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REVIEW PAPER

Facultative crassulacean acid metabolism (CAM) plants: powerful tools for unravelling the functional elements of CAM photosynthesis

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Abstract

Facultative crassulacean acid metabolism (CAM) describes the optional use of CAM photosynthesis, typically under conditions of drought stress, in plants that otherwise employ C_3 or C_4 photosynthesis. In its cleanest form, the upregulation of CAM is fully reversible upon removal of stress. Reversibility distinguishes facultative CAM from ontogenetically programmed unidirectional C_3-to-CAM shifts inherent in constitutive CAM plants. Using mainly measurements of 24 h CO_2 exchange, defining features of facultative CAM are highlighted in five terrestrial species, *Clusia pratensis*, *Calandrinia polyandra*, *Mesembryanthemum crystallinum*, *Portulaca oleracea* and *Talinum triangulare.* For these, we provide detailed chronologies of the shifts between photosynthetic modes and comment on their usefulness as experimental systems. Photosynthetic flexibility is also reviewed in an aquatic CAM plant, *Isoetes howellii*. Through comparisons of C_3 and CAM states in facultative CAM species, many fundamental biochemical principles of the CAM pathway have been uncovered. Facultative CAM species will be of even greater relevance now that new sequencing technologies facilitate the mapping of genomes and tracking of the expression patterns of multiple genes. These technologies and facultative CAM systems, when joined, are expected to contribute in a major way towards our goal of understanding the essence of CAM.

Key words: C4/CAM, *Calandrinia*, *Clusia*, constitutive CAM, crassulacean acid metabolism, inducible CAM, *Isoetes*, *Mesembryanthemum*, *Portulaca*, *Talinum*.

Introduction

The report that crassulacean acid metabolism (CAM) was induced by high salinity in the annual halophyte *Mesembryanthemum crystallinum* L. (Aizoaceae) described for the first time the ability of a plant to switch its pathway of CO_2 assimilation from C_3 to CAM in response to water-deficit stress (Winter and von Willert, 1972). The expression of CAM in *M. crystallinum* is facultative, i.e. optional, because plants can revert in the main to a C_3 phenotype when the environmental stress is removed.

In many species with CAM, its expression is not optional. CAM is part of the constitutive pre-set processes of development and growth (Kluge and Ting, 1978; Osmond, 1978, 2007; Nobel, 1988; Winter and Smith, 1996a, b). As photosynthetic tissues mature, CAM always eventually develops, irrespective of environmental conditions. But even in these constitutive CAM plants, a small facultative CAM component may be detectable. In young tissues with still minimal expression of CAM, drought stress can accelerate the ontogenetic increase

Abbreviations: CAM, crassulacean acid metabolism; PEPC, phosphoenolpyruvate carboxylase; PFD, photon flux density; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; VPD, vapour pressure difference.

in dark CO_2 fixation in a reversible manner (Winter *et al.*, 2008, 2011), demonstrating that the categories of constitutive and facultative CAM are, in reality, endpoints of a continuum between CAM that is fully controlled by ontogeny and CAM that is controlled by environmental stress.

Prior to the report of CAM induction in *M. crystallinum*, it had long been known that, in the photoperiod-sensitive dwarf cultivar of *Kalanchoë blossfeldiana* Poelln., cv. Tom Thumb, young leaves would become succulent and express strong CAM, and flowering would be initiated when long-day-grown plants were subjected to short days (Bode, 1942; Gregory *et al.*, 1954). The CAM induction process in *K. blossfeldiana* differs fundamentally from that in *M. crystallinum* and other facultative CAM species considered in this review. In *K. blossfeldiana* grown under long days, leaves will normally develop CAM as they age (Queiroz and Brulfert, 1982), suggesting that the induction of CAM following short-day treatments is essentially an acceleration of underlying ontogeny. Leaves of *K. blossfeldiana* that exhibit CAM after short-day treatments retain CAM when the plants are returned to long days (Queiroz and Brulfert, 1982). The expression of CAM in this species is thus not optional.

Forty years after the initial *M. crystallinum* report, the number of species for which there is good experimental evidence that CAM is optional remains small but is increasing. By good evidence, we mean demonstration that CAM is elicited or upregulated following drought stress of C_3 tissues, and that there is at least a substantial reversion to C_3 photosynthesis following the removal of stress. In addition to *M. crystallinum*, facultative CAM has been demonstrated in species in the Bromeliaceae [*Guzmania monostachia* (L.) Rusby ex Mez (Medina *et al.*, 1977)], Crassulaceae [e.g. *Sedum mite* Gilib. (Schuber and Kluge, 1981); *Sedum album* L. (Castillo, 1996); *Sedum telephium* L. (Smirnoff, 1996)], Montiaceae [e.g. *Calandrinia* (*Parakeelya*) *polyandra* Benth. (Winter *et al.*, 1981; Winter and Holtum, 2011)], Piperaceae [e.g. *Peperomia scandens* Ruiz and Pav. (Ting *et al.*, 1996)], Portulacaceae [e.g. *Portulaca oleracea* L. (Koch and Kennedy, 1982; this publication)], Talinaceae [e.g. *Talinum triangulare* (Jacq.) Willd. (Herrera *et al.*, 1991)] and also in perennial woody species of *Clusia* [e.g. *Clusia cylindrica* Hammel, *C. minor* L., and *C. pratensis* Seem. (Borland *et al.*, 1992, 1998; Lüttge, 1999, 2006, 2007; Holtum *et al.*, 2004; Winter *et al.*, 2009)].

Species in which an increase in dark CO_2 uptake or nocturnal acidification has been shown in response to reduced soil water availability but where the increases are extremely small or, if large, reversibility has yet to be demonstrated, include species in the Aizoaceae [e.g. *Aptenia cordifolia* (L.f.) Schwantes (Treichel, 1975; *Carpobrotus edulis* (L.) N.E.Br. (Winter, 1973); *Mesembryanthemum nodiflorum* L. (Treichel and Bauer, 1974; Winter and Troughton, 1978); *Delosperma tradescantioides* (P.J. Bergius) L. Bolus (Herppich *et al.*, 1996)], Araceae [*Zamioculcas zamiifolia* (Lodd.) Engl. (Holtum *et al.*, 2007)], Bromeliaceae (*Werauhia sanguinolenta* (Cogn. & Marchal) J.R. Grant (Beltrán *et al.*, 2013)], Commelinaceae [*Callisia fragrans* (Lindl.) Woodson, *Tripogandra multiflora* (Sw.) Raf., and *Tradescantia brevifolia* (Torr.) Rose (Martin *et al.*, 1994)], Crassulaceae [e.g. *Sedum* spp. (Kluge, 1977; Gravatt and Martin, 1992); *Umbilicus rupestris* (Salisb.) Dandy (Daniel *et al.*, 1985); *Tylecodon paniculatus* (L.f.) Toelken (Veste *et al.*, 2001)], Didiereaceae [e.g. *Portulacaria afra* Jacq. (Ting and Hanscom, 1977; Guralnick and Ting, 1986)], Euphorbiaceae [*Pedilanthus* (=*Euphorbia*) *tithymaloides* L. (Reddy *et al.*, 2003)], Gesneriaceae [e.g. *Haberlea rhodopensis* Friv. (Markovska 1999)], Piperaceae [*Peperomia* spp. (Holthe *et al.*, 1992)], Portulacaceae [*Ceraria fruticulosa* H. Pearson & Stephens (Veste *et al.*, 2001); *Anacampseros* spp., *Grahamia* spp., *Portulaca grandiflora* Hook., *Talinum paniculatum* (Jacq.) Gaertn. (Guralnick *et al.*, 2002, 2008)] and Vitaceae [*Cissus trifoliata* (L.) L. (Olivares *et al.*, 1984); *Cissus quadrangularis* L. (Virzo De Santo and Bartoli, 1996)].

We know that the abiotic factors that induce, enhance, or reduce the expression of CAM are associated with transcriptional, post-transcriptional, and post-translational regulatory events (Taybi and Cushman, 1999; Cushman and Borland, 2002; Cushman *et al.*, 2008b), but we lack a detailed description of the sequence of the molecular events that result in the changed photosynthetic phenotype. What is the physical signal that is detected as drought and what is the cascade of events that lead to the upregulation of CAM? For *M. crystallinum*, it has been suggested that molecular responses to different stresses such as drought stress and high soil salinity may be triggered by multiple signals, and that parallel response pathways and gene-regulatory mechanisms enable a plant to upregulate specific genes or subsets of genes in response to a range of environmental stimuli (Vernon *et al.* 1993). Ca^{2+}, Ca^{2+}-dependent kinases, abscisic acid and other hormones, nitric oxide, and H_2O_2 have all been variously implicated in signalling (Chu *et al.*, 1990; Taybi and Cushman, 1999, 2002; Slesak *et al.*, 2008; Freschi and Mercier, 2012). It may also well be that the different stresses that elicit CAM all adversely affect a particular aspect of plant water relations and operate via a common signal and transduction pathway. This dearth of connectivity between basic physical, biochemical, and molecular information is in part the result of the complexity of whole-organism physiology and the changes that occur as CAM plants, particularly facultative CAM plants, simultaneously grow and respond to stress. From an experimental viewpoint, preferred 'clean' model systems of facultative CAM are clearly those in which complicating developmental side effects are minimal.

The aim of this review is to highlight the well-documented reversible facultative CAM systems that might best be used to tease apart the elements of CAM at the molecular level. We do not exhaustively review or list the entire literature on facultative CAM; rather, we have selected six species with characteristics that change markedly in ways that should be conducive to molecular experimental exploration. To this end, we highlight *Clusia pratensis*, a tropical evergreen tree; *Talinum triangulare*, a herbaceous eudicot; *Calandrinia polyandra*, an annual eudicot; *M. crystallinum*, a halophytic annual eudicot; *Portulaca oleracea*, an annual C_4 eudicot; and *Isoetes howellii* Engelm., a seasonally aquatic species that can switch from CAM to C_3. Perhaps surprisingly, the preceding list contains no monocots. Despite the large number of CAM species in the Agavoideae, and particularly the Bromeliaceae

and Orchidaceae, only one species in the monocot clade, *Guzmania monostachia*, has been identified as a facultative CAM species *sensu strictu* (Medina *et al.*, 1977).

Clusia pratensis: a tropical tree

The tropical genus *Clusia* (Clusiaceae) is a rich genetic resource for the study of the functional and ecological elements of CAM. In general, *Clusia* species are plastic in habit, with life forms that include trees, shrubs, epiphytes, and hemiepiphytes, and are ecologically catholic, inhabiting wet forests, seasonally dry forests, savannahs, and rocky landscapes from sea level to over 2000 m (reviewed by Lüttge, 2007). The genus is monophyletic, yet within it CAM has appeared and has possibly been lost several times (Gehrig *et al.*, 2003; Holtum *et al.* 2004; Gustafsson *et al.*, 2007). It is speciose and physiologically diverse, with 300–370 species (The Plant List, 2010, version 1: http://www.theplantlist.org/) that include apparently purely C_3 taxa (e.g. *C. multiflora* Kunth; Grams *et al.*, 1998), constitutive strong CAM species (e.g. *C. rosea* Jacq. and C. *hilariana* Schltdl.; Popp *et al.*, 1987; Herzog *et al.*, 1999) and many species that can switch to lesser or greater degrees between C_3 and CAM (Lüttge, 2007). Well-characterized *Clusia* species with facultative CAM include *C. minor* (Borland *et al.*, 1992, 1993, 1994), *C. uvitana* (Winter *et al.*, 1992; Zotz and Winter, 1993, 1994*a*, *b*), *C. cylindrica* (Winter *et al.*, 2009), and *C. pratensis* (Winter *et al.*, 2008), although *C. minor* and *C. uvitana* Pittier do not represent 'clean' facultative CAM systems because considerable CAM activity may be present in mature leaves of well-watered plants (Borland *et al.*, 1998; de Mattos and Lüttge, 2001; Winter *et al.*, 1992).

Clusia pratensis (Fig. 1A) is remarkably flexible photosynthetically. Fully reversible induction of CAM in response to water stress has been demonstrated in whole plants (Winter *et al.*, 2008). Fig. 2 shows how an individual leaf downregulates CO_2 uptake during the day and shifts from net CO_2 loss to net CO_2 uptake in the dark in response to drought stress, with the four phases of CAM CO_2 exchange present after 4 d. As is evident from this and other induction experiments, a strong transient reduction of CO_2 uptake during the light often precedes

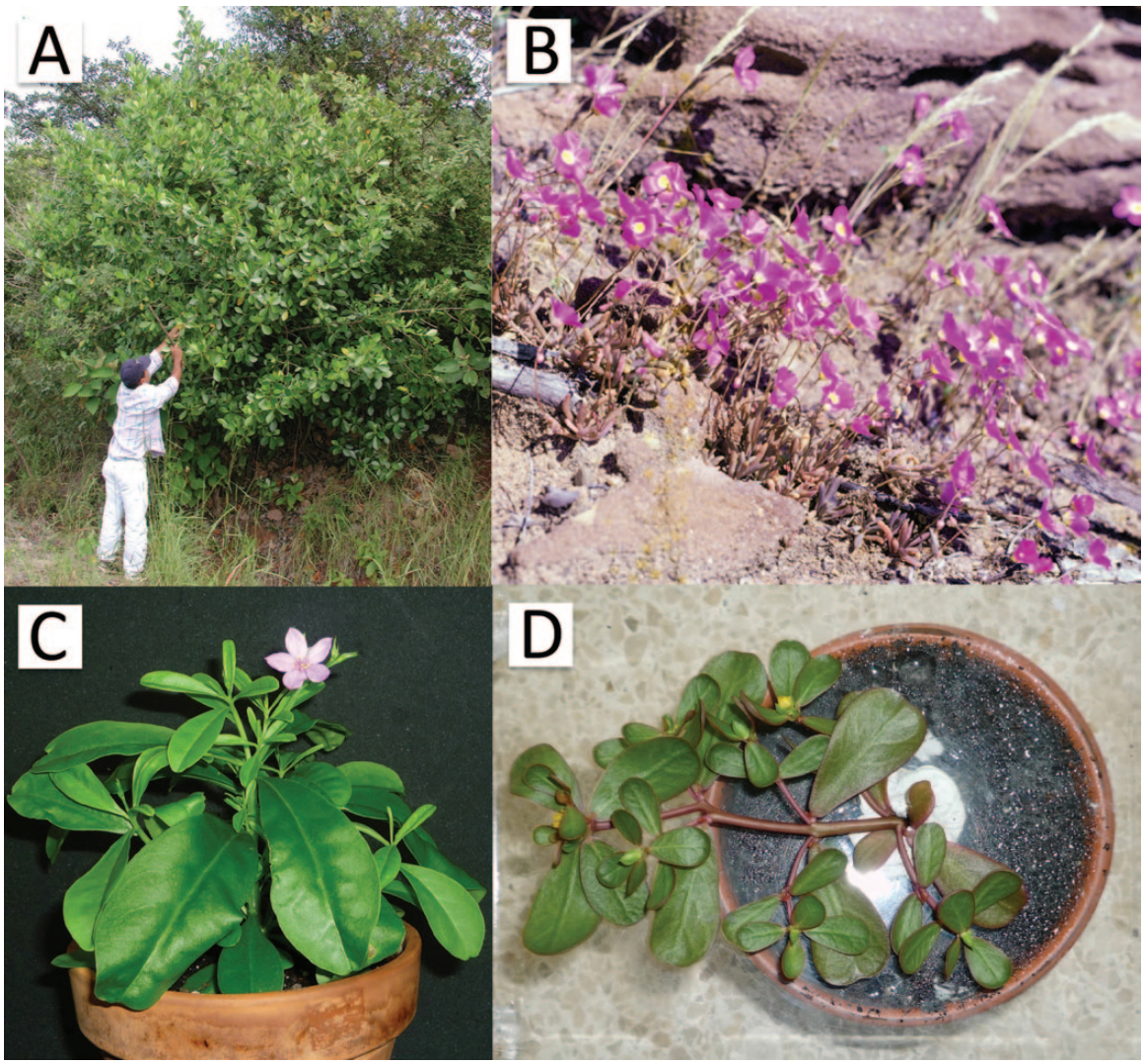

Fig. 1. Four species that exhibit facultative CAM. (A) *Clusia pratensis*, about 4 m tall, at Santa Fé, Veraguas Province, Republic of Panama. (B) *Calandrinia polyandra* in its native habitat among sandstone outcrops near Kalbarri, Western Australia. (C) *Talinum triangulare* in flower. (D) The C_4-CAM plant *Portulaca oleracea* growing through the base plate of a gas-exchange cuvette. (This figure is available in colour at *JXB* online.)

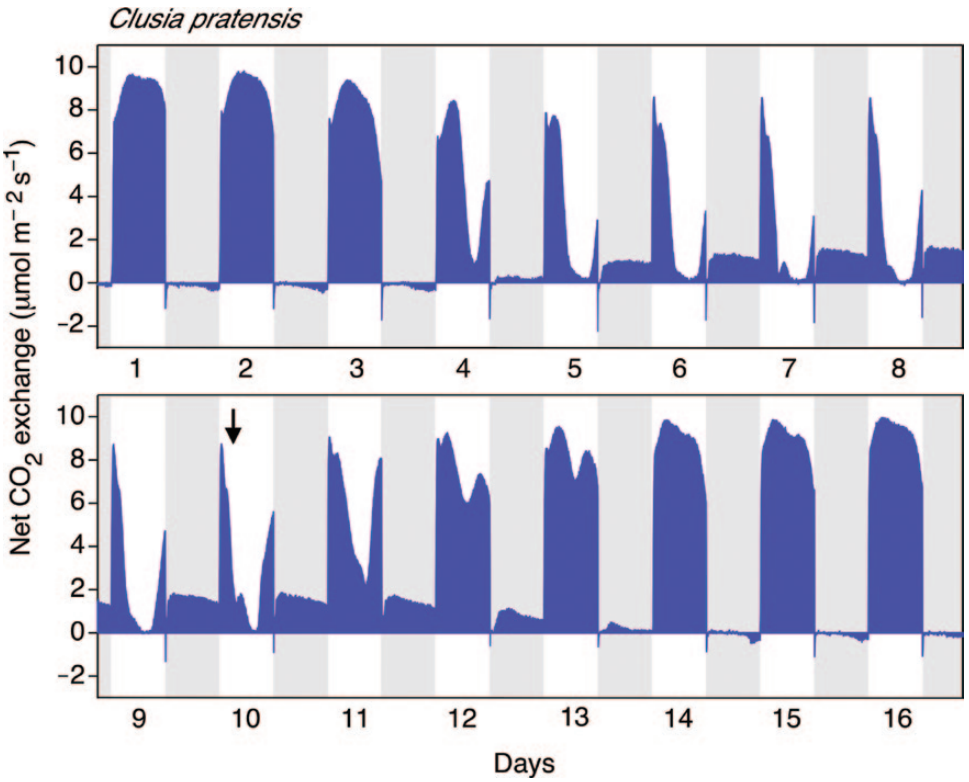

Fig. 2. Sixteen days of net CO_2 exchange by a fully developed leaf attached to a 25 cm tall potted *Clusia pratensis*. Measurements were performed at 400 ppm CO_2 in a controlled environment chamber maintained under 12 h light (28 °C)/12 h dark (22 °C) cycles. Photon flux density (PFD) at leaf level was 500 µmol m^{-2} s^{-1}. Watering was withheld on d 1 and recommenced on d 10 (arrow indicates rewatering). Shaded areas represent the dark periods. (This figure is available in colour at *JXB* online.)

the appearance of net CO_2 fixation in the dark. Maximum rates of dark fixation of close to 2 µmol m^{-2} s^{-1} are about 20% of the rates of day-time CO_2 fixation in non-stressed leaves. Upon rewatering, the leaf reverts to the pre-treatment rates of CO_2 uptake during the day and CO_2 loss at night. Indeed, *Clusia pratensis* is the only CAM species to date in which whole shoots of seedlings have been demonstrated to fully switch twice from C_3 to CAM and back during a period that is shorter than the lifetime of individual leaves (Winter *et al.*, 2008).

In order to test whether a reduction in day-time carbon gain is not just an intermediary step or consequence of CAM but can trigger CAM induction, we lowered the CO_2 concentration surrounding a leaf of a well-watered *Clusia pratensis* from 400 to 100 ppm for 2–8 h during the middle of the day (Fig. 3A). Consistent with previous studies with *M. crystallinum*, this treatment had no effect on nocturnal CO_2 exchange (Winter, 1979). To explore whether the high internal CO_2 concentrations that are characteristic of CAM tissues reinforce CAM induction, we exposed a leaf of a well-watered *Clusia pratensis* to 800 ppm for 8 h of the light period. The rise in CO_2 concentration strongly increased day-time carbon gain but was also without effect on nocturnal CO_2 exchange (Fig. 3B). Following severance of the stem, the leaf inside the gas-exchange cuvette immediately ceased CO_2 uptake in the light, and respiratory net CO_2 loss in the dark continued but at a much higher rate. There was no evidence of CAM, not even of recycling of respiratory CO_2, at least not during the 2 d experimental period, highlighting the potential role of root-to-shoot communication in the CAM induction process. Drought-stressed induction of CAM occurs not only under the current ambient CO_2 concentration but also under elevated CO_2 concentration (Fig. 4), suggesting that facultative CAM characteristics will be conserved as global CO_2 concentration increases.

Leaf-to-air vapour pressure difference (VPD) has been inferred to play a major role in the photosynthetic pathway control of *Clusia minor*. Schmitt *et al.* (1988) showed that, following rewatering of water-stressed CAM-performing *Clusia minor*, the reversion to C_3 photosynthesis occurred more rapidly when VPD was low (6.2 vs 13.1 mbar). This experiment has been erroneously interpreted to suggest that high VPD can rapidly induce CAM (Dodd *et al.*, 2002; Matiz *et al.*, 2013). Fig. 5 shows, for a leaf of a well-watered *Clusia pratensis*, increasing the leaf-to-air VPD from 14.1 to 44.3 mbar during the light markedly decreased day-time CO_2 uptake but had no effect on nocturnal CO_2 exchange.

It has been mooted that the seasonal water-use characteristics of *Clusia pratensis*, and its ability to switch reversibly between C_3 and CAM photosynthesis, make it a potential tree species for reforestation in the Panama Canal watershed where too much water in the Canal at the end of the wet season and too little water during the dry season can be problematic (Fig. 6). In order to reduce superfluous water runoff into the Canal during the wet season but maximize drainage into the Canal during the dry season, tree species are being trialled that transpire profusely when soils are wet and transpire minimally during the dry season.

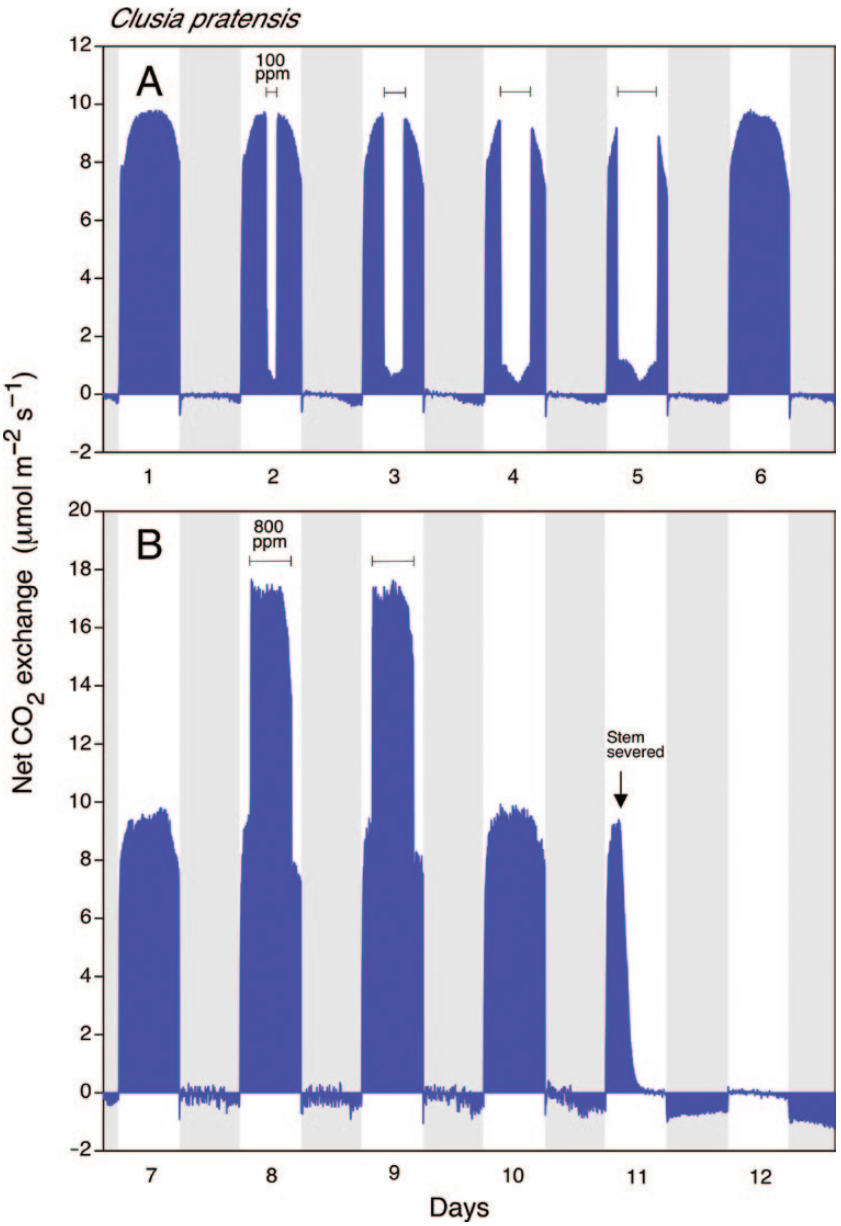

Fig. 3. Effect of lowering (A) or raising (B) the CO_2 concentration during the middle of the day on net CO_2 exchange by a fully expanded leaf of a well-watered *Clusia pratensis* in the C_3 mode. Measurements were performed under the conditions described in Fig. 2 except that the leaf was exposed to 100 ppm CO_2 during the middle of the day for 2 h (d 2), 4 h (d 3), 6 h (d 4) or 8 h (d 5), or exposed to 800 ppm CO_2 for 8 h on d 8 and 9. The shoot was severed from the roots on d 11. (This figure is available in colour at *JXB* online.)

With respect to *Clusia* species as experimental systems for biochemical and molecular research, the extraction of metabolites, RNA, and DNA can be complicated by the presence of latex and polyphenols in leaf extracts, but methods have been developed to surmount this problem (Gehrig *et al.*, 2000). Plants have been established from tissue culture (Ball *et al.*, 1991; Saleh, 1999). To our knowledge, no molecular transformation has been published for members of the genus *Clusia*.

Calandrinia polyandra: a succulent annual

In contrast to the long-lived *Clusia*, most facultative CAM species are small in stature and relatively short-lived. One of these species is *Calandrinia polyandra* (Montiaceae). In a series of continuous whole-plant lifetime CO_2 exchange measurements, Winter and Holtum (2011) demonstrated exemplary facultative CAM in this succulent-leafed prolifically seeding annual that is native to low-nutrient sandy soils in coastal and inland Western Australia (Fig. 1B; Kapitany, 2007). Reducing soil water availability not only induces a shift from solely daytime CO_2 fixation to mainly nocturnal net CO_2 fixation in *Calandrinia polyandra* (Fig. 7), but the C_3 to CAM shift is also fully reversible either upon rewatering or upon a combination of rewatering and the addition of nutrients.

No complete life-cycle studies have been reported for *Calandrinia* in the field, but gas-exchange patterns of *Calandrinia polyandra* in the laboratory are consistent with strategies predicted for annuals that begin life as C_3 plants when soil water is available and then switch to the

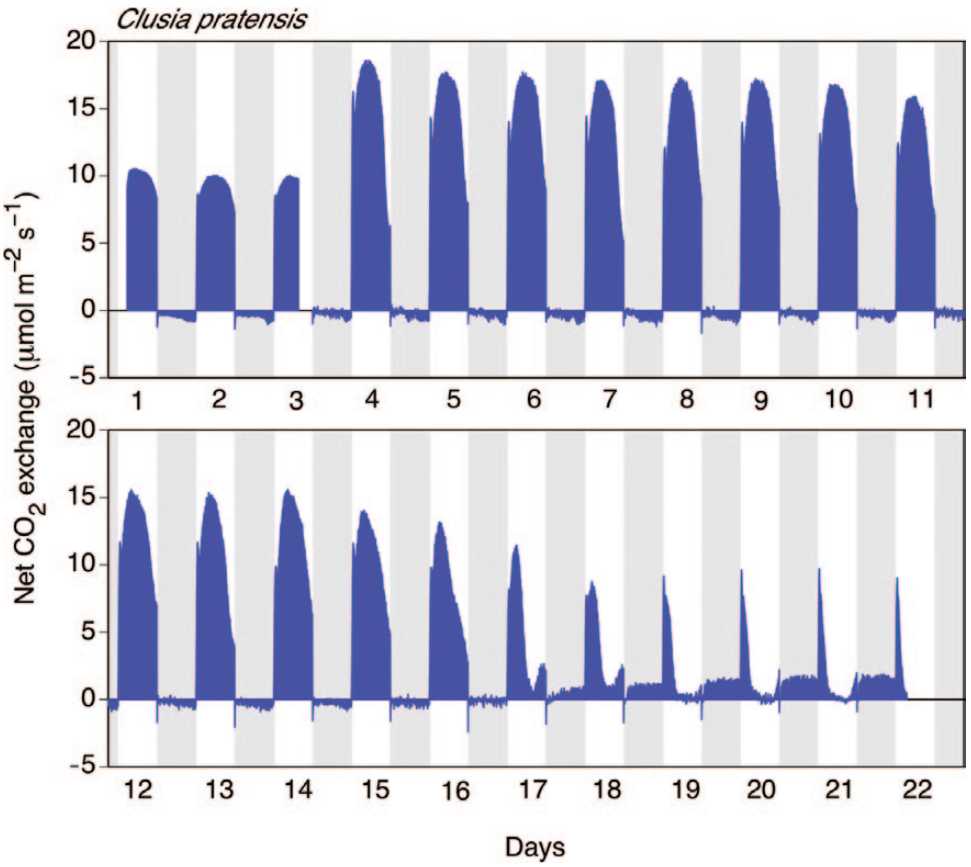

Fig. 4. Induction of CAM in a fully expanded leaf of *Clusia pratensis* grown at 400 ppm CO_2 and exposed to 800 ppm CO_2 from the afternoon of d 3 onwards. Other conditions were as described in Fig. 2. Watering was withheld from d 4 onwards. (This figure is available in colour at *JXB* online.)

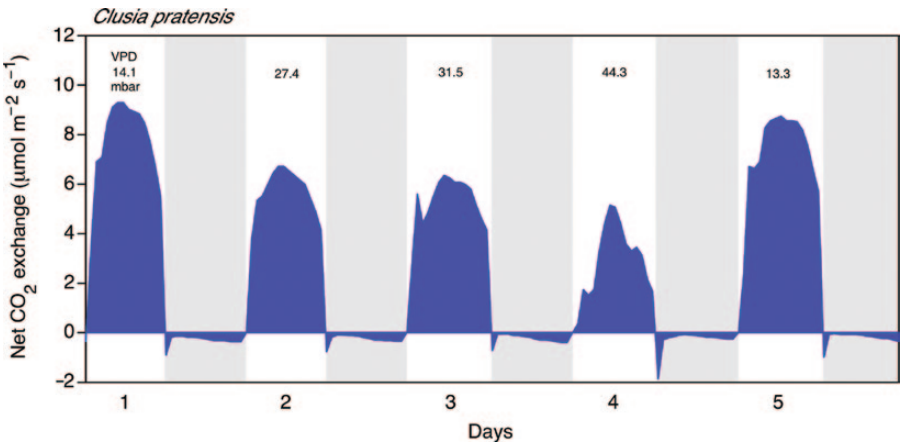

Fig. 5. Response to variations in leaf-to-air VPD in the light of a fully expanded leaf of a well-watered *Clusia pratensis* in the C_3 mode. Measurements were performed at 400 ppm CO_2. Temperature during the 12 h light period (PFD of 600 µmol m^{-2} s^{-1}) was 30 °C except for d 4 when the temperature was 35 °C. Temperature at night was 22 °C. The leaf was exposed to midday VPDs of 14.1 (d 1), 27.4 (d 2), 31.5 (d 3), 44.3 (d 4), and 13.3 mbar (d 5). (This figure is available in colour at *JXB* online.)

water-conserving CAM mode as water becomes limiting (Winter and Holtum, 2011). Leaf $\delta^{13}C$ values of between –22.5‰ and –22.2‰, and significant nocturnal acidification in leaves collected from *Calandrinia polyandra* near the site shown in Fig. 1B, are consistent with the operation of CAM during the later part of the life cycle of plants in the field. In an unidentified perennial *Calandrinia* that dries back to root tubers during the dry season (*Calandrinia* sp. I; Winter *et al.*, 1981), leaf $\delta^{13}C$ values shifted from –23.5‰ in September to –19.3‰ at the late flowering stage 3 months later. At that time, root tubers exhibited $\delta^{13}C$ values of –15.9‰, suggesting that CAM contributed carbon not only to seed production but also specifically to tuber filling.

In all probability, other Australian *Calandrinia* species will also prove to exhibit CAM, and possibly facultative CAM (Winter *et al.*, 1981). The large number of *Calandrinia* species, roughly 70 (Australian Virtual Herbarium 2013, http://avh.chah.org.au, Canberra: Council of Heads of Australasian

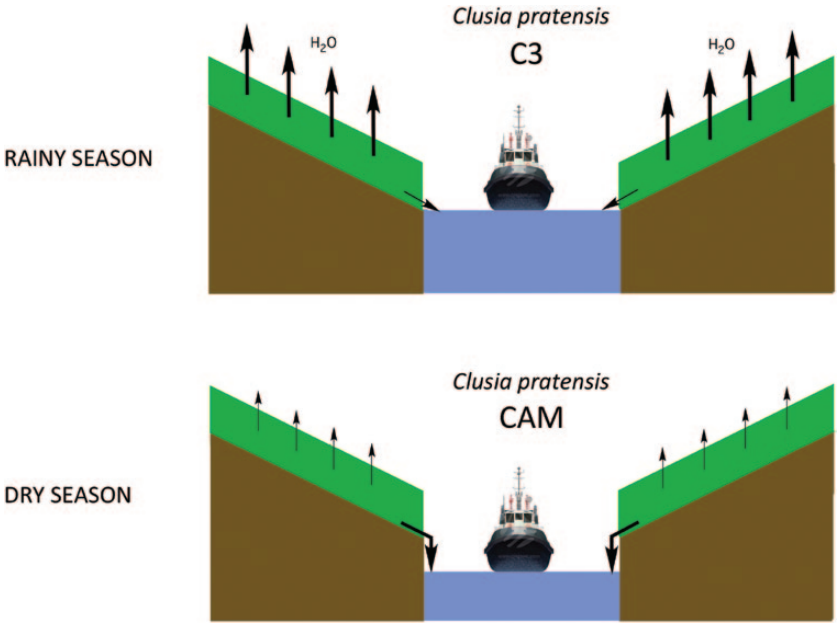

Fig. 6. Hypothetical water fluxes into the atmosphere and into the Panama Canal during the rainy season (upper panel) and the dry season (lower panel) assuming *Clusia pratensis* was planted in the Canal watershed and functioned as a C_3 plant during the rainy season and as a CAM plant during the dry season. (This figure is available in colour at *JXB* online.)

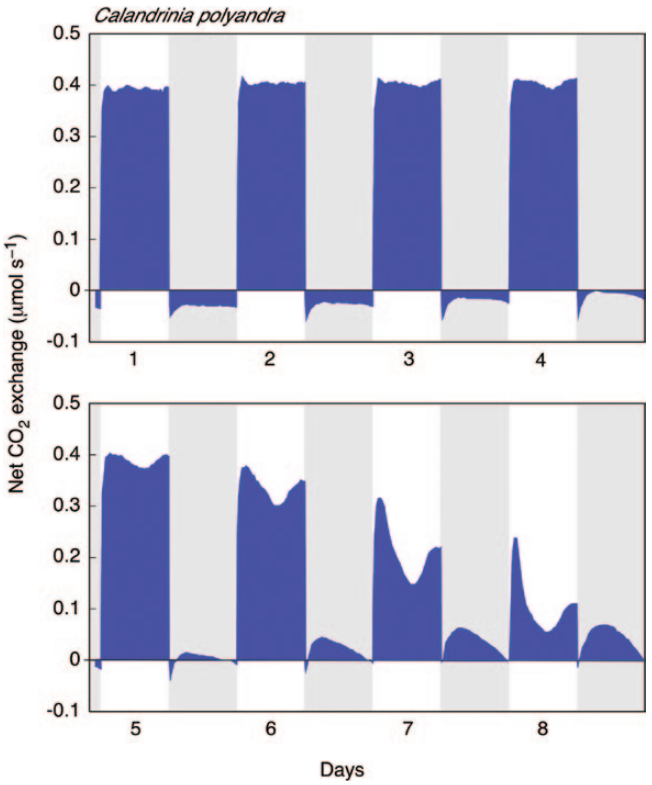

Fig. 7. Eight days of net CO_2 exchange by a branch of a 2-month-old *Calandrinia polyandra* plant exposed to drought. Measurements were performed at 400 ppm CO_2 in a controlled environment chamber maintained under 12 h light (25 °C)/12 h dark (17 °C) cycles. PFD at leaf level was 500 µmol m^{-2} s^{-1}. Watering was withheld from d 1 onwards. Shaded areas indicate the dark periods. (This figure is available in colour at *JXB* online.)

Herbaria Inc.; F. Obbens, Western Australian Herbarium, personal communication), which include annual, perennial, and tuberous herbs (Pate and Dixon, 1982; Obbens, 2006, 2011), and the range of climates in which they are found, from temperate, arid, and tropical Australia, indicate that *Calandrinia* will become a useful resource for interrogating CAM, even more so when systematic issues are resolved. The Australian *Calandrinia* species are not monophyletic with the New World *Calandrinia* species (Carolin, 1993), and even though a new genus name, *Parakeelya*, has been published for the Australian clade, monophyly within it has yet to be demonstrated (Hershkovitz, 1998). Species identification is difficult, as many species are overtly similar or exhibit considerable phenotypic variation across their ranges. Three hundred years after the collection in 1699 of the *Calandrinia polyandra* that is the oldest pressed Australian plant specimen in a herbarium (Dampier, 1703; George, 1999), new species continue to be described (Obbens, 2006, 2011; Tahir and Carolin, 2011).

We are unaware of any *Calandrinia* transformation system, but *Calandrinia mirabilis* (West and Chinnock, 2013) (formerly *Calandrinia* sp. Mt. Clere), a species with horticultural potential (Harrison *et al.*, 2009; Cave *et al.*, 2011), which like *Calandrinia polyandra* can self-cross, has been grown in tissue culture (Wickramasinghe *et al.* 2009), and pigment genes have been expressed transiently in *Antirrhinum majus* L. (Hsiao-Hang, 2012).

Talinum triangulare: a herbaceous weed

CO_2 fixation in *T. triangulare* (Fig. 1C), used as a leaf vegetable in some tropical regions, has been studied extensively in the laboratory of Herrera (Herrera *et al.*, 1991; Herrera 1999, 2009). Fig. 8 is a detailed chronology of how a leaf of *T. triangulare* changes from C_3 to CAM and back to C_3. In the well-watered state, the day/night pattern of CO_2 exchange with essentially constant net CO_2 loss at night indicates

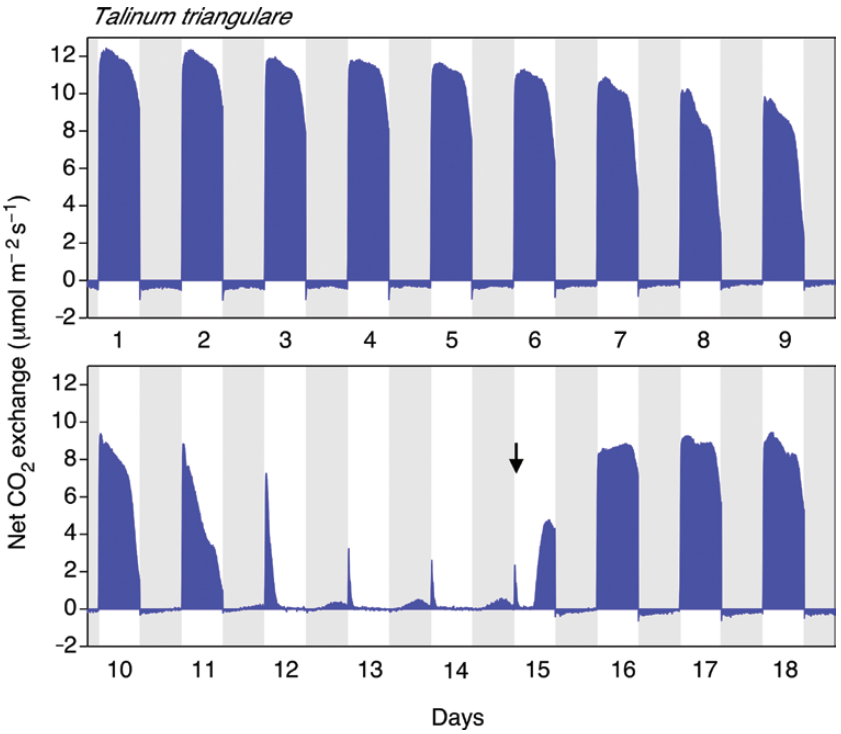

Fig. 8. Eighteen days of net CO_2 exchange by a fully developed leaf attached to a 20 cm tall potted *Talinum triangulare* plant. Measurements were performed under the conditions described in Fig. 2. Watering was withheld from d 1 and recommenced on d 15 (arrow indicates rewatering). Shaded areas indicate the dark periods. (This figure is available in colour at *JXB* online.)

entirely C_3 photosynthesis, more so than for example in well-watered *Clusia pratensis* in which CO_2 loss may be reduced during the early dark period (Fig. 2). As the *T. triangulare* leaf transitions to CAM, it traverses a tipping point during the night of day 11 when low rates of net CO_2 loss during the first part of the night are balanced by low rates of net CO_2 gain during the latter half of the night. Three days later, maximum CAM activity is reached, and day-time CO_2 fixation is restricted to a short period at the beginning of the light period. As with all facultative CAM species, the overall CO_2 fluxes in the CAM mode are low in comparison with the initial well-watered C_3 state, partly because, in the CAM state, these facultative CAM plants are drought stressed. The difference in CO_2 uptake rates between C_3 and CAM states in *T. triangulare* is particularly pronounced. Upon rewatering, CO_2 fluxes rapidly increase, and within 4 d, a C_3 CO_2 exchange pattern is observed.

Of the approximately 60 species in the genus *Talinum* (Talinaceae), seven are known to exhibit nocturnal acid accumulation in response to drought conditions, but acid accumulation that is accompanied by net CO_2 uptake in the dark has only been reported for *T. triangulare* (Herrera *et al.*, 1991) and *T. paniculatum* (Güerere *et al.*, 1996), two relatively weedy erect species with lignified stems and broad slightly succulent leaves. The remaining five species that have been reported to exhibit features of weakly expressed CAM frequent shallow soils on rock outcrops in northern America and are all small with succulent sausage-shaped leaves (Martin and Zee, 1983; Harris and Martin, 1991).

There are no published reports of *Talinum* transformation, but procedures for converting leaf discs and transverse thin cell layers of internodal explants via callus to plants have been established (Swarna and Ravindhran, 2012, 2013). Regenerated plants that successfully established under field conditions exhibited phenotypes similar to the mother plants. The advantages of *T. triangulare* as a model facultative CAM system include full reversibility of CAM and large evenly green leaves that are conducive to gas-exchange studies and partial leaf sampling. Furthermore, growth is rapid, the life cycle is relatively short, and plants can self-cross.

Portulaca oleracea: an annual C_4 plant

While the majority of facultative CAM species use C_3 photosynthesis to fix CO_2 in the light, *P. oleracea* (Fig. 1D) stands out as a C_4 plant with an ability to induce CAM (Koch and Kennedy, 1980, 1982). Fig. 9 shows net CO_2 exchange for the above-ground tissues of a young *P. oleracea* as it grows rapidly inside a gas-exchange cuvette. Watering was withheld from day 3, but, supplied by water still present in the pot, the plant continued to grow, exhibiting increasing net CO_2 uptake in the light and increasing respiratory CO_2 loss in the dark. On d 7, as water availability became limiting, CO_2 uptake started to decrease and CO_2 exchange was zero for most of the night. A pronounced midday depression in CO_2 exchange dominated d 8, and nocturnal net CO_2 uptake was observed for the first time. By d 10, CO_2 exchange in the light was limited to a short burst following the onset of the light period, but net CO_2 uptake in the dark persisted. Upon rewatering on d 11, the CO_2 fluxes increased during the light and the dark, reverting to a non-CAM pattern within 24 h, although the transient reductions in the rate of CO_2 loss at night suggest

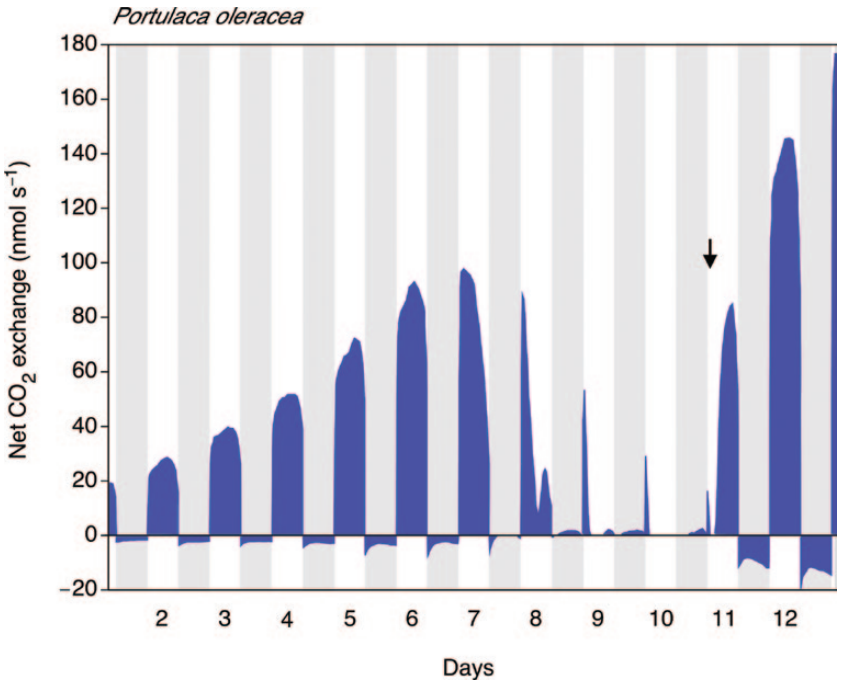

Fig. 9. Twelve days of net CO_2 exchange by a developing shoot of *P. oleracea* (the shoot at the end of the experiment is shown in Fig. 1D). Measurements were performed under the conditions described in Fig. 2 except that PFD was 800 μmol m^{-2} s^{-1}. Watering was withheld from d 3 and recommenced on d 11 (arrow indicates rewatering). Shaded areas indicate the dark periods. (This figure is available in colour at *JXB* online.)

residual CAM activity. Again, as in *T. triangulare*, net CO_2 uptake at night in the CAM state is, although clearly present, low, and therefore is unlikely to support vegetative growth. Its functional significance is presumably to prolong survival by minimizing carbon and water loss, and to assist reproduction.

The transitional state of zero nocturnal CO_2 exchange for most of the night of d 7 (Fig. 9) is typical of what is commonly referred to as CAM cycling. This term tacitly assumes that CO_2 released by mitochondrial respiration is quantitatively refixed into the CAM cycle. However, the actual CO_2 species, respiratory or atmospheric, that is converted to malic acid under these conditions has not been determined experimentally, for example through mass spectrometry. All we know at this point is that, when nocturnal exchange is zero, CO_2 fluxes into and out of the leaf are balanced. In all cases, the transition from respiratory CO_2 loss in the C_3 state to net CO_2 uptake in the CAM state is presumably accompanied by a shift in the respiratory baseline that is attributable to reduced rates of mitochondrial respiration in response to drought stress. For example, in seedlings of a tropical C_3 tree, *Calophyllum longifolium* Willd., the decrease in daily carbon gain by 69% during drought stress was accompanied by a 66% decrease in net respiratory CO_2 loss (Fig. 10). Thus, decreased mitochondrial respiration, independent of CAM, contributes to the nocturnal CO_2 signal during C_3-to-CAM transitions. The proportional contribution of respiration is largest in plants that shift to low-level CAM. However, a shift in the respiratory baseline by itself could never result in net CO_2 uptake.

A diversion in the facultative CAM story of *P. oleracea* is that not only can leaves perform CAM but also stems that lack C_4 photosynthesis. This is also the case in the closely related C_4-CAM *Portulaca grandiflora* (Guralnick *et al.*, 2002). Stems either have very few stomata (*P. oleracea*) or are believed to lack stomata (*P. grandiflora*). We do not know the extent to which stems, which were included in the measurements shown in Fig. 9 (see the plant depicted in Fig. 1D), contributed to net CO_2 exchange. If the stems merely recycle respiratory CO_2 (Guralnick *et al.*, 2002), net CO_2 exchange would not be affected.

Because most of the reactions in CAM and C_4 photosynthesis are identical, the leaves of *P. oleracea* are a potent system for differentiating CAM and C_4 isogenes using expression studies, particularly as the C_4 cycle is downregulated during drought stress and the CAM pathway is upregulated.

An intriguing question in *Portulaca* is how the C_4 and CAM pathways are compartmented within a single leaf. The two pathways are believed to be spatially separated in leaves of both *P. oleracea* and *P. grandiflora*, with C_4 occurring in the bundle-sheath and mesophyll cells, and CAM occurring in succulent chloroplast-containing water-storage cells (Sage, 2002). The hypothesis is reasonably supported for *P. grandiflora*, but experimental proof is equivocal for *P. oleracea* (Lara *et al.*, 2003, 2004). In the fleshy lanceolate leaves of *P. oleracea*, the water-storage cells are located between the epidermis and the mesophyll cells that encircle the bundle sheaths (atriplocoid type; Voznesenskaya *et al.*, 2010; Edwards and Voznesenskaya, 2011). As expected, immunolocalization placed phosphoenolpyruvate carboxylase (PEPC) in the mesophyll and the water-storage cells but not in bundle-sheath cells (Lara *et al.*, 2003, 2004). Unexpectedly, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was not detected in the water-storage cells but was solely in bundle-sheath cells. If these separate locations of PEPC and Rubisco are correct,

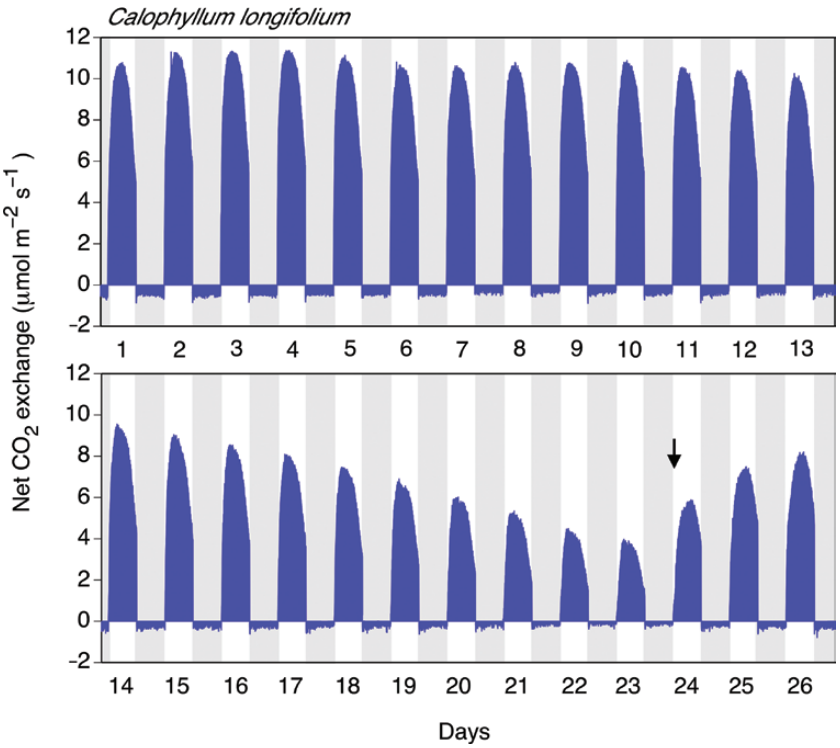

Fig. 10. Net CO_2 exchange by a fully developed leaf of a 24 cm tall seedling of the tropical C_3 tree *Calophyllum longifolium* (Calophyllaceae) during a 26 d drought-recovery cycle. Measurements were performed under the conditions described in Fig. 2. Watering was withheld on d 1 and recommenced on d 24 (arrow indicates rewatering). Shaded areas indicate the dark periods. (This figure is available in colour at *JXB* online.)

then CAM may not be a single-cell pathway in *P. oleracea*. One would have to propose that CO_2 released during de-acidification in the light is refixed in the C_4 bundle-sheath cells by Rubisco (Lara *et al.*, 2004).

The anatomy of *P. grandiflora* differs substantially from *P. oleracea*. *P. grandiflora* has semi-terete leaves with a peripheral distribution of vascular bundles surrounded by C_4-type bundle-sheath and mesophyll cells, with a main vein located at the leaf centre. The central vein is separated from the outer photosynthetic cells by three to four layers of large cells with scattered chloroplasts that increase in abundance towards the central vein (pilosoid type; Nishioka *et al.*, 1996; Voznesenskaya *et al.*, 2010; Edwards and Voznesenskaya, 2011). Based on immunolabelling, there is unequivocal evidence for Rubisco in bundle-sheath cells and for PEPC in mesophyll cells (Guralnick *et al.*, 2002). For central water-storage tissue, Nishioka *et al.* (1996) demonstrated Rubisco presence in chloroplasts, and Guralnick *et al.* (2002) used antibody-based tissue prints to provide evidence for increased presence of PEPC following water stress. C_4 and CAM photosynthesis may thus be spatially separated in leaves of *P. grandiflora*, with C_4 occurring in the leaf periphery and CAM in the central water-storage cells. It should be feasible to demonstrate the cellular location of CAM in both *P. oleracea* and *P. grandiflora* by tracking vacuolar acidification using pH-sensitive fluorescent probes.

P. grandiflora has been grown in tissue culture, transformed with *Agrobacterium tumifaciens*, and transformants grown through callus to plants (Rossi-Hassani *et al.*, 1995; Safdari and Kazemitabar, 2010), whereas *P. oleracea* has been grown in tissue culture and has undergone hairy root transformation using *Agrobacterium rhizogenes* (Kennedy *et al.*, 1977; Safdari and Kazemitabar, 2009).

Mesembryanthemum crystallinum: an annual halophyte

M. crystallinum is the most comprehensively studied facultative CAM plant, with more than 300 papers published with its name in the title since the first report of CAM in the species (Winter and von Willert, 1972; Web of Science, 2014: http://thomsonreuters.com/thomson-reuters-web-of-science/). In most studies, high soil salinity has been the experimental treatment used to induce CAM. Early conjecture that the shift to CAM in *M. crystallinum* is not facultative but represents an acceleration of normal developmental processes (Osmond, 1978) was dispelled when it was demonstrated that plants grown under appropriately non-stressful conditions undergo their entire life cycle as C_3 plants, traversing all developmental stages including setting viable seed (Winter and Holtum, 2007).

Reversibility of CAM has been demonstrated (Winter, 1974; Vernon *et al.* 1988; Schmitt, 1990), although the short life span of leaves can make it difficult to attain full reversion. Furthermore, if high soil salinity is the stressor, abrupt transfer from highly saline to non-saline growth medium can osmotically damage roots and prolong stress. Fig. 11 shows the net CO_2 exchange of a *M. crystallinum* plant grown under non-saline conditions and exposed to drought. As day-time

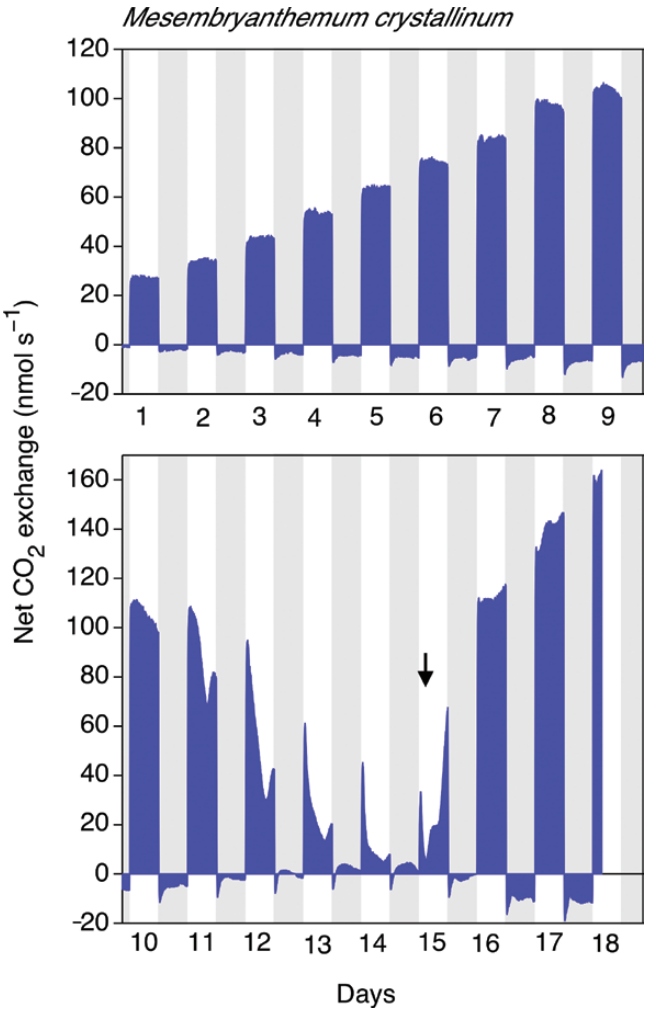

Fig. 11. Net CO_2 exchange for a shoot of the halophyte *M. crystallinum* grown under non-saline conditions and exposed to a drought-recovery cycle. The plant was 6 weeks old at the beginning of the experiment. Watering was withheld on d 3 and recommenced on d 15 (arrow indicates rewatering). Measurements were performed at 400 ppm CO_2 in a controlled environment chamber maintained under 12 h light (25 °C)/12 h dark (17 °C) cycles. PFD at leaf level was 350 µmol m^{-2} s^{-1}. Shaded areas indicate the dark periods. (This figure is available in colour at *JXB* online.)

CO_2 fixation decreases, nocturnal CO_2 exchange switches from CO_2 loss to CO_2 uptake within a few days. Upon rewatering, day-time CO_2 fixation increases rapidly and nocturnal CO_2 balance becomes negative again.

Comparing *Mesembryanthemum* tissues in the C_3 and CAM states, studies of extractable activities of enzymes and their intracellular location (Holtum and Winter, 1982; Winter *et al.*, 1982; Paul *et al.*, 1993), of transporters (Häusler *et al.*, 2000), and of isolated chloroplasts (Demmig and Winter, 1983; Monson *et al.*, 1983; Neuhaus *et al.*, 1988), mitochondria (Winter *et al.*, 1986), and vacuoles (Struve *et al.*, 1985) have provided the bases of many details of our current concepts of the CAM cycle (Edwards *et al.*, 1982; Winter and Smith, 1996b; Holtum *et al.*, 2005). For example, large increases in the activity of enolase, phosphoglyceromutase, and NAD:glyceraldehyde-3-phosphate dehydrogenase in the cytoplasm of leaves operating in the CAM mode suggest that, during the decarboxylation of malic acid, PEP formed from pyruvate inside the chloroplasts has to return to the cytosol before re-entering the chloroplasts as triose phosphate for the final steps of gluconeogenesis (Holtum and Winter, 1982; Winter *et al.*, 1982).

The first-generation molecular analyses were built on the above-mentioned biochemical observations (Bohnert *et al.*, 1988; Ostrem *et al.*, 1990; Cushman 1993; Cushman and Bohnert, 1999). For instance, the gene encoding the CAM isoform of PEPC was identified (Cushman *et al.*, 1989), and the increase in enolase activity was shown to be associated with greater transcription but not more protein, indicating that the increase in activity was the result of post-translational changes to existing protein (Forsthoefel *et al.*, 1995). Transcriptome, proteome, and mutant-based analyses are now superseding earlier molecular approaches (e.g. Bohnert and Cushman, 2000; Kore-eda *et al.*, 2004; Cushman *et al.*, 2008a; Barkla *et al.*, 2009; Haider *et al.*, 2012; Cosentino *et al.*, 2013).

Development of a successful transformation system for *M. crystallinum* remains problematic. Whole-plant regeneration from callus has been achieved by somatic embryogenesis and organogenesis (Meiners *et al.*, 1991; Cushman *et al.*, 2000; Libik *et al.*, 2005), and *M. crystallinum* roots and hypocotyls have been successfully transformed and grown as stable callus (Andolfatto *et al.*, 1994; Ishimaru, 1999; Ishimaru *et al.*, 1999). Shoots or somatic embryos have yet to be generated from transformed callus, possibly because of a problem with hormonal homeostasis (Konieczny *et al.*, 2011).

Isoetes howellii: an aquatic species

It may surprise at first sight that *Isoetes* is included in this review, because facultative CAM is typically associated with drought stress in terrestrial habitats, while *I. howellii* is an aquatic CAM species generally considered a constitutive CAM plant. In shallow, marshy, or seasonally inundated freshwater environments, submerged plants in the lycophyte genus *Isoetes* and the anthophyte genera *Crassula*, *Littorella*, *Sagittaria*, and *Vallisneria* can express appreciable CAM (Keeley, 1981, 1982, 1998; Keeley and Morton, 1982; Aulio, 1986; Keeley and Rundel, 2003). When water levels recede, and leaves (microphylls) emerge into the atmosphere, the capacity for CAM may be lost and C_3 photosynthesis becomes essentially the only pathway of carbon acquisition (Keeley *et al.*, 1983*a*, *b*; Groenhof *et al.*, 1988; Keeley and Sandquist, 1991).This change includes stages when the submerged leaf bases still exhibit substantial CAM, whereas tips of the same leaves do not (Keeley and Busch, 1984; Keeley, 1996). These aquatic species differ from terrestrial facultative species in that the initial switch is from CAM to C_3, rather than from C_3 to CAM. In *I. howellii*, the most extensively studied aquatic species, CAM may contribute 40% of total carbon gain in submerged leaves but less than 1% under aerial conditions (Keeley and Sandquist, 1991). The switch from CAM to C_3 in *I. howellii* is at least partially reversible (Keeley, 1983).

CO_2 assimilation in submerged habitats is strongly limited by the slow diffusion of CO_2, even though the combined

inorganic carbon pool can be high. The trigger for the emergence-associated loss of CAM in *I. howellii* is not known. The most widely held opinion is that this loss of CAM is a response to an enhanced availability of CO_2 under aerial conditions, eliminating the ecological advantage of dark CO_2 fixation (Keeley, 1998). However, it is unclear how to reconcile this view with observations that in *I. howellii* and two other aquatic CAM species, *Isoetes setacea* Lam. and *Littorella uniflora* Asch., the loss of CAM upon emergence is prevented by high relative air humidity (Aulio, 1986; Keeley, 1988; Gacia and Ballesteros, 1993), a result that suggests that the change in photosynthetic metabolism upon exposure to air is related to water status of the exposed tissues. Assuming that higher humidity aids in maintaining favourable leaf hydration, the humidity response contrasts with the responses in non-aquatic plants where aridity favours CAM. These observations clearly warrant clarification. When emergent for extensive periods, the leaves of *Isoetes* die back to the corm, which lies dormant until resubmerged. As the leaves die back, there is no dehydration-induced reversion to CAM.

Critical assessment and perspective

Facultative CAM does not seem to be uncommon. Although reversibility is often not tested, many of the species with confirmed or suspected facultative CAM cluster in the order Caryophyllales, which houses, for example, the Aizoaceae, Montiaceae, Portulaceae, and Talinaceae. To better understand the evolutionary trajectories of facultative CAM relative to constitutive CAM, more species need to be screened for the ability to reversibly induce CAM, and this trait overlaid on detailed and robust phylogenies. In two of the major facultative CAM-containing genera, *Clusia* (order Malpighiales) and *Calandrinia*, many species remain undescribed and these genera require revision.

In answer to the question 'What is facultative CAM good for?'(Herrera, 2009), it seems clear that, in annuals such as *M. crystallinum* and *Calandrinia polyandra*, the induction of CAM with the onset of the dry season prolongs net carbon gain at low water cost, thereby aiding reproduction. For example, in salt- and drought-stressed *M. crystallinum* that was prevented from taking full advantage of CAM by exposure to CO_2 during the day and not at night, seed production was only 10% of that in plants that were provided with CO_2 throughout the 24 h cycle (Winter and Ziegler, 1992). In these annuals, CAM can contribute substantially to total carbon gain as evidenced by $\delta^{13}C$ values as high as –14‰ (Winter *et al.*, 1978; Cernusak *et al.*, 2013). Conversely, the absence of CAM and the full engagement in C_3 photosynthesis during the first part of the life cycle in the wet season allows high rates of CO_2 fixation and rapid vegetative growth.

In perennial *Clusia*, the connection between the optional use of CAM and seasonal phenology is not well understood. Carbon isotope ratios more negative than about –24‰ suggest that, on an annual basis, the contribution of CAM to carbon gain is moderate. This could still mean that plants seasonally engage in strong CAM if only for a limited but crucial period. *Clusia uvitana*, a weak CAM plant with the ability to reversibly upregulate nocturnal CO_2 uptake in the laboratory (Winter *et al.*, 1992), is the only *Clusia* species that has been studied continuously for over a year in its natural tropical habitat of Panama (Zotz and Winter, 1994b). C_3 photosynthesis was the major contributor to carbon gain at all times, and, surprisingly, CAM was present throughout the year. Nonetheless, consistent with our definition of facultative CAM, the contribution of CAM increased from 27 to 42% during the transition from wet to dry season. Enhanced CAM activity at the onset of the dry season was also observed during a 2-month field study of *Clusia minor* in Trinidad (Borland *et al.*, 1992). These *in situ* studies need to be extended to *Clusia* species, such as *Clusia pratensis*, which, at least under controlled conditions, exhibit greater amplitude of C_3 and CAM usage than *Clusia minor* and *Clusia uvitana*.

In the laboratory, the degree of upregulation of dark CO_2 fixation in facultative CAM varies and maximum rates of CO_2 uptake in the dark are generally lower than in constitutive CAM plants because the inducing conditions are at the same time stress conditions. The extent of nocturnal uptake is species specific and also reflects differences in the severity and speed at which water deficit develops. In *Clusia pratensis* and *Calandrinia polyandra*, CO_2 uptake rates in the dark were approximately 20% of the rates in the light prior to a drought treatment (Figs 2 and 7), whereas maximum dark fixation rates were 5% or less in *T. triangulare*, *P. oleracea*, *and M. crystallinum* (Figs. 8, 9 and 11).

The mechanisms by which stress is perceived and the signal translated into a biochemical response, the induction of CAM, remain poorly understood in all species with facultative CAM. The challenge in defining the water-relation parameters that induce and maintain CAM was apparent in early studies of the halophyte *M. crystallinum*. In the experiment shown in Fig. 11, *M. crystallinum* was cultivated under non-saline conditions, and drought stress led to pronounced leaf wilting, inducing and constraining nocturnal CO_2 fixation. When high soil salinity is used to engender CAM, higher rates of dark CO_2 fixation are achieved than with induction by drought. After transient decreases in leaf water content, the absorption of NaCl allows osmotic adjustment such that leaf turgor in salt-treated plants may eventually exceed the level in well-watered plants under non-saline conditions (Winter and Gademann, 1991). The observation that CAM is nevertheless retained shows that high salinity is recognized by the plant as water-deficit stress. In spite of leaf osmotic adjustment and turgor maintenance in salt-treated plants, root cell turgor decreased under high soil salinity and the roots may thus be involved in stress signalling (Rygol and Zimmermann, 1990), possibly via abscisic acid (Chu *et al.*, 1990).

When CAM is induced in response to environmental stress, it is inevitable that the upregulation of CAM genes will be accompanied by other gene responses to drought or salinity. An alternative C_3-CAM study system that avoids these stress-related complications is the ontogenetic upregulation of CAM in constitutive CAM species (Gehrig *et al.*, 2005; Winter *et al.*, 2011). However, mature tissues with CAM are source tissues, while very young tissues in which CAM is absent are strong carbon sinks, introducing a different type of

complication. In contrast to ontogenetic C_3-to-CAM shifts, the timing of the onset of CAM can be controlled in facultative CAM systems and responses can also be studied during CAM-to-C_3 reversals.

With the impressive biological research power provided by species with genomes that, depending upon environmental conditions, lead to vastly contrasting phenomes of photosynthetic carbon assimilation, facultative CAM plants have been key contributors to investigations that have sketched the pathway of CAM, uncovered CAM enzyme regulation as well as mechanisms of metabolic control, and made substantial inroads into understanding the roles of CAM in natural environments. Research into facultative CAM may aid attempts to introduce CAM into C_3 crop species in order to improve their tolerance to drought.

Facultative systems in combination with new sequencing technologies, modern comparative genomics, and technologies that can locate and quantify metabolites in cellular compartments should provide the resolving power to reveal the signal-transduction cascades that underlie the induction and reversion processes. Moreover, close monitoring of the up- and downregulation patterns of genes in facultative CAM plants will pinpoint CAM-specific isogenes and their control elements that constitute the CAM pathway. The quality of the knowledge provided will markedly improve when such molecular examination is phylogenetically informed and tightly coupled with whole-plant physiological approaches as highlighted in this review. Comparisons of stress responses in closely related species with and without facultative CAM will be particularly promising. There is reason to believe that in the not-too-distant future we will be able to answer the question of where C_3 ends and where CAM begins.

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