntia engelmanii and O. oricola showed accelerated growth in the middle part of the day (phase III of CAM) and little or no growth at night (Gouws et al., 2005). It was suggested that the markedly different diel growth patterns in CAM species as compared with C3 can be explained in terms of both the distinctive turgor relations and carbon supply of CAM plants (Gouws et al., 2005). It has been reported for a range of C3 species that leaf growth occurs predominantly at night, and studies on Arabidopsis have indicated a close coupling between the rate of growth and the rate of nocturnal starch degradation (Walter and Schurr 2005; Smith and Stitt, 2007). In contrast, nocturnal stomatal opening and transpiration in CAM plants can cause leaf turgor to be low for much of the dark period, with maximum turgor not occurring until stomata are closed in phase III (Smith and Lütte, 1985). The uncoupling of leaf expansion growth from nocturnal carbohydrate degradation could provide a means of reconciling potential conflicts of demand between accumulation of carbohydrate reserves for CAM (or bioethanol production) and partitioning of resources for growth.

**Starch degradation in CAM plants**

The enzymatic route by which starch is degraded at night in CAM plants could have important implications for understanding the mechanisms that uncouple nocturnal starch breakdown from the synthesis of sucrose for growth. In leaves of Arabidopsis, the hydrolytic pathway has been shown to be of prime importance for the
nocturnal conversion of starch to sucrose, with maltose being the major export product from chloroplasts degrading starch at night (Niittyla et al., 2004). In contrast, the phosphorolytic pathway of starch degradation has been proposed to supply carbon for metabolism inside the chloroplast of Arabidopsis, with starch phosphorylase providing glucose-6-P as substrate for the oxidative pentose phosphate pathway, particularly under conditions of stress and when photorespiration is elevated (Zeeman et al., 2004; Weise et al., 2006).

The induction of CAM in M. crystallinum by exposure to salinity is accompanied by increased activities of a range of starch-degrading enzymes implicated in both the hydrolytic and phosphorolytic pathways (Paul et al., 1993). However, a change in transport activities across the chloroplast envelope has been reported with the switch to CAM in M. crystallinum. Chloroplasts isolated from C₃ M. crystallinum exported mainly maltose, whilst chloroplasts isolated from plants in the CAM mode exported mainly glucose-6-P (Neuhauß and Schulte, 1996). Two isoenzymes encoding glucose-6-P/P, translocators have been isolated from M. crystallinum (McGPT1 and McGPT2); CAM induction is accompanied by an increase in transcript abundance of both isoenzymes, which also show robust circadian patterns of abundance, implying a key role for these transporters in CAM (Koreeda et al., 2005). The nocturnal export of glucose-6-P from the chloroplast to the cytosol in CAM plants might be predicted to cause allosteric activation of PEPC (Osmond and Holtum, 1982), whilst glycolytic conversion of glucose-6-P in the cytosol would provide ATP by substrate-level phosphorylation and thus help to energize the nocturnal accumulation of malate in the vacuole (Holtum et al., 2005). Further indications that the phosphorolytic route may predominate over the hydrolytic route for starch degradation in CAM plants is provided by the very high extractable activities of chloroplastic starch phosphorylase from leaves of M. crystallinum, K. fedtschenkoi, and A. comosus, with activities in the CAM species 10-fold higher than that in Arabidopsis (T Taybi and AM Borland, unpublished observation). In contrast, glucanotransferase (DPE2), which in Arabidopsis acts on cytosolic maltose to provide a substrate for sucrose synthesis (Smith et al., 2005), shows very low activity in CAM-performing M. crystallinum (AM Borland, unpublished observation). Interrogation of an expressed sequence tag (EST) database for M. crystallinum containing 25,000 ESTs failed to identify any candidate chloroplastic maltose transporters (J Hartwell, unpublished observation), which might suggest that maltose export from the chloroplast in M. crystallinum (and indeed other CAM species) is quantitatively less important than that in Arabidopsis.

Lütтge et al. (1981) first pointed out that use of the phosphorolytic pathway for starch breakdown would reduce the energetic cost of nocturnal malic acid accumulation in CAM plants compared with the hydrolytic pathway. A full understanding of the relevance of utilizing the phosphorolytic pathway for directing carbon skeletons towards the synthesis of PEP rather than sucrose will require genetic manipulation of key enzymes and transporters implicated in these pathways in a CAM species.

### Day/night turnover and storage of sugars in CAM plants

For CAM species such as pineapple and agave in which soluble sugars are the major storage carbohydrate and the source of PEP for nocturnal carboxylation, the vacuole is likely to play a key role in regulating partitioning of sugars between growth and CAM. The photosynthetically active cells of CAM plants are typically dominated by a large central vacuole that occupies ~95% of the cell volume. Isolated vacuoles of A. comosus (pineapple) contain mainly glucose and fructose (Kenyon et al., 1985; Christopher and Holtum, 1998), whilst whole-leaf extracts contain substantial amounts of sucrose (Kenyon et al., 1985). Given the very high extractable activities of acid invertase reported for pineapple leaves (Black et al., 1996), it has been proposed that sucrose is synthesized in the cytoplasm during the day, transported across the tonoplast into the vacuole, and hydrolysed in the vacuolar lumen by acid invertase (Smith and Bryce, 1992). To avoid futile cycling, the hexoses thus produced would need to be stored in the vacuole and not released to the cytoplasm until the following dark period, when they would be metabolized via glycolysis to provide the C₃ substrate for nocturnal malate synthesis. This model is supported by the finding that the tonoplast of pineapple possesses a sucrose transport system with kinetics appropriate to catalyse sucrose fluxes of the required magnitude. McRae et al. (2002) demonstrated that sucrose uptake by isolated tonoplast-enriched vesicles prepared from pineapple leaves exhibits concentration-dependent saturation kinetics, ATP independence and trans stimulation by internal sucrose, characteristics that are consistent with the operation of a carrier type of sucrose transporter. Recently, a specific hexose transport system has also been observed in isolated pineapple tonoplast vesicles (D Haines and JAM Holtum, personal communication). Thus, sugar transporters located at the tonoplast could play a strategic role in controlling the supply and demand for carbon over the day–night cycle in sugar-accumulating CAM plants such as pineapple (Antony and Borland, 2008).

The mechanisms that control import and export of photoassimilates from the vacuole during the day–night cycle are largely unexplored for any plant species, and the first plant tonoplast sugar carriers have only recently been identified at the molecular level. Currently, the only vacuolar sucrose transporters to be identified at the molecular level (HvSUT2 from barley and AtSUC4 from Arabidopsis; Endler et al., 2006) are believed to facilitate sucrose export from the acidic lumen of the vacuole (Neuhauß, 2007). The first tonoplast monosaccharide transporters were recently identified from Arabidopsis (AtTMT) and are believed to operate via an H⁺-coupled antiport mechanism that would allow import of glucose and fructose in the vacuole by a secondary active transport mechanism, possibly in response to stimuli (i.e. cold, drought, salinity), that promotes sugar accumulation in Arabidopsis (Wormit...
et al., 2006). The model proposed for vacuolar sugar transport in the leaves of *A. comosus* (Smith and Bryce, 1992; McRae et al., 2002) implies the existence of a tonoplast hexose transport system to permit efflux of glucose and fructose at night to provide substrates for dark CO\textsubscript{2} uptake. A putative hexose transporter (AcMST1) recently identified from pineapple leaves that localized to the tonoplast of tobacco epidermal cells is a potential candidate for the energy-independent export of hexoses from the vacuole (Antony et al., 2008). However, the crucial kinetic mechanism that restricts efflux of vacuolar hexose to the dark period (thus avoiding futile cycling during the light period) is still completely unknown. There is no evidence for diurnal or circadian control of transcript abundance of this pineapple tonoplast hexose transporter, nor was there any difference in transcript abundance of AcMST1 between pineapple cultivars that differed in the magnitude of CAM (Antony et al., 2008). Recent examinations of the phosphoproteome of the tonoplast from rice and Arabidopsis suggest that phosphorylation of sugar transporters could be an important mechanism for controlling the day/night loading and unloading of sugars across the vacuolar membrane (Whiteman et al., 2008a, b).

In *Agave*, fructans stored in the stems and leaf bases are the major source of ethanol from this plant and as such represent an important vacuolar sink for photoassimilate. In mature leaves of *Agave deserti*, fructan biosynthesis was restricted to the vascular tissue (Wang and Nobel, 1998). Long-distance transport in the phloem of fructans with a low degree of polymerization has been reported for a number of plant species, but the mechanisms whereby fructans are unloaded into sink tissues and subsequently accumulated in the vacuole are unknown. Generally, it is considered that fructans are synthesized in the vacuole via the enzyme fructosyl transferase using imported sucrose as substrate (Valluru and Van den Ende, 2008). Fructans are believed to protect membranes in plants under abiotic stress and may also participate in the scavenging of reactive oxygen species in the vacuole (Van den Ende and Valluru, 2009). Substantial variation in sugar and fructan content has been reported for different cultivars of *Agave* (Vargas-Ponce et al., 2007) which could provide opportunities for selecting varieties for enhanced bioethanol production and stress tolerance. To maximize such potential will require a better understanding of the biochemical and molecular basis of carbohydrate partitioning in *Agave* alongside a systems-level approach to identify control points for sugar partitioning. Genes that encode vacuolar sugar transporters could be appropriate candidates for increasing sugar content in plants grown for bioethanol production. Indeed, metabolic control analysis of the kinetics of sucrose accumulation in the maturing culm tissue of sugar cane identified the rate of sucrose uptake by the vacuole as a key control element in the magnitude of sucrose accumulation (Uys et al., 2007). Given that leaf succulence and vacuolar capacity are particularly high in CAM species, knowledge of the tonoplast proteome in a sugar-accumulating CAM species could offer potential for increasing leaf sugar content whilst maintaining photosynthetic carbon assimilation by avoiding feedback repression of primary carbon metabolism and providing substrate for nocturnal carboxylation.

**Genetic models for CAM**

An important step towards maximizing the yield potential of CAM species as feed stock for biofuel production will be the development of a systems-level understanding of the molecular and metabolic controls over the pathway. Tractable model CAM species will be key to dissecting the pathway at molecular and biochemical levels. Early attempts to develop a molecular–genetic model for the study of CAM centred on *M. crystallinum* (Bohnert and Cushman, 2000). CAM may be induced on a C\textsubscript{3} background via the imposition of salinity or drought in *M. crystallinum* (Winter and Holtum 2007), and this metabolic switch has proved a very attractive system for identifying CAM-associated genes and proteins (Cushman and Bohnert, 1989, 1999; Cushman, 1992; Boxall et al., 2005). Other beneficial attributes of *M. crystallinum* include: a relatively rapid life cycle (7–14 weeks depending on growth conditions); a relatively small genome, perhaps the smallest known amongst CAM species measured to date (~390 Mbp; De Rocher et al., 1990); and the setting of thousands of small seeds which are ideal for mutagenesis, allowing traditional forward genetic screens to be undertaken. Mutant populations of *M. crystallinum* have been developed and screened to identify CAM-deficient mutants (Cushman et al., 2008a). A database of *M. crystallinum* ESTs containing some 27 000 sequences (Kore-eda et al., 2004) remains the largest readily accessible gene index for a CAM species. This gene index has been used to generate a Nimblegen oligonucleotide microarray with representation of 8455 unique genes, which was used to investigate both the induction and circadian regulation of CAM (Cushman et al., 2008b).

The further development of *M. crystallinum* as a model CAM system is presently hampered by the lack of an efficient and stable transformation system to facilitate *in vivo* testing of gene function using both overexpression, and gene silencing/RNAi (RNA interference) approaches. In addition, the study of CAM in *M. crystallinum* is complicated by the requirement for drought or salt stress to induce CAM, and it is therefore very difficult to separate CAM genes from those genes that are more directly involved in resisting the osmotic stress imposed by the drought or salt. Given such constraints, attention is now shifting towards the Madagascan endemic *K. fedtschenkoi* as the next major genetic model for CAM based on the following rationale. (i) *K. fedtschenkoi* is an obligate CAM species that displays a clear developmental progression from C\textsubscript{3} to CAM, even under well-watered conditions. The complication of the drought or salt stress response is therefore avoided, and it is thus regarded as a much ‘cleaner’ model for the study of CAM. (ii) *K. fedtschenkoi*
has the key advantage of a simple and efficient stable transformation system, permitting transgenic approaches to deciphering gene function. (iii) *K. fedtschenkoi* has a reasonably small genome at ~858 Mbp (De Rocher et al., 1990), which places it well within the capabilities of the latest DNA sequencing technologies. (iv) *K. fedtschenkoi* is very easy to grow and develops rapidly from cuttings or leaf plantlets, reaching a size suitable for CAM experiments within 2 months. The ease of clonal propagation means that large quantities of developmentally coordinated plants can be generated very quickly from individual transformants. (v) *K. fedtschenkoi* has the further advantage that a wealth of background literature exists providing detailed characterization of the physiology and biochemistry of the circadian control of CAM (Wilkins, 1992; Hartwell et al., 2002; Hartwell, 2005b).

The characteristics of *K. fedtschenkoi* described above have led to a new project in the Hartwell laboratory (University of Liverpool, UK) to perform in-depth sequencing of the transcriptome of this species. The project will also establish a large-insert bacterial artificial chromosome (BAC) library and perform pilot BAC sequencing in readiness for whole genome sequencing. Digital transcriptomics (the use of the number of sequence reads per gene as a quantitative digital read-out of transcript abundance in the original RNA sample) is being employed to identify CAM-associated genes whose transcript abundance is up-regulated in CAM leaves relative to *C₃* leaves. Candidate CAM genes are subjected to more detailed real-time reverse transcription-PCR (RT-PCR) analysis to test for circadian regulation in CAM leaves, and the most interesting candidates will be overexpressed and silenced in transgenic *K. fedtschenkoi* to test the in vivo function of each candidate CAM gene. Thus, this project aims to identify all of the genes that *K. fedtschenkoi* uses to perform CAM, and set the stage for future proteomic and metabolomic analysis of CAM in *K. fedtschenkoi*. The ultimate goal is to combine data concerning transcripts, proteins, and metabolites into predictive models, which can be used to identify the key control points for optimal performance and yield associated with CAM.

The phylogenetic position of *K. fedtschenkoi* within the family Crassulaceae and order Saxifragales means that this species shared its last common ancestor with *M. crystallinum* (Aizoaceae, Caryophyllales) some ~80–90 million years ago, and thus evolved the CAM pathway completely independently. Ongoing genomics projects with *M. crystallinum* and *K. fedtschenkoi* will thus permit comparative analysis of the functional genomics of CAM in divergent species that evolved the CAM pathway independently. Such an approach may reveal differences in the regulatory and enzymatic steps that have been co-opted into a CAM-specific role during the evolution of this pathway in different taxonomic groups. This is important in light of the fact that *M. crystallinum* and *K. fedtschenkoi* are not closely related to the high-yielding CAM species of cacti and agaves that appear to be suited to bioenergy production on marginal lands.

**Carbon uptake and sequestration—from leaf to canopy**

Carbon uptake by leaf or stem succulent CAM plants represents a classic example of conflict resolution in physiological ecology, whereby the potential for carbon gain across a 24 h cycle is traded against diffusive constraints imposed on CO₂ supply by succulent tissues (Dodd et al., 2002; Pierce et al., 2002; Griffiths et al., 2007). The growth characteristics of CAM plants, as summarized in Table 1, present a number of favourable attributes when considering the cultivation of dedicated bioenergy crops in semi-arid regions of the world. However, the practicalities for maximizing CAM biomass and carbon sequestration need to be informed by underlying molecular, physiological, and ecological processes. Understanding the integration of molecular and cellular signals and their regulation by environmental conditions will allow chemical and structural composition to be targeted towards a specific end-product, provided that any potential market is sustainable and the local community supports the initiative (Cowling et al., 2008). Additionally, there are uncertainties over the maintenance of potential productivity for the future, because a changing climate will lead to altered precipitation patterns and tensions between food and fuel production, which are current even in today’s marginal habitats. However, it should be noted that the exceptional degree of stress tolerance of typical CAM plants towards restricted water availability, high temperatures, and high light intensities (Table 1) would be expected to make them relatively robust to the impact of future climate change.

**Stomatal versus mesophyll (internal) constraints to carbon gain**

At the scale of individual photosynthetic organs, stomata occur in relatively low densities and have low conductances to water vapour in CAM plants, reflecting the high water-storage capacity, low external surface area:volume ratio, and high WUEs, for leaves, cladodes, and stems of CAM plants (Osmond, 1978; Nobel, 1988). The compromise between maximizing day- and night-time uptake is exemplified in comparisons of two *Kalanchoë* species (*K. daigremontiana* and *K. pinnata*) which contrast in the degree of leaf succulence (Griffiths et al., 2008; von Caemmerer and Griffiths, 2009). Stomatal densities were lower in the more succulent species (*K. daigremontiana*), which was more committed to the conventional CAM cycle, with higher rates of acid accumulation, CO₂ uptake, and higher stomatal conductance at night. In contrast, the less succulent *K. pinnata* showed the more *C₃*-like expression of the CAM phases by day, with a higher proportion of integrated 24 h net CO₂ uptake mediated directly by Rubisco during phases II and IV. Overall, the ratios of internal:external CO₂ concentration were similar at night for the two species, and actually higher during phase IV for *K. daigremontiana*. However, the sensitivity of stomata to transient changes in external CO₂ concentration during the day–night cycle...
suggested that internal CO₂ concentration is not the only effector regulating the CAM diel stomatal cycle (von Caemmerer and Griffiths 2009).

These data help to explain the compromise between the constraints imposed by succulent tissues, and the potential for internal metabolism facilitated via high PEPC and Rubisco carboxylation capacities as a means of overcoming external diffusion limitation (Griffiths et al., 2002). However, with the succulent photosynthetic tissues of typical CAM plants containing only ~5% airspace (Smith and Heuer, 1981; Maxwell et al., 1997), there are significant constraints imposed on the internal diffusive supply of CO₂ (outside the ‘regenerative’ phase III, when internal CO₂ concentrations are likely to saturate Rubisco). Thus, at night, the carbon isotope signals associated with PEPC were strongly suggestive of diffusion limitation in *K. daigremontiana* (Griffiths et al., 2007). Additionally, mesophyll conductances derived during phase IV of gas exchange in the light (and for a range of *Clusia* species) reflected the degree of succulence, with internal CO₂ supply at Rubisco potentially as low as 110 μmol mol⁻¹ (Griffiths et al., 1999).

Given such diffusive constraints, the interpretation of carbon isotope composition of bulk organic material as being representative of the balance between daytime (C₃) and night-time (C₄) carboxylation processes is complicated. When carbon isotope composition is predicted by stomatal conductance and carboxylase fractionations, lower discrimination is likely to be associated with CO₂ uptake during phase IV in succulent tissues by day, and higher discrimination may be predicted at night (Griffiths et al., 1990, 2007, 2008; Roberts et al., 1997). The use of carbon isotope composition to predict ‘furtive’ CAM species (or C₃–CAM intermediates) in a given population may be confounded both by internal constraints to diffusion and by the extent of carbon gain actually undertaken for the short period that CAM may be active during extreme conditions (Pierce et al., 2002; Winter and Holtum, 2002; Silvera et al., 2005).

Additionally, the isotopic composition of CAM plants tends to be fairly conservative across a wide range of habitats (Griffiths, 1992), and may not always be indicative of precipitation gradients (Amundson et al., 1994). This may limit the use of carbon isotopes as a tool in selecting improved productivity and water use of bioenergy crop cultivars, which are mostly as yet unimproved. However, when combined with oxygen isotopes, which provide a marker for precipitation inputs and water sources within the soil profile, carbon isotope composition may be more revealing. It has recently been shown that oxygen isotopes can resolve water vapour exchanges by epiphytic CAM plants (Helliker and Griffiths, 2007; Reyes-Garcia et al., 2008) and act as a means of climate reconstruction from saguaro spines (English et al., 2007).

**Tolerance of water deficits, water use and productivity**

Despite the metabolic limitations of high gas-phase resistances associated with succulent tissues, high productivities can be achieved by CAM plants in habitats where precipitation is intermittent, but regular (i.e. seasonal) and reasonably predictable on an annual basis (Ellenberg, 1981). With their relatively shallow root systems, CAM plants are able to exploit efficiently the small amounts of water available even from intermittent rainfall events delivering <10 mm, which may be sufficient to moisten only the uppermost soil layers. Coupled with this efficient harvesting of incident precipitation, leaf- and stem-succulent CAM plants also have very high water-storage capacitance in their above-ground tissues, meaning that they are able to make

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**Table 1. Growth characteristics of CAM plants favourable for cultivation as a bioenergy crop in semi-arid regions**


<table>
<thead>
<tr>
<th>Trait</th>
<th>Example</th>
<th>Comment</th>
</tr>
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<tbody>
<tr>
<td>High water-use efficiency</td>
<td>5–16 mmol CO₂ per mol H₂O on an annual basis</td>
<td>Typically 4- to 10-fold higher than C₃ plants</td>
</tr>
<tr>
<td>High drought tolerance</td>
<td>Can grow in areas with as little as 25 mm year⁻¹ precipitation</td>
<td>Tissues can tolerate up to 90% loss of water content (cacti)</td>
</tr>
<tr>
<td>Tolerance of high temperatures</td>
<td>Up to 70 °C, based on 50% loss of cell viability after 1 h; can survive exposure to 74 °C</td>
<td>Typically upper limit of 50–55 °C in C₃ plants</td>
</tr>
<tr>
<td>Tolerance of high PPFD</td>
<td>Can tolerate &gt;1000 μmol m⁻² s⁻¹ (or &gt;40 mol m⁻² d⁻¹) without photoinhibition</td>
<td>Generally more tolerant of high PPFD than agronomically important C₃ plants</td>
</tr>
<tr>
<td>Tolerance of UV-B radiation</td>
<td>Only 1% incident UV-B transmitted through epidermis of <em>Yucca filamentos</em> (Agavaceae)</td>
<td>Generally thick epidermis and high foliar concentrations of phenolics in CAM plants</td>
</tr>
<tr>
<td>Entire shoot surface typically</td>
<td>Whole shoot photosynthetic in both leaf- and stem-succulent species; limited bark formation even on stems of arborescent cacti</td>
<td>Many C₃ species deciduous (shedding photosynthetic organs for part of year) or woody (limited stem photosynthesis)</td>
</tr>
<tr>
<td>High shoot:root ratio and harvest index</td>
<td>Shoot:root ratio as high as 10:1; above-ground biomass readily harvested</td>
<td></td>
</tr>
<tr>
<td>High resistance to herbivores</td>
<td>Effective physical defences (stem succulents) and chemical defences (leaf succulents)</td>
<td></td>
</tr>
<tr>
<td>High content of non-structural carbohydrate</td>
<td>Especially monocotyledons (~20% dry weight); ready conversion of soluble sugars to bioethanol</td>
<td></td>
</tr>
<tr>
<td>Low lignin content</td>
<td>Weak secondary thickening and lack of true wood formation</td>
<td></td>
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maximum benefit of the water absorbed by their root systems in improving tissue water relations. At the cellular level, this high storage capacitance is a consequence of two important characteristics of CAM plants. First, compared with other drought-tolerant plants, they typically have rather dilute cell sap, with osmotic pressures almost invariably <1.0 MPa (Walter, 1960; Smith and Lüttge, 1985; Smith et al., 1986). Secondly, the large parenchymatous cells in the shoot tend to have thin cell walls, and consequently are relatively elastic (technically, they have a low volumetric elastic modulus: Steudle et al., 1980; Smith et al., 1987). These properties combine to confer on the tissues of the shoot a high water-storage capacitance, as capacitance is inversely proportional to both cell osmotic pressure and elastic modulus (Steudle et al., 1980; Smith et al., 1987). Furthermore, at the level of the whole organ, CAM plants can withstand large changes in relative volume: whereas loss of one-fifth of their relative water content is lethal for many plants species, some CAM plants can tolerate loss of 80–90% of their water content and still survive, as may occur in exceptional periods of several years without rainfall (Nobel, 1988).

There is an evident contrast between tolerance of water deficits at cellular and ecological levels, whereby CAM water and solute potentials are close to −1 MPa, whilst nearby C3 shrubs may approach −4 MPa or lower (Smith et al., 1986). Avoidance of soil water deficits seems to be a convergent property of CAM in many phylogenetic groups, and the classic work by Park Nobel and colleagues has revealed the mechanisms which allow roots to become effectively isolated from the soil (Nobel, 1988). Shrinkage of the root cortex occurs even at modest soil water deficits (approximately −0.1 MPa), which, together with cavitation of root xylem, helps to protect any reverse flux of water from storage tissues to soil (Nobel, 1988; North et al., 2004). In older roots, a sclerified exodermis also helps to prevent water loss, and aquaporins in the cortex and endodermis provide short-term control of root hydraulic conductance (North et al., 2004). In contrast, in younger roots (and presumably ‘rain’ roots, which may take 3–4 d to reach maximum hydraulic conductance (Nobel 1988), aquaporins in the epidermis are quantitatively more significant in regulating water fluxes (North et al., 2004).

By controlling connectivity with the soil, the advantages conferred by a high water-storage capacitance in CAM plants translate into an ability to buffer fluctuations in environmental water availability. This is manifested in high WUEs for growth compared with other plant life forms and, in some species, a considerable degree of flexibility in the partitioning of gas exchange and CO2 uptake between the day and night. Seasonal measurements of 24 h gas exchange for six CAM species that were cultivated in semiarid plantations in Mexico without addition of water or nutrients indicated that 75–97% of total daily net CO2 uptake occurred at night and that total daily net CO2 uptake averaged 823 mmol m−2 d−1 (Nobel et al., 2002). The highest values of daily net CO2 uptake reported for these CAM species exceed that of nearly all productive C3 and C4 crops and occurred under rain-fed as well as dry conditions when moderate day/night temperatures prevailed (Nobel et al., 2002). Thus, CAM plants that have been exploited agronomically in marginal habitats can display annual productivities close to those found in the most productive C3 or C4 agronomic systems (Nobel, 1988, 1991a). For instance, maximal above-ground dry-biomass productivities of 47–50 Mg ha−1 year−1 have been recorded for platyopuntias in Chile and Mexico, and of 42 Mg ha−1 year−1 for agaves in Mexico (Nobel, 1996). For pineapple, the most commercially important CAM plant, a maximal dry-biomass productivity of 35 Mg ha−1 year−1 has been observed in Hawaii. These values compare with average above-ground productivities of ∼35 Mg ha−1 year−1 and 50 Mg ha−1 year−1 for the most productive C3 and C4 crops, respectively (Nobel, 1991a).

To put this in an agronomic context, we can convert the standard physiological term WUE (CO2 assimilated per mol H2O transpired) and figures for above-ground carbon sequestration into crop water demand (as g H2O required per g C sequestered in biomass, thereby ignoring water evaporated directly from soils). Using the crop productivities identified above and typical ranges of WUE reported for crops with different photosynthetic pathways, Table 2 allows a comparison of calculated values of crop water demand for CAM, C3 and C4 crops. Thus, CAM crops use ∼20% of the water required for cultivation of the most water-use efficient C3 or C4 crops (although it should be noted that WUE values may be somewhat higher for CAM plants growing under cultivation compared with native CAM species growing in their natural habitats; Nobel, 1994). Agronomists calculate WUE as the combined evaporative loss from the crop and soil, and hence these figures only equate to the minimum water use, but the

<table>
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<th>Agronomic traits</th>
<th>Photosynthetic pathway</th>
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<tr>
<td>Average above-ground productivity [Mg (tonnes) ha−1 year−1]</td>
<td>CAM: 43</td>
</tr>
<tr>
<td>Water use efficiency (mmol CO2 per mol H2O)</td>
<td>4–10</td>
</tr>
<tr>
<td>Crop water demand (Mg H2O ha−1 year−1)</td>
<td>2580–6450</td>
</tr>
</tbody>
</table>

Table 2. A comparison of typical agronomic traits relating to above-ground dry biomass productivity, water-use efficiency (integrated over 24 h), and crop water demand for cultivated crops belonging to the different photosynthetic pathways.

Data for average above-ground productivity are taken from Nobel (1991a).
Climate change and CAM productivity

Formulating best agronomic practice and selection of appropriate cultivars of CAM plants such as eucalypts and agaves for biofuel production on marginal land in the 21st century will require an understanding of how atmospheric [CO2] may modify plant responses to precipitation variability/abundance and increases in temperature. For C3 plants, rising CO2 offers the potential to stimulate crop productivity and offset crop losses caused by greater water and temperature stress, since Rubisco is not saturated at current atmospheric [CO2] and photorespiration can result in significant reductions in plant yield (Bowes, 1991). Whilst C4 photosynthesis is saturated under ambient [CO2], stomatal conductance may be reduced by up to 20% under elevated [CO2], and yield improvements in maize grown under 550–600 ppm CO2 were achieved through an amelioration of short-term drought stress via the conservation of soil moisture (Leakey et al., 2004). Given that CAM is considered to suppress photorespiration (at least for the middle/hottest part of the day) and is an adaptation to conserving plant water status under arid and semi-arid conditions, it might be predicted that elevated CO2 would have negligible impact on carbon gain and biomass productivity in plants with this photosynthetic pathway. In reality, a wide and seemingly contradictory range of responses to elevated [CO2] have been reported in terms of day/night patterns of gas exchange and WUE in CAM species. Responses to elevated [CO2] include: decreased nocturnal CO2 uptake (e.g. Portulacaria afra, Clusia uvitana: Huerta and Ting, 1988; Winter et al., 1992); no change in 24 h carbon gain (e.g. K. daigremontiana: Holtum et al., 1983); an increase in daytime CO2 uptake (e.g. K. pinnata, Aechmea maya: Winter et al., 1997; Ceusters et al., 2008); an increase in nocturnal CO2 uptake (A. deserti, A. salmiana: Graham and Nobel, 1996; Nobel et al., 1996); and an increase in both day and nocturnal CO2 uptake (e.g. A. comosus, O. ficus-indica: Nobel and Israel, 1994; Zhu et al., 1999). The contrasting responses to elevated [CO2] are a reflection of the inherent photosynthetic plasticity of CAM. However, for a range of desert CAM succulents, Drennan and Nobel (2000) demonstrated that alongside increases in temperature and drought, the daily net CO2 uptake increased by 1% as the atmospheric [CO2] increased by 10 ppm, and dry biomass production of O. ficus-indica was stimulated by 40% over a 1-year period of exposure to 750 ppm [CO2] (Nobel and Israel, 1994). Sustained increases in productivity may be facilitated by the high succulence of CAM species which can accommodate large increases in chlorenchyma thickness and accumulation of photosynthate without feedback inhibition of photosynthesis (Nobel, 2000). The succulent nature of the photosynthetic organs of CAM plants and the associated diffusional constraints to CO2 imposed by densely packed cells could also be a key factor underpinning the various reports of stimulation of diurnal and nocturnal CO2 uptake by elevated [CO2].

Carbon sequestration and ecosystem services

From a climate change perspective and for the sustainable management of ecosystem services, detailed budgets comparing above-ground productivity, water use, and soil organic carbon are urgently required for potential bioenergy crops and their contrasting photosynthetic pathways. This is particularly relevant for the dense canopies of C4 bioenergy crops, for which water use as well as light limitation needs to be taken into consideration (Kromdijk et al., 2008), and for the sustainability of any irrigation needed to maximize CAM productivity. Carbon sequestration below ground by perennial crops is also an important positive gain from a climate change perspective, and many desert soils have a low soil organic matter (SOM) (Amundson et al., 1994).

To date, there has been limited use of eddy covariance techniques to evaluate carbon sequestration of bioenergy crops (but see Kromdijk et al., 2008). However, one highly innovative study has characterized the carbon balance and net ecosystem productivity for a pineapple crop (San José et al., 2007a, b). Here, it was evident that rates of carbon uptake matched those predicted by Nobel (1991a), whilst carbon sequestration was affected by both climatic extremes associated with ENSO (El Niño Southern Oscillation) events and the seasonality of rainfall. Thus, the eddy flux system could distinguish the balance between day- and nighttime carbon uptake on a daily and seasonal basis, as well as resolving crop and soil water use as a function of soil water availability, stomatal sensitivity, and atmospheric water deficit (San José et al., 2007a, b). For future studies, the use of carbon isotopes could help to partition plant and soil respiration (Kromdijk et al., 2008), as well as determining the contribution that CAM or C4 pathways make to SOM.

The contribution of CAM to soil carbon sequestration can be inferred from the isotope composition of SOM, as shown for sites along the Californian Baja peninsula (Amundson et al., 1994). The SOM δ13C values ranged from −23.3‰ to −20.2‰ along a north to south transect, which represented a change in vegetation dominance from 27% to 49% CAM inputs, as inferred from isotopic mass balances. There seem to be no other isotopic analyses of SOM from CAM-dominated habitats: it would be interesting to know how much carbon from the saguaro-dominated landscapes of the Sonoran Desert is captured in the soil. Whilst Carnegia gigantea provides such a striking visual
dominance of this landscape, it is probable that soil carbon sequestration in such habitats is mainly derived from the C3 shrubs that dominate the understorey. Habitats supporting a higher proportion of succulents in the above-ground vegetation in arid-zone regions of southern Africa and Madagascar, for example, might provide much more compelling examples of soil carbon storage being dominated by CAM plants. Indeed, the distinct semi-deciduous thicket, or ‘spiny forest’, of southern and south-western Madagascar (rainfall <600 mm year\(^{-1}\)) probably represents the highest standing biomass of CAM vegetation on earth, dominated by woody members of the endemic family Didiereaceae, together with numerous species of CAM plants from the genera Aloë, Euphorbia, and Kalanchoë, amongst others (Rauh, 1973; Winter 1979; Grubb, 2003; Cowling et al., 2005).

In the Eastern Cape of South Africa, the natural vegetation is dominated by the C3–CAM intermediate Portulacaria afr\(\)a, with leaf carbon isotope ratios ranging from −17.4\% to −20.5\% (Mills et al., 2005), suggesting that CAM is more important under natural conditions than was inferred from early laboratory studies (Guralnick et al., 1994). The vegetation in this area of South Africa, formerly known as ‘valley bush veld’ or more recently as ‘subtropical thicket’, is another striking example of a habitat consisting predominantly of stem- and leaf-succulent CAM plants. The subtropical thicket occupies 17% of the land area, equivalent to some 1.4 M ha\(^{-1}\) (or 14 000 km\(^{2}\)) of which 45% is degraded to grassland savanna, and another 35\% subject to degradation due to overgrazing (Mills et al., 2005; Mills and Cowling, 2006). In pristine thicket, the succulents make up nearly half of the above-ground dry biomass, with the inducible CAM plant P. afr\(\)a forming a dense impenetrable matrix, and acting (probably) as a nurse plant for a variety of CAM succulents, which all then help to protect the ~5\% of species which are C\(_3\). However, P. afr\(\)a is particularly palatable, previously sustaining the diverse native megafauna, but is readily overgrazed by goats, leading to the conversion into grassland savanna (Mills et al., 2005).

The ecosystem carbon storage in pristine examples of this biome can be partitioned into 76 Mg (C) ha\(^{-1}\) (biomass) and 133 Mg (C) ha\(^{-1}\) (upper soil profile), and because P. afr\(\)a can be readily regenerated from cuttings, it has been suggested that up to 80 Mg (C) ha\(^{-1}\) could be sequestered by restored thicket (Mills et al., 2005). The focus for this innovative programme of work undertaken in South Africa has partly been to provide a scientific basis for evaluating biodiversity and conservation of natural vegetation (Rouget et al., 2006). An additional framework has been the opportunities provided by the Clean Development Mechanism of the Kyoto protocol, which allows such biomes to receive carbon trading credits (Mills et al., 2005; Mills and Cowling, 2006). The need to evaluate such developments in terms of ‘ecosystem services’ has also been pioneered by these researchers (Cowling et al., 2008; Rouget et al., 2008). Cowling et al. (2008) show the importance of including all stakeholders in decision support systems (users, land-owners, researchers, and policy makers, as well as the benefits for local communities) during the assessment, planning/implementation, and management of developments that may have implications for land and water use at regional scales. Carbon sequestration by this subtropical thicket biome also has potential for biofuels and bioenergy, particularly when local solutions in energy generation are made available for local people. Bioethanol generation plants, or combined heat and power plants, could be allied with bioenergy crop plantations to ensure the involvement (literally the empowerment!) of local communities. Thus, notions of ecosystem services are equally relevant for developments involving biomass crops and bioenergy generation, particularly for marginal landscapes where the tensions between food, fuel, and water availability may be all too tangible for the future.

**Conclusions**

To exploit the yield potential of CAM species as feedstock for biofuel production, a systems-level understanding is required that spans the molecular and metabolic controls over the pathway through to the agronomic performance of CAM in marginal ecosystems. Whilst M. crystallinum and K. fedtschenkoi are tractable model systems that will allow great advances in our understanding of CAM, they are not closely related to CAM species such as Agave that could potentially achieve very high yields for bioethanol production. Genetic diversity for bioethanol production is reported to exist within A. tequiliana, but the relationship between such quality-related aspects and photosynthetic performance is not known for Agave or indeed for any CAM species. Comprehensive transcriptome sequencing, proteomic and metabolomic analysis of high-yielding CAM species would be an important step towards maximizing the potential of such species as biofuel feedstocks through selection and breeding.

The application of predictive models of biomass production as part of a whole-system approach to land use and natural resource management will also play a crucial role in exploiting the potential of CAM for carbon sequestration and biofuel production on marginal lands. The power of predictive biology was harnessed >20 years ago for CAM plants by Nobel (1988; 1991b) who developed an environmental productivity index (EPI) to inform and improve agronomic practice for CAM cultivation, as well as to predict the geographical regions that CAM plants might successfully exploit in a changing environment. Using EPI, it was recognized not only that the global range of CAM cultivation could be extended but also that CAM plants could play a significant role in terrestrial sequestration of atmospheric CO2 in arid and semi-arid regions of the world. Desertification is a worldwide problem directly affecting 250 million people and a third of the earth’s land surface (i.e. >4 billion hectares). In addition, the livelihoods of some 1 billion people who depend on land for most of their needs and usually the world’s poorest in >100 countries are
threatened by land degradation. Such regions are poorly suited to C₃ and C₄ crops without irrigation. The substantial biomass increases reported for CAM species under elevated CO₂ on marginal lands indicate that serious consideration should be directed towards exploring the potential of CAM plants as a low-input source of bioenergy and as a means of stimulating sustainable economic growth in developing countries.

Acknowledgements

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