Chapter 10

Functional Anatomical Traits of the Photosynthetic Organs of Plants with Crassulacean Acid Metabolism

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Summary

Crassulacean acid metabolism (CAM) is a photosynthetic adaptation to water and/or CO_2 limited environments that has evolved in 400 genera from 36 families of higher plants. Despite the taxonomic and ecological diversity of CAM, plants with this photosynthetic specialization share a number of common anatomical traits that impinge on the physiological processes underpinning photosynthetic CO_2 assimilation and water use. Thick, succulent leaves and/or stems are typical for terrestrial CAM plants. The large cells within these succulent tissues serve to accommodate the overnight vacuolar accumulation of malic acid that defines CAM and also increase water storage capacity. Significant morphological and anatomical diversity exists among leaf and stem succulents that impact on water-use strategies and thus the predisposition towards CAM. We provide an overview of CAM diversity in terms of leaf and stem anatomy, leaf venation and stomatal patterning. We consider the physiological implications of these anatomical traits in terms of water use and leaf hydraulic properties as well as the impacts on CO_2 uptake and carbon gain. We also discuss which anatomical traits are likely to be important determinants for the mode and level of CAM that might be engineered into non-CAM species as a means of improving plant water use efficiency.

I. Introduction

Among the three modes of photosynthesis found in higher plants, the C_3 pathway is ancestral and most common, accounting for approximately 90% of all higher plant species. Crassulacean acid metabolism (CAM) is a specialized mode of photosynthesis, found in ~ 6% of higher plants, that evolved from C_3 photosynthesis in response to water and CO_2 limitation (Yamori et al. 2014). While C_3 photosynthesis uses the three-carbon molecule 3-phosphoglycerate (3-PGA) for the fixation of CO_2 captured by Rubisco during the day, CAM generates a four-carbon organic acid from the fixation of CO_2 at night. In CAM, this nocturnal carboxylation reaction is catalyzed outside the chloroplast by phosphoenolpyruvate carboxylase (PPC), which uses the 3-carbon substrate phosphoenolpyruvate (PEP) supplied by the glycolytic breakdown of carbohydrate formed during the previous day (Fig. 10.1). PPC is activated at night by phosphorylation which is catalysed by a dedicated phoshoenolpyruvate carboxylase kinase (PPCK; Nimmo 2000). The nocturnally accumulated malic acid is stored overnight in a large central vacuole and, during the subsequent day, malate is decarboxylated to release CO_2 at an elevated concentration for Rubisco in the chloroplast. The decarboxylation of malate may be catalysed by NAD-malic enzyme (NAD-ME), NADP-ME or phosphoenolpyruvate carboxykinase (PEPCK) depending on the plant species (Holtum et al. 2005). Transgenic silencing of mitochondrial NAD-ME has indicated this as the major decarboxylase in the model CAM species *Kalanchoë fedstchenkoi* (Dever et al. 2015; Hartwell et al. 2016) and pyruvate produced from decarboxylation is subsequently processed to PEP either in the cytosol or the chloroplast (Kondo et al. 2001; reactions summarized in Fig. 10.1).

The diel separation of carboxylases in CAM is accompanied by an inverse (compared to C_3 and C_4) day/night pattern of stomatal closure/opening. The main periods of stomatal opening (night, Phase I) and stomatal closure (middle part of day, Phase III) may be punctuated by periods of variable duration where stomata open for direct uptake of CO_2 at the start (Phase II) and end (Phase IV) of the day, giving rise to four

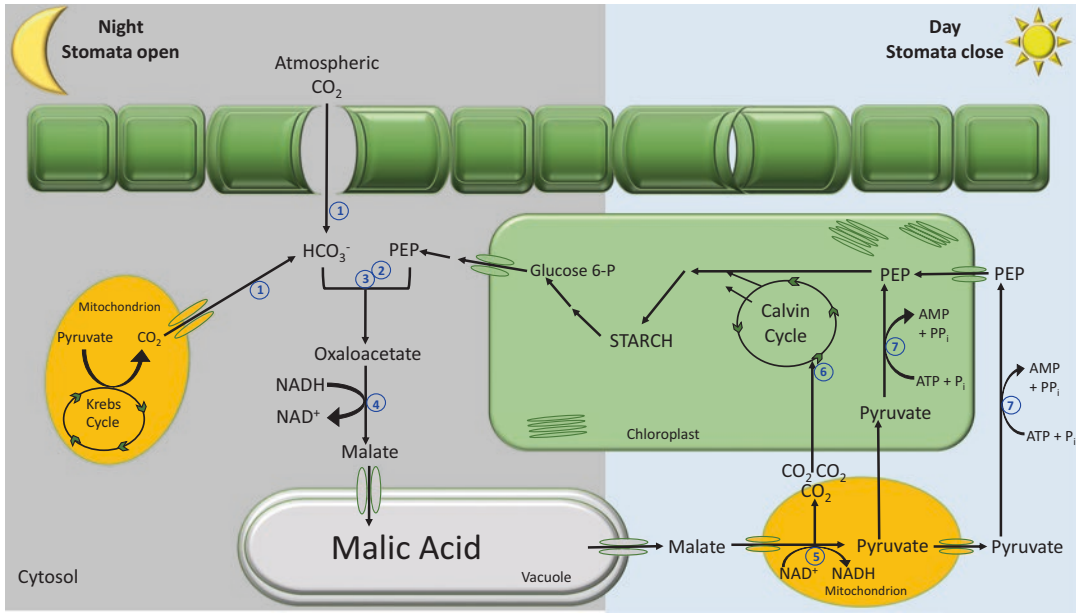

Fig. 10.1. Summary outline of the nocturnal and daytime metabolic reactions of crassulacean acid metabolism (CAM). At night, atmospheric CO_2 enters through open stomata and is converted to HCO_3^- via the enzyme carbonic anhydrase (CA). Respiratory CO_2 produced by the mitochondria can also be converted to HCO_3. Phosphoenolpyruvate (PEP) is produced via the glycolytic breakdown of storage carbohydrate (e.g. starch). PEP and HCO_3 are substrates for phosphoenolpyruvate carboxylase (PPC) that is activated (phosphorylated) by a dedicated kinase (PPCK). The product oxaloacetate is converted to malate via nicotinamide dinucleotide (NAD) malate dehydrogenase (MDH). The malate is pumped into the central vacuole and stored overnight as malic acid. During the day while stomata are closed, malate exits the vacuole and, in the case of NAD malic enzyme (NAD-ME) type CAM plants such as *Kalanchoë fedstchenkoi*, enters the mitochondrion where it is decarboxylated to produce pyruvate. The CO_2 released is re-fixed by Rubisco and processed via the Calvin cycle. Pyruvate is converted to PEP via the enzyme pyruvate phosphate dikinase (PPDK), a reaction which can occur in the cytosol and/or chloroplast. Carbohydrate (starch) is recovered by the gluconeogenic processing of PEP. Enzyme-catalyzed steps are represented by blue numbering where 1 = CA, 2 = PPC, 3 = PPCK, 4 = MDH, 5 = NAD-ME, 6 = Rubisco, and 7 = PPDK

'typical' phases of leaf gas exchange in the CAM mode (Osmond 1978; Fig. 10.2). Closing stomata for much of the day and opening stomata predominantly at night when temperature is generally lower and humidity higher than during the day, enhances water-use efficiency (WUE: i.e., the ratio of moles of CO_2 fixed to moles of water lost by transpiration). The WUE of CAM plants can be 6-fold higher than that of C_3 plants and 3-fold higher than C_4 plants under comparable conditions (Borland et al. 2009).

The water conserving properties of CAM have highlighted the potential of succulent CAM genera like *Agave* and *Opuntia* as dedicated bioenergy feedstocks that can be grown on semi-arid land with minimal inputs (Owen and Griffiths 2014; Cushman et al. 2015). The CAM pathway has also been identified as a target for synthetic biology to engineer improved water use efficiency into C_3 crops that are grown for food, feed and as sources of bioenergy (Borland and Yang 2013; Borland et al. 2014; DePaoli et al. 2014). In principle, CAM-into-C_3 engineering is realistic because all of the enzymes required for CAM appear to be homologs of ancestral forms found in C_3 species (West-Eberhard et al. 2011). The existence of facultative CAM species, which can change photosynthetic physiology from C_3 to CAM in response to drought or salinity

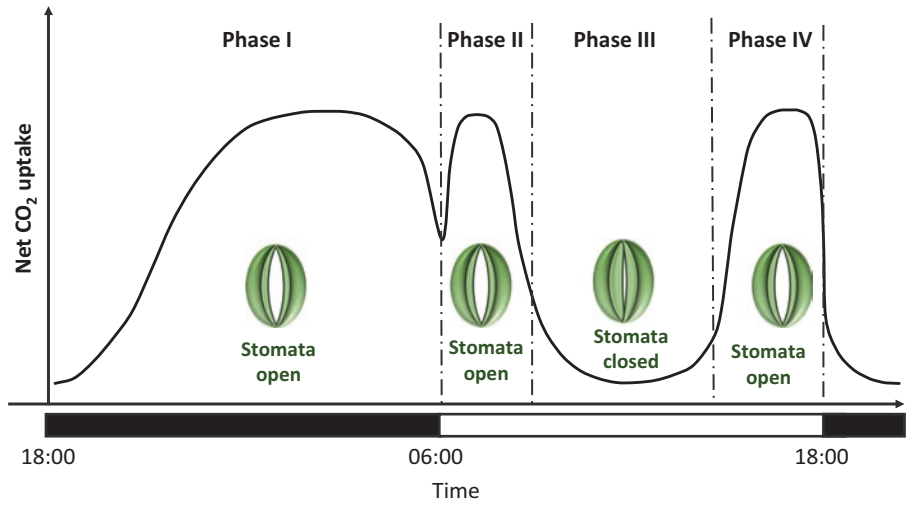

Fig. 10.2. The 4 phases of crassulacean acid metabolism (CAM) that occur over a 24 h dark-light cycle. At night (Phase I), stomata open in response to the draw-down in internal [CO_2] as HCO_3^- is fixed by PPC. The transition of PPC from the dephosphorylated form (with low activity) at the start of the night to the phosphorylated (active) form as the night progresses, allows continued CO_2 uptake from the atmosphere until the vacuoles begin to reach their limit for holding malic acid leading to decreased CO_2 uptake as dawn approaches. At the start of the photoperiod (Phase II), stomata remain open and a surge of net CO_2 uptake occurs as Rubisco becomes the principle carboxylase. During the middle part of the photoperiod (Phase III), large amounts of CO_2 are released inside the tissues as malic acid leaves the vacuoles and is decarboxylated. This elevation of internal [CO_2] is believed to be a key stimulus for stomatal closure and throughout Phase III internally generated CO_2 is fixed in the chloroplasts behind closed stomata. Towards the end of the photoperiod (Phase IV), the internal store of malic acid is exhausted and the internal concentration of CO_2 falls allowing stomata to open and CO_2 to be fixed directly from the atmosphere by Rubisco (Based on Osmond 1978).

(Winter and Holtum 2014), also implies that there are no metabolic incompatibilities between the operation of CAM and C_3 photosynthesis at the organismal level. Moreover, CAM is a single-cell carbon concentrating mechanism, meaning that, in principle, plants will concentrate and assimilate carbon in the same cell. Therefore, engineering the CAM pathway into C_3 species does not require differentiated mesophyll and bundle sheath cell types, each with their own specialized metabolic adaptations, as is the case with C_4 photosynthesis (Reeves et al. 2017; see Chap. 9). However, the photosynthetic organs of CAM species do possess particular anatomical characteristics that impinge on the physiological processes underpinning photosynthetic CO_2 assimilation and water use. Within the context of the convergence of the CAM phenotype across diverse taxonomic groups, the aim of this chapter is to present an overview of the anatomical traits typically associated with the photosynthetic organs of CAM plants, to discuss the physiological consequences of these anatomical traits and to consider the implications for bioengineering CAM into non-CAM species.

II. Convergence of CAM Across Diverse Phylogenies

Convergent evolution (when unrelated lineages independently evolve similar morphological and/or functional features) is thought to underpin the repeated emergence of CAM from C_3 ancestors in response to similar ecological selection pressures (Edwards and Ogburn 2012). CAM has evolved in more than 400 distinct genera from over 36 families (Yang et al. 2015) and is found in terrestrial, epiphytic, and aquatic species. Terrestrial

CAM plants live in semi-arid, seasonally arid or other water limited environments (Herrera 2009). They have independently evolved similar physiological characteristics to adapt to water-deficient conditions thus enhancing WUE relative to C_3 plants (Borland et al. 2014; Yang et al. 2015). For example, *Kalanchoë laxiflora*, *Lithops hookeri*, *Phalaenopsis equestris* (orchid) and *Ananas comosus* (pineapple) are terrestrial obligate CAM plant species belonging to four different orders (Saxifragales, Caryophyllales, Asparagales, and Poales) (Fig. 10.3a). Specifically, all of these species open their stomata at night for net CO_2 uptake and keep stomata closed for much of the day. The daytime closure of stomata is possible due to the decarboxylation of vacuolar stores of malic acid that generates CO_2 for assimilation via Rubisco and the Calvin cycle while transpirational water loss is curtailed. In addition to the terrestrial plants, CAM photosynthesis is also present in some aquatic plants such as species in the genera of *Isoëtes*, *Crassula*, *Littorella* and *Sagittaria* (Griffiths 1992; Keeley 1998). Both *Littorella* and *Sagittaria* have evolved CAM traits despite these genera being very distantly related (i.e., part of the dicot Lamiales and monocot Alismatales orders, respectively; Fig. 10.3a). In contrast to the terrestrial CAM plants, the convergent evolution of CAM in aquatic plants is driven by an adaptation to alleviate reduced availability of CO_2. The slow diffusion of CO_2 in water as well as competition for CO_2 from other aquatic photosynthetic species employing C_3 photosynthesis during the day means that CO_2 availability can be limiting in aquatic environments (Keeley 1998; Klavsen et al. 2011). Thus, the primary selective advantage of CAM in aquatic plants is to provide an internal source of CO_2 during the day when external levels of CO_2 are potentially limiting for photosynthesis (Keeley 1998).

Phenotypic and functional convergence is underpinned by changes in genomic sequences (Pfenning et al. 2014). Although CAM convergence is widely recognized at the physiological level, our knowledge about the molecular basis underlying the convergent evolution of CAM photosynthesis is limited. Recently, the evolution of the key carboxylation enzyme PPC was studied by Deng et al. (2016) using phylogenetic analysis of the PPC gene family in 60 species. A CAM-specific isoform of PPC was found to be shared among five different CAM species in the monocot lineage (Fig. 10.3b). This supports the hypothesis that convergence in PPC gene evolution contributes to emergence of the CAM syndrome. To test this hypothesis, future studies on convergent evolution of PPC and other CAM genes should include both monocot and dicot lineages. A more comprehensive genome-wide comparative analysis of the newly published orchid (Cai et al. 2015) and pineapple genome sequences (Ming et al. 2015) as well as the genomics data generated from the ongoing *Kalanchoë* genome project (Yang et al. 2015, 2017) will provide new insights into the genomic basis of CAM convergence.

III. Succulence and Diversity in Anatomy and Morphology of CAM Species

A. The Succulence Syndrome

Despite the taxonomic and ecological diversity that underpins CAM, plants with this photosynthetic specialization share a number of common anatomical traits. Thick, succulent leaves and/or stems are typical for terrestrial CAM plants (Sayed 1998). At the cellular level, succulence is manifest as multiple numbers of large cells with greatly enlarged vacuoles that occupy 90% or more of the cell volume. The enhanced vacuolar storage capacity of succulent leaves/stems is a key factor for accommodating the nocturnal storage of malic acid that defines CAM (Martin and Siedow 1981). Positive relationships have been demonstrated between succulence and the magnitude of CAM expression and

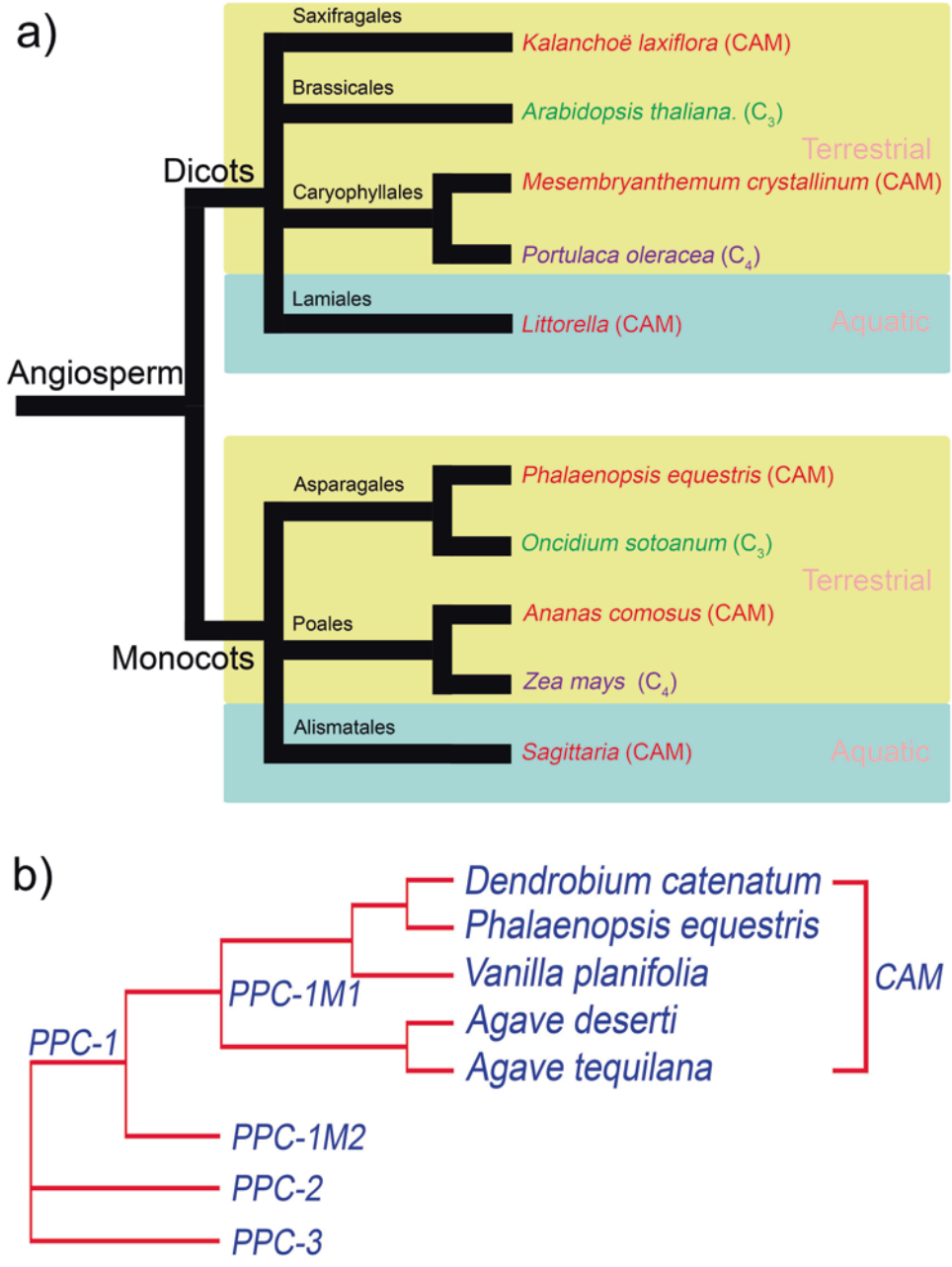

Fig. 10.3. CAM convergence at physiological and molecular levels. (**a)** Physiological convergence of CAM species from both dicot and monocot lineages. Species names of C_3, C_4, and CAM plants are shown in green, purple and red fonts, respectively. The yellow box indicates terrestrial plants whilst the blue box indicates aquatic plants. (**b)** Molecular evolution of the PPC gene family can be divided into three subfamilies: PPC-1, PPC-2 and PPC-3. Within the subfamily PPC-1, the clade PPC-1 M1 is shared by five CAM species in the CAM lineage, including three orchid species (*Dendrobium catenatum*, *Phalaenopsis equestris* and *Vanilla planiolia*) and two *Agave* species (*Agave deserti* and *A. tequilana*). (Adapted from Deng et al. 2016) (Colour figure online)

between leaf thickness and CAM-like ^{13}C discrimination in diverse phylogenetic lineages that include the Crassulaceae and tropical tress of the genus *Clusia* (Teeri et al. 1981; Winter et al. 1983; Kluge et al. 1991; Borland et al. 1998; Holtum et al. 2004; Vargas-Soto et al. 2009). Across nine species of *Clusia* with photosynthetic characteristics ranging from obligate C_3 through C_3-CAM intermediate to constitutive CAM, the species with more succulent and thicker, denser leaves (lower specific leaf area) engaged in a greater amount of dark CO_2 uptake than the thinner-leaved species (Barrera-Zambrano et al. 2014).

In addition to enhancing the nocturnal storage capacity for malic acid, succulence increases water storage and is often linked with general adaptation to water-limited habitats (Ogburn and Edwards 2010). In most leaf or stem succulents, water is stored either in or immediately adjacent to photosynthetic tissues, indicating an intimate relationship between succulence and daily carbon uptake and growth. It has been suggested that possession of leaf or stem succulence might have predisposed ancestral CAM taxa towards the evolution of this photosynthetic specialization in water-limited habitats (Sage 2002). However, great morphological and phylogenetic diversity exists among leaf and stem succulents (Eggli and Nyffeler 2009) and it is worth considering how anatomical variants of the succulence trait in photosynthetic organs might impact water-use strategies and thus the predisposition towards CAM.

B. Succulent Traits Have Been Incorporated into a Variety of Different Leaf Anatomies Across CAM Lineages

One type of leaf succulence can be classified as 'all-cell succulence' where all cells contain chloroplasts and store water simultaneously. Examples of CAM families with this type of succulence include the Crassulaceae and Aizoaceae and within the genus *Kalanchoë,* in which the largely undifferentiated mesophyll is made up of tightly compacted cells (Fig. 10.4a; Balsamo and Uribe 1988; Nelson et al. 2005). In contrast, storage succulence is a situation in which specialised, achlorophyllous water storage cells are adjacent to, but clearly differentiated from, the photosynthetic cells (e.g., as found in the CAM genera *Aloe* and *Lithops;* Eggli and Nyffeler 2009). In *Aloe*, large non-photosynthetic cells (or hydrenchyma) with a water storage function are found in the central core of the leaf, and are surrounded by a layer of tightly packed photosynthetic mesophyll cells (chlorenchyma) (Fig. 10.4c; Ni and Tizard 2004). In other CAM genera, there is more heterogeneity across the leaf, with several distinct layers of chloroplast-containing mesophyll, as that found in the genera *Peperomia* and *Clusia* (Fig. 10.4b; Nelson et al. 2005; Barrera-Zambrano et al. 2014). In the leaves of tropical trees of the genus *Clusia*, the photosynthetic tissues are differentiated into a layer of palisade mesophyll comprised of tightly compacted and elongated cells and a layer of spongy mesophyll that is generally less tightly packed (Barrera-Zambrano et al. 2014; Fig. 10.4b). Water storage tissue or hydrenchyma is found just below the epidermis in *Clusia* as is also the case for *Peperomia* (Kaul 1977). The anatomical complexity of *Clusia* leaves is compounded by the arrangement of chlorenchyma cells surrounding the mid-vein, which are often tightly compacted, rather like the mesophyll cells of *Kalanchoë* (Lüttge 2008a; Fig. 10.4b).

C. CAM Is Found in Specific Cell Types Within the Leaf

In many succulent CAM species, the leaf mesophyll tissues are usually not strongly differentiated into palisade and spongy layers (Nelson et al. 2005; Nelson and Sage 2008). However, those CAM species that do possess differentiated mesophyll cells present the potential for variations both in cell size and for accommodating differential distribution of PPC and Rubisco proteins within the leaf. A comparative study between C_3 and CAM

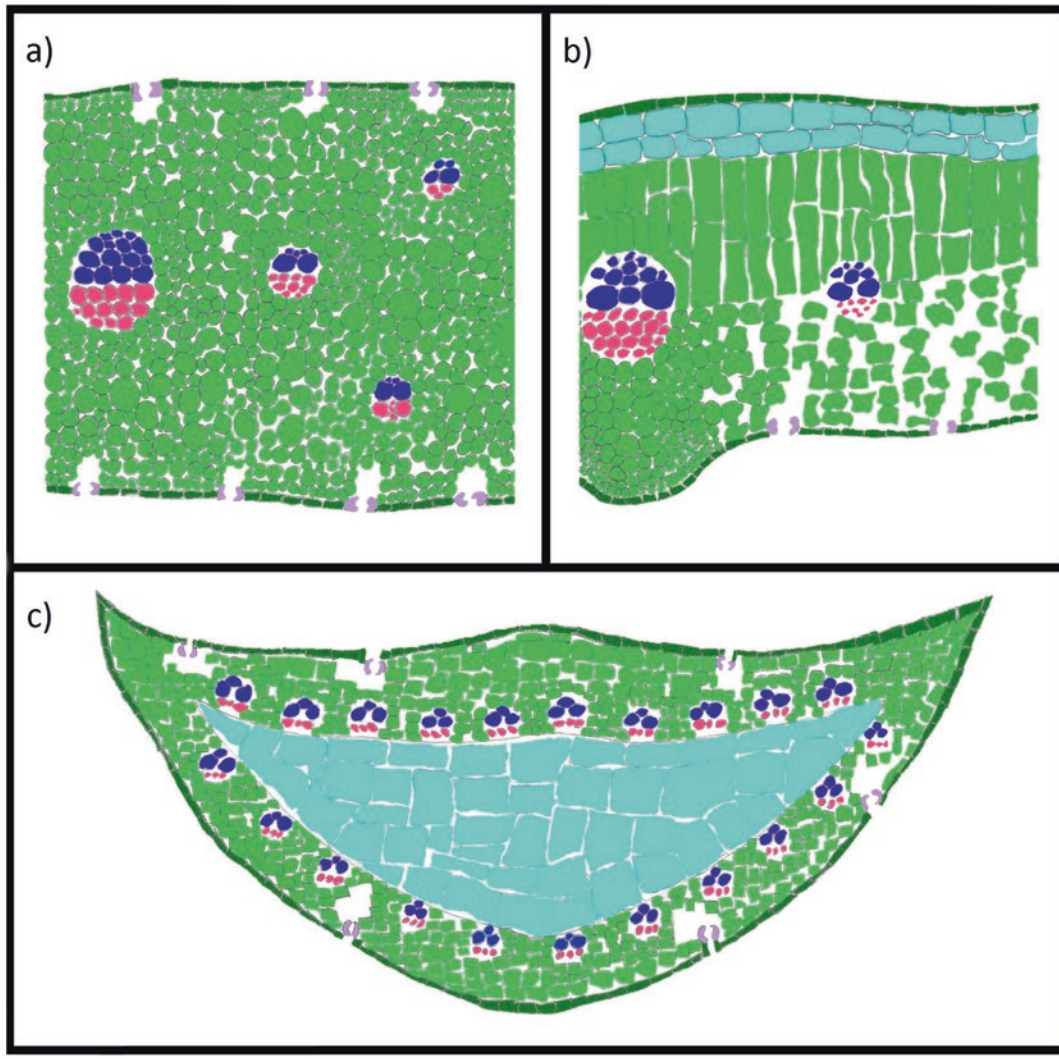

Fig. 10.4. Morphological diversity of succulent leaf forms in CAM plants. (**a**) In *Kalanchoë*, tightly packed and largely undifferentiated cells make up the mesophyll. A 3-dimensional arrangement of veins is found both above and below the central plane running through the middle of the leaf. These 3D veins are suspected to be necessary for the transport of water and photosynthate into highly succulent tissue. Stomata are located within the epidermis on both surfaces of the leaf. (**b**) In *Clusia*, a differentiated leaf anatomy is comprised of a palisade mesophyll layer, a spongy mesophyll layer and a layer of hydrenchyma below the upper epidermis. It has been suggested that CAM activity is more prominent in the tightly packed cells of the palisade mesophyll and the chlorenchyma surrounding the veins than in the spongy mesophyll in *Clusia*. Stomata are located on the abaxial leaf surface. (**c**) In *Aloe*, the mesophyll is made up of tightly compacted chlorenchyma cells surrounding a central core of hydrenchyma cells that function in water storage. The veins of *Aloe* are found in a 3D arrangement and stomata are distributed throughout the epidermis

Clusia species demonstrated not only that leaves were thicker in CAM performing species, but also that cells of the palisade mesophyll were significantly larger and constituted a greater % of leaf thickness in species that undertake a greater amount of nocturnal net CO_2 uptake (Barrera-Zambrano et al. 2014; Fig. 10.5). In addition, the abundance of PPC protein in CAM species of *Clusia* was found to be three times higher in cells of the palisade

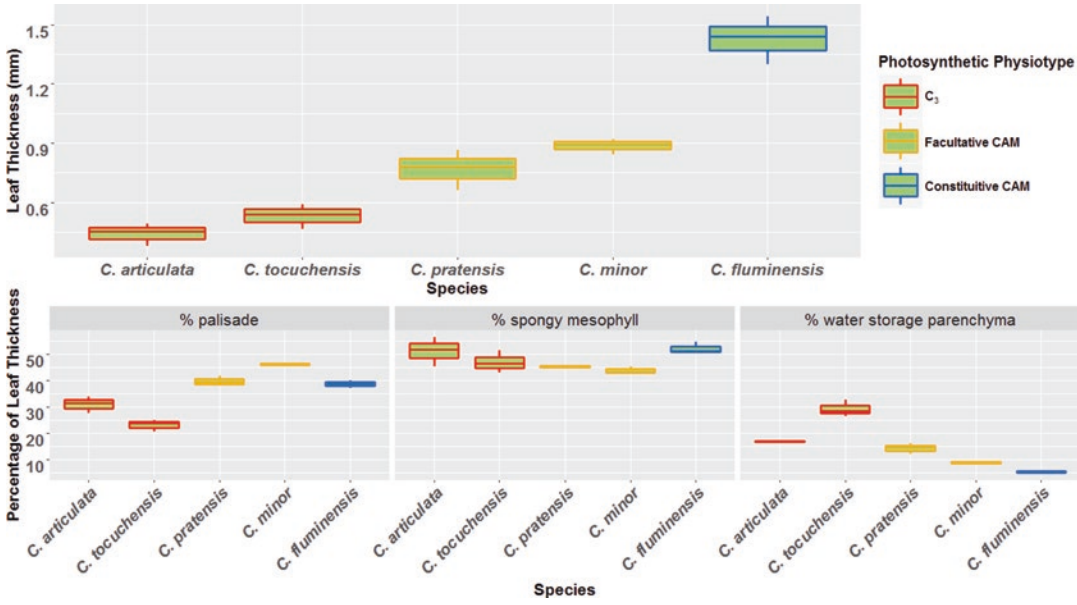

Fig. 10.5. Leaf anatomical traits are related to the capacity for CAM in *Clusia.* (**a**) Species that undertake a greater amount of night time CO_2 uptake have thicker leaves. The putative C_3 and obligate C_3 species *C. articulata* and *C. tocuchensis* have the thinnest leaves. Facultative CAM species, *C. pratensis* and *C. minor* have an intermediate leaf thickness. The obligate CAM species, *C. fluminensis* has the thickest leaves. Three separate groups for obligate C_3, facultative CAM and constitutive CAM are supported by ANOVA and post hock Tukey-Kramer test. (**b**) The proportion of the leaf made up by the palisade mesophyll layer is higher for species that undertake more night time CO_2 uptake. The proportion of the leaf made up by the spongy mesophyll (**c**) and hydrenchyma (**d**) (water storage parenchyma) does not show a direct relationship with the capacity for nocturnal CO_2 uptake. For all species, measurements were made on a single mature leaf from each of 3 different plants, with 3 technical measurements made for each leaf. Data are presented using box and whisker plots where the upper and lower edge of box represents the 75% and 25% quantile respectively, and the middle line is the median value. Upper and lower whiskers extend to the largest or smallest value, respectively

mesophyll compared to the spongy mesophyll (Barrera-Zambrano et al. 2014). Furthermore, a study of the facultative CAM species *Clusia minor* suggested that the tightly packed chlorenchyma cells that surround the leaf mid vein can perform CAM when the rest of the leaf is C_3 (Lüttge 2008b). An uneven distribution of CAM activity across the differentiated leaves of *Clusia* may explain how this photosynthetic specialization is able to evolve in different morphological backgrounds and could have important implications for bioengineering CAM into non-CAM crops (see Sect. 7).

The compartmentalization of CAM into specific cell types could, in principle, facilitate the evolution of CAM and C_4 photosynthesis in the same leaf (Sage 2002). However, it has been observed that very few species possess the capacity for both C_4 and CAM, despite evidence showing that both modes of photosynthesis probably evolved from similar clades in the phylogeny of angiosperms (Edwards and Ogburn 2012). This is likely to be because of metabolic and anatomical incompatibilities between CAM and C_4 photosynthesis (Sage 2002). Anatomical incompatibilities are due to the fact that CAM requires large succulent cells, specialised for intracellular storage functions, whereas C_4 leaves require cells that can efficiently undertake intercellular transport of metabolites (Sage 2002). *Portulaca* is the only genus known to show both CAM and C_4 characteristics and this has probably been possible due to the evolution of CAM in specific cell

types (Guralnick et al. 2008). In *P. grandiflora* leaves, for example, CAM activity is localised to the central water storage tissue, whereas C_4 activity occurs in the mesophyll and bundle sheath cells surrounding the vasculature (Winter and Holtum 2014). The evolution of CAM, which is believed to have originated before C_4 in *Portulaca*, occurred in cells most predisposed to succulent characteristics (i.e., large cells with big vacuoles; Christin et al. 2014). This allowed the subsequent evolution of C_4 Kranz anatomy in the mesophyll, adding to the diversity of leaf forms in which CAM is found.

D. CAM in Photosynthetic Stems

Many species have evolved CAM in organs besides the leaf, such as stems (Hastilestari et al. 2013; Kocurek et al. 2015; Winter and Holtum 2015). Across the plant kingdom there is a spectrum of CAM photosynthetic physiologies in stems. Some species, such as *Kalanchoë pinnata* and *Clusia rosea* which show strong CAM in the leaves, are able to carry out weak CAM in their stems, while *Jatropha curcas* can show weak CAM in both stems and leaves (Kocurek et al. 2015; Winter and Holtum 2015). The overnight accumulation of low levels of malate in these stems is probably an adaptation to recover endogenous CO_2 that would otherwise be lost via nocturnal respiratory processes (Kocurek et al. 2015).

In other species, the stem dominates carbon gain, having taken over from the leaf as the major organ for photosynthesis. CAM has emerged polyphyletically in several taxa of stem succulents that include the Cactaceae, Asclepiadaceae, Apocynaceae, Asteraceae, Didieraceae, Euphorbiaceae and Vitaceae phylogeny (Eggli and Nyfeller 2009; Hastilestari et al. 2013; Kocurek et al. 2015). In the majority of stem succulents with CAM, an external photosynthetically active chlorenchyma surrounds an internal water storing hydrenchyma of mainly non-green cells built up from cortex and pith of the stems (Eggli and Nyffeler 2009). An iconic example of CAM in stems evolving to have an analogous role to CAM in leaves is found within the genus *Opuntia*, which belongs to the Cactaceae (Pimienta-Barrios et al. 2012; Mason et al. 2015). During the evolution of *Opuntia*, leaves have reduced in size or disappeared altogether. In their place the stems have evolved into thick, flat, succulent organs called cladodes that use strong CAM to take up CO_2 almost exclusively at night. A retardation in the developmental rate of woody tissues (allometric neoteny) has been proposed as the main mechanism for the development of stem succulence in cacti (Altesor et al. 1994).

Some stem succulents in the Opuntioideae, Euphorbiaceae and Didieraceae that perform CAM will also undertake seasonal production of less succulent leaves. The production of these leaves allows a photosynthetic division of labour between stems and leaves on the same plant that serves to optimize carbon gain and water use in response to changing environmental conditions (Lüttge 2008b). In *Euphorbia tirucalli*, the C_3 performing leaves are shed in response to drought and the stem increases the amount of malate accumulated overnight (Hastilestari et al. 2013). In most leaf-producing stem succulents, CAM activity is generally highest in the stems (Lüttge 2008b), which most likely reflects the higher vacuolar capacity for nocturnal storage of malic acid in the more succulent stems compared to leaves. As found in leaf succulent CAM species, a full range of CAM photosynthetic physiologies may be found in stem succulents, ranging from weak scavenging of respiratory CO_2 to substantial night-time net CO_2 uptake.

E. CAM in Other Photosynthetic Organs

Perhaps the most extreme example of CAM evolving outside of the leaf is in the epiphytic orchids (Kerbauy et al. 2012). The origin of an epiphytic lifestyle is often associated with the evolution of CAM that serves to conserve

water in the variable and potentially drought-prone epiphytic habitat (Silvera et al. 2009). Within the epiphytic orchids, there are examples of CAM in every different photosynthetic organ. CAM is found in leaves, pseudobulbs (enlarged succulent internodal regions of the stem), photosynthetic roots and even flowers (Martin et al. 2010; Kerbauy et al. 2012). Furthermore, during their evolutionary history, some species have lost their leaves altogether. In these species, such as *Bulbophyllum minutissimum* and *Campylocentrum tyrridion*, the role of leaf photosynthesis as the major source of fixed carbon has been replaced by CAM pseudobulbs (Winter et al. 1983) or CAM photosynthetic roots (Winter et al. 1985), respectively. While CAM is usually considered an adaptation in the leaf, it can occur in any photosynthetically active succulent tissue, either to recover CO_2 lost from respiration or to increase water retention across the entire plant body.

In summary, a great deal of morphological and anatomical variation in photosynthetic organs exists across CAM species. The fact that CAM is found in such diverse forms has implications for the evolution of this carbon concentrating mechanism as it means that succulent traits that facilitate CAM can originate in a number of different anatomical 'starting points'.

IV. Physiological Consequences of Succulence

A. Water Use

Succulence and CAM are commonly considered as traits that are characteristic of plants found in desert habitats, but in reality both traits are largely lacking in plants of extremely xeric environments (Schmida 1985). Leaf and stem CAM succulents are more commonly found in semi-deserts, semi-arid scrub or rainforests, the latter exemplified by over 10,000 species of epiphytic orchids and bromeliads that can be subject to variable and potentially limiting water supply (Zotz and Hietz 2001). In ecophysiological terms, succulence represents a mechanism to avoid drought, rather than being physiologically tolerant of extreme water deficits, and succulent CAM species rarely develop water potentials less than −1 MPa while nearby C_3 shrubs may approach −4 MPa or lower (Ogburn and Edwards 2010). Moreover, in many species of cacti and agave, the roots rapidly dehydrate and shrink to lose contact in soil water potentials between −0.03 and −0.3 MPa (Nobel 1988; North et al. 2004).

The general mechanism by which succulents avoid drought at the cellular level can be described by the physiological trait of hydraulic capacitance (C), the change in volume of a cell or tissue per unit change in water potential (Nobel 1999). Capacitance is closely related to cell wall elasticity. Succulent cells or tissues, such as the specialized hydrenchyma cells, tend to have high values of C (Ogburn and Edwards 2010). Thus, hydrenchyma cells can take up or lose large volumes of water for a given change in C relative to cells or tissues with lower values of C. This ability to maintain turgor during tissue desiccation is one factor explaining the tendency of succulents to have relatively high tissue water potentials, even when droughted (Ogburn and Edwards 2010).

The modulus of elasticity (e), which provides an estimate of cell wall rigidity, is closely related to the inverse of capacitance (Nobel 1999) and has important implications for the movement of water between neighbouring tissues. In the case of storage succulence, where tissues within water-storing leaves and stems are divided into large celled, achlorophyllous hydrenchyma and smaller-celled chlorenchyma, there can be a tendency for hydrenchyma cells to lose water and buckle during desiccation and for chlorenchyma cells to stay hydrated at their expense (Schmidt and Kaiser 1987). Direct measurements on some tissue succulents have shown

that cell wall thickness of chlorenchyma cells can be twice as high as that of the hydrenchyma cells (Goldstein et al. 1991). Thus, if hydrenchyma tissues have cell walls with lower values of e (i.e., less rigid cell walls), they will better maintain turgor, and hence higher water potentials, when compared with the more rigid chlorenchyma cells for a given amount of drying across all tissues (Goldstein et al. 1991). This differential decrease in water potentials provides a driving force for water flow from hydrenchyma to chlorenchyma that requires no expenditure of energy. Thus, if water availability decreases, the water potential of photosynthetically active tissue is buffered. Such preferential hydration of chlorenchyma at the expense of hydrenchyma has been documented for a range of CAM succulents that include *Carnegiea gigantea, Opuntia basilaris* and *Peperomia magnoliaefolia* (Barcikowski and Nobel 1984; Schmidt and Kaiser 1987). Modelling of hydrenchyma water storage as an electrical analog of capacitance is consistent with reports that these succulent cells deliver between 34% (*Ferocactus*) and 37% (*Agave*) of daily transpiration demand (Smith et al. 1987; Schulte et al. 1989). The role of the hydrenchyma as an internal water reservoir is particularly important during CAM-idling, a situation in which stomata remain closed during both day and night and the plant internally recycles respiratory CO_2 via PPC under severe drought stress. CAM-idling has been well documented for cacti of the American semideserts (Szarek et al. 1973; Holthe and Szarek 1985). During CAM-idling, any water lost by cuticular transpiration can be replaced in the chlorenchyma by reserves in the hydrenchyma, and thus plants can survive for up to several months via CAM idling.

The vacuolar storage capacity for malate, a feature that is positively correlated with succulence and CAM activity, has been linked with plant water uptake from the soil in several species. Nocturnally accumulated malate functions as a solute in vacuoles of the chlorenchyma, increasing osmotic pressure and providing a stronger driving gradient for soil water uptake. This effect has been demonstrated in the leaf succulents *Kalanchoë daigremontana* (Smith and Lüttge 1985), *Clusia minor* (Herrera et al. 2008), and *Senecio medley-woodii* (Ruess and Eller 1985). In contrast, diel malate fluctuations were found to be relatively unimportant in driving soil water uptake in *Agave deserti* (Smith et al. 1987; Tissue et al. 1991). Since malate is consumed during the day in CAM plants and osmotic potential becomes higher again, the water so gained becomes thermodynamically more available to the tissues (Lüttge 2004).

B. Division of Labor Between Hydrenchyma and Chlorenchyma: Implications for CAM and Water Use

In most stem succulents that have been examined, the non-photosynthetic central hydrenchyma does not appear to participate in the diel oscillations of organic acid levels that define CAM (Lüttge et al. 1989). In the peripheral stem chlorenchyma, nocturnal malate accumulation can increase osmotic pressure within these cells so that they take up water from the hydrenchyma and thus turgor pressure of the chlororenchyma increases (Lüttge and Nobel 1984). Dynamic diel cycles of radial internal water distribution in the stem succulent cacti are such that water moves more readily towards the water storage tissue at dusk and towards the chlorenchyma at dawn. These assertions are supported by detailed quantitative assessments of water relation parameters such as cell osmotic pressure and turgor pressure and through use of hydrogen isotopes ^3H and ^2H (tritium and deuterium, respectively) to assess mixing of water between the two tissues (Goldstein et al. 1991; Tissue et al. 1991). Such diel changes in water relation parameters appear to determine diel timing of growth cycles such that in cladodes of

Opuntia growth is maximal at midday when turgor is still high while malate mobilisation also provides a source for production of carbohydrates to fuel the carbon and energetic demands of growth (Gouws et al. 2005). This contrasts with the situation for many C_3 species where growth of the photosynthetic organs generally occurs at night (Gouws et al. 2005). In CAM plants, this uncoupling of leaf expansion growth from nocturnal carbohydrate degradation has been proposed as a means of reconciling potential conflicts of demand between accumulation of carbohydrate reserves required for PPC-mediated CO_2 uptake at night and partitioning of resources for growth during the day (Borland et al. 2009, 2016).

In some leaf succulents in which the hydrenchyma is formed by layers of cells below the epidermis, there is a negative relationship between thickness of the hydrenchyma layer and CAM expression, a situation reported within the genus *Peperomia* (Sipes and Ting 1985). Leaves of the C_3-CAM intermediate *P. obtusifolia* possessed a thicker hydrenchyma (i.e., 63% of total mesophyll cross-section) compared with *P. macrostachya* a constitutive CAM species where hydrenchyma thickness represented 19% of total mesophyll (Fondom et al. 2009). The same study also indicated that the drought-induced switch to CAM in *P. obtusifolia* was accompanied by a shrinkage of the hydrenchyma and palisade mesophyll but this species still presented a higher water use efficiency compared with the constitutive CAM *P. macrostachya* under the same conditions of drought (Fondom et al. 2009). It was thus suggested that, even when *P. obtusifolia* performed CAM, the hydrenchyma could act to conserve water.

In a comparative study of leaf anatomy across nine species of tropical trees of the genus *Clusia*, the presence of a thick layer of hydrenchyma was particularly evident in two C_3 species, with the thickness of this layer determined principally by cell size and number of cell layers (Barrera-Zambrano et al. 2014). It is tempting to speculate that by acting as a means of buffering against water shortage, the hydrenchyma layer that is present in these obligate C_3 *Clusias* (section Anadrogyne of the Clusiaceae) might obviate the need for CAM. However, the presence of hydrenchyma in a strong CAM species of *Clusia* (*C. alata*) indicates that the presence of hydrenchyma and CAM are not mutually exclusive within the *Clusia* genus. It is possible that the thickness of hydrenchyma is determined more by phylogeny than photosynthetic mode since *C. grandiflora*, a C_3 species within Section Chlamydoclusia of the Clusia phylogeny had a reduced hydrenchyma compared to the other C_3 species examined in this study but which was of comparable depth to the hydrenchyma in the CAM species *C. rosea*, also located within this section (Barrera-Zambrano et al. 2014). A more comprehensive survey of leaf anatomy encompassing species within all sections of the *Clusia* genus would be informative in terms of the evolutionary origins of tissue succulence and CAM within this photosynthetically diverse genus.

C. CO_2 Uptake and Carbon Gain

Leaf and stem succulence is generally accompanied by an increase in mesophyll cell size that leads to low internal air space (IAS) as a result of the tightly packed cells (Smith and Heuer 1981). A reduced IAS and the concomitant reduction in the length of mesophyll cells that are exposed to the IAS (L_{mes}/area) will increase resistance to CO_2 efflux from the leaf (Nelson et al. 2005). It has been proposed that, alongside the daytime reduction in stomatal conductance, a reduced IAS improves the carbon economy of CAM during the day-time decarboxylation of malate (Phase III of CAM, Fig. 10.2) because net efflux of CO_2 from the leaf will be curtailed (Maxwell et al. 1997). Moreover, a low internal conductance to CO_2 will reduce the diffusion out of mesophyll cells and this could enhance the recapture of respiratory CO_2 at night (Phase I of CAM)

via PPC (Griffiths 1992). Recapture of respiratory CO_2 at night is thought to have been an early step in the evolutionary process by which CAM evolved from C_3 photosynthesis. Hence, it has been suggested that low IAS may be a trait that was selected for to enhance the efficiency of CAM rather than simply being the unavoidable consequence of the large, tightly packed cells that characterize succulent leaves or stems (Maxwell et al. 1997). This assertion is supported by a study of 18 CAM plants belonging to 13 families and six C_3 and four C_4 plants that found a close association between the degree of succulence, IAS and L_{mes}/areas and with all 3 traits being substantially lower in the CAM species (Nelson et al. 2005). Thus, tight cell packing appears to be a trait that is common in all CAM lineages and that reflects evolutionary convergence of leaf anatomy within the CAM functional type.

While a reduced IAS and L_{mes}/area would seem to improve the carbon economy of CAM by minimizing net CO_2 efflux from the leaf, a reduction in leaf internal conductance to CO_2 will curtail direct uptake of atmospheric CO_2, particularly during the latter part of the photoperiod when stomata may re-open and direct Rubisco-mediated CO_2 uptake occurs, i.e., Phase IV in Fig. 10.2 (Griffiths 1992; Nelson and Sage 2008). In 'weak CAM' plants, which rely heavily on Phase IV uptake of CO_2, (as opposed to 'strong CAM' in which Phase I nocturnal uptake of CO_2 dominates diel carbon gain), a low IAS could limit photosynthetic efficiency since diffusion through mesophyll limits carbon availability for Rubisco (Evans and von Caemmerer 1996; Maxwell et al. 1997; Nelson and Sage 2008). Thus, it would appear that photosynthetic divergence between weak and strong CAM is mediated by % IAS and L_{mes}/area that collectively present a functional threshold for predominantly Rubisco- or predominantly PPC-mediated net CO_2 uptake (Nelson and Sage 2008). The compromise between maximizing day- or night-time uptake of CO_2 was exemplified by a comparison of two *Kalanchoë* species (*K. daigremontiana* and *K. pinnata*) that differed in the magnitude of leaf succulence and % IAS (Griffiths et al. 2008; von Caemmerer and Griffiths 2009). The more succulent species (*K. daigremontiana*) was more committed to the conventional CAM cycle, with higher rates of acid accumulation and dark net CO_2 uptake as well as a higher stomatal conductance at night. In contrast, the less succulent *K. pinnata* showed a more C_3-like expression with a higher proportion of integrated 24-h net CO_2 uptake mediated directly by Rubisco during Phases II and IV (Griffiths et al. 2008). A perceived incompatibility between the optimal anatomy for high nocturnal PPC activity and the internal structure ideal for C_3 photosynthesis may account for the bimodal distribution of weak and strong CAM plants that is indicated by carbon isotope ratios ($\delta^{13}C$) across various families known to contain both C_3 and CAM species (Winter and Holtum 2002; Crayn et al. 2004; Silvera et al. 2005).

V. Vasculature and Hydraulic Traits of Photosynthetic Organs of CAM Plants

A. Venation Patterns

The typically succulent leaves and stems of CAM species could potentially present a high cell hydraulic path length for water flow or the transfer of sugars between vascular bundles and metabolic tissues. However, it seems that highly succulent species have circumvented limitations to hydraulic connectivity by evolving 3D venation (Balsamo and Uribe 1988; Cutler 2004; Ogburn and Edwards 2013). Most leaves have their vasculature arranged in two dimensions, with all of the veins arranged in a flat plane that ramifies through the central portion of the mesophyll. However, some succulent CAM lineages, like the monocot *Aloe*, have 3D

vasculature, with veins running through the leaf mesophyll both above and below the central hydrenchyma tissue (Fig. 10.4c). Such 3D vein architecture is also found in dicots where the veins have a fractal-like appearance with a primary vein (the midrib) giving rise to smaller secondary veins, which in turn give rise to tertiary veins, and so on. In the CAM species *Kalanchoë daigremontiana*, the fifth order veins branch at different angles, moving into the adaxial (upper) and abaxial (lower) portions of the mesophyll (Fig.10.4a; Balsamo and Uribe 1988). These fifth order veins are able to cross over higher order veins in order to reach new portions of the mesophyll, and provide water to this tissue. The evolution of 3D venation is believed to release the constraints on succulence, allowing plants to maintain adequate levels of water across the leaf, even in extremely thick leaves (Ogburn and Edwards 2013). It is intriguing to consider how the multiple independent origins of 3D venation across phylogenetically diverse lineages of plants might have contributed to the convergent evolution of CAM (Griffiths 2013).

B. Hydraulic Traits

For plants growing in environments where evaporative demands are high, excessive negative pressures within the xylem can cause the sap to change from liquid to gas in a process called cavitation (Lens et al. 2013a). Cavitation results in the formation of air emboli that often occur at the pits between xylem vessels, thus breaking the column of water in the xylem and resulting in hydraulic failure (Christman et al. 2012; Lens et al. 2013b). Given the hot and water-limiting habitats where CAM plants are competitive, it might seem intuitive that these species should be able to withstand highly negative pressures in their xylem vessels. However, the facultative CAM species *Clusia uvitana* was found to be less tolerant than sympatric C_3 species to highly negative pressures in the xylem vessels, as indicated by measurements of $\Psi 50$, the xylem pressure (negative water potential) at which a 50% loss in hydraulic conductivity can occur (Lüttge and Duarte 2007). However, as described above (Sect. 4.1) and elsewhere, CAM is a trait that evolved as a mechanism for drought avoidance (Griffiths 2013; Borland et al. 2015). Thus, the xylem vessels of CAM species are probably rarely exposed to pressures as negative as those experienced by C_3 species. Consequently it can be hypothesized that evolution has not driven the development of vasculature that is resistant to cavitation in CAM plants. Anecdotal support for this hypothesis comes from documented reports of low lignin content in leaves/stems of CAM species such as *Agave* and *Opuntia* (Cushman et al. 2015) which could be related to a low tolerance to xylem cavitation. Low lignin content is known to increase a plant's susceptibility to cavitation by affecting the permeability or thickness of vessel pits, the location where most xylem embolisms begin to form (Awad et al. 2012). Thus, the drought avoiding strategy of CAM may have reduced the requirement for lignification of the xylem.

The evolutionary relationship between CAM and the vasculature of plants may extend beyond these findings. One hypothesis is that CAM species are less likely to evolve secondary woodiness (i.e., evolve woodiness from an herbaceous ancestor). Evidence is emerging to suggest that the evolution of even small amounts of woodiness may increase tolerance to highly negative xylem pressures, either because strong vessels are resistant to cavitation, or because thicker vessels are less likely to form microfractures that nucleate embolisms (Lens et al. 2012, 2013b). Since the evolution of woodiness is believed to increase a plant's ability to withstand the negative pressures associated with high transpiration rates, it might be hypothesized that CAM lineages, which are known to have low transpiration rates, will rarely evolve from an herbaceous to woody growth habit. Furthermore, evidence from time calibrated phylogenies sug-

gests that aridity has driven the adaptive radiations of succulent CAM lineages as well as lineages that have recently evolved secondary woodiness in the last 9 million years (Arakaki et al. 2011; Lens et al. 2013a). It is intriguing to speculate that these adaptations are alternative solutions to tolerating drought, which may be mutually exclusive; i.e., CAM lineages avoid drought by changing the leaf to reduce transpiration whereas secondary woody lineages change the xylem structure to tolerate drought.

VI. Stomatal Traits in CAM Plants

A. Stomatal Patterning

In general, more succulent species show lower stomatal densities than less succulent species (Sayed 1998; Lüttge 2008b). This holds true for the photosynthetic organs of CAM species, which typically have low stomatal densities and subsequent low conductance to water vapor (Barrera-Zambrano et al. 2014; Males and Griffiths 2017). Such stomatal patterning concurs with the high water-storage capacity, low external surface area:volume ratio, and high water-use efficiencies for leaves and cladodes of CAM species (Osmond 1978; Nobel 1988). In stem succulent CAM species where ribs are obvious, stomata tend to be located at the base between ribs and in many cases the stomata are sunken. In leaf succulent CAM species, an amphistomatic (stomata on both upper and lower leaf surfaces) or hypostamatic (stomata only on the lower leaf surface) location of stomata appears to be related to leaf thickness. For instance, thick-leaved species of the Agavaceae, Crassulaceae and Aizoaceace, such as *Agave tequilana*, *Kalanchoe fedtschenkoi* and *Mesembryanthemum crystallinum,* respectively, are amphistomatic (Moreira et al. 2012; Monja-Mio et al. 2015). In contrast, the relatively thinner leaved *Clusias* are hypostomatic, regardless of the propensity for CAM (Barrera-Zambrano et al. 2014). Amphistomaty is considered an evolutionary adaptation to increase maximum leaf CO_2 conductance by the reduction of its diffusion pathway to the mesophyll, which is advantageous in thicker leaves (Mott et al. 1982), while hypostomaty is considered primarily an adaptive trait to avoid water loss (de Faria et al. 2012).

A negative correlation between stomatal density and size seems to hold for many C_3 species, where plants with lower stomatal densities show a greater mean stomatal size, and smaller stomata are found in leaves with higher stomatal densities (Doheny-Adams et al. 2012; Lawson and Blatt 2014). Such a relationship is attributed to spatial limits in the placing of stomata on the leaf surface that constrains the maximum size and density of stomata (Beaulieu et al. 2008; Franks et al. 2009). In a comparative study of stomatal patterning across nine species of *Clusia* that possess varying capacities for CAM, it was found that stomata were present in lower densities in the thicker-leaved CAM-performing species (Barrera-Zambrano et al. 2014). However, the stomatal pore areas tended to be larger in CAM *Clusias* compared to C_3 *Clusias,* which supports the spatial limitation view described above for C_3 species (Barrera-Zambrano et al. 2014).

As well as affecting the rate of transpiration, the size of the stomata exert a strong influence on the speed with which stomata open and close (Hetherington and Woodward 2003). Smaller stomata may have faster response times when opening and closing compared with larger stomata due to their high membrane surface area to volume ratio. This means that smaller guard cells require less water movement, relative to their size, to inflate or deflate, and affect the pore-facing membrane. It has been suggested that smaller stomata are better at improving water use efficiency due to their more rapid response to changes in environmental conditions such as humidity (Hetherington and Woodward 2003). Therefore, it is reasonable to predict

that CAM species, which are often subject to fluctuating levels of water availability, might have many small stomata. A comparative study of *Clusia* species with different photosynthetic physiologies, however, did not find this to be true. In fact, *Clusia* species that undertake a greater amount of night-time photosynthesis tend to have fewer, large stomata (Barrera-Zambrano et al. 2014). This is believed to be because the production of more, smaller stomata for a given leaf area may incur additional metabolic costs due to higher rates of guard cell respiration (Srivastava et al. 1995; Franks et al. 2009). These extra costs could be compensated for with high CO_2 assimilation rates, but only if environmental resources such as water and light are not limiting (Franks et al. 2009). Given that CAM-performing species of *Clusia* commonly inhabit water-limited environments the possession of larger stomata in lower densities might be the most appropriate strategy in terms of resource use. Furthermore, the positive correlation found within *Clusia* in terms of leaf thickness, mesophyll cell size and guard cell size may be a consequence of common genetic control of cell sizes (Beaulieu et al. 2008).

B. Physiological Implications of Stomatal Patterning

In C_3 plants, it has been proposed that size correlations between different cell types in the leaf (e.g., guard cells, epidermal cells, mesophyll and xylem) provide a highly efficient match between potential maximum water loss (determined by stomatal conductance) and the leaf vascular system's capacity to replace that water (which is determined by vein density; Brodribb et al. 2013). Ultimately, the anatomical potential for diffusive exchange across leaves may be calculated as a function of stomatal density and pore area (i.e., anatomical G_{smax}; (Lawson et al. 1998). Calculations of anatomical G_{smax} across nine *Clusia* species showed no clear trend between photosynthetic mode or the potential for water loss (Barrera-Zambrano et al. 2014). Similarly, a comparison of two facultative CAM species of *Clusia*, *C. minor* and *C. pratensis*, showed comparable anatomical potential for stomatal water loss compared to a related constitutive C_3 species (*C. tocuchensis*; Fig. 10.6). Comparing G_{smax} for these three *Clusia* species with that of *Kalanchoë fedtschenkoi* indicated a significantly reduced anatomical potential for stomatal water loss in the thicker leaved constitutive CAM *Kalanchoë* (Fig. 10.6d). However, in all three species, the calculated G_{smax} was at least 10-fold higher than the measured stomatal conductance (data not shown). Thus, endogenous control over stomatal conductance appears to be more important than stomatal patterning in determining the potential for water loss across different CAM species (Barrera-Zambrano et al. 2014).

In CAM plants, the diel process of malate turnover results in profound shifts in the leaf internal partial pressure of CO_2 (pCO_2), which in turn is believed to underpin the CAM-defining daytime closure and opening of stomata (Cockburn et al. 1979; Wyka et al. 2005). However, other stimuli/regulators are known to influence stomatal conductance in CAM plants either independently or in conjunction with the endogenous CAM cycle of malate turnover. Stomata are subject to regulation by light intensity, light quality, osmolyte concentration, humidity, temperature and the circadian clock, (von Caemmerer and Griffiths 2009; Males and Griffiths 2017). Recent data has demonstrated temporal reprogramming of the expression of several genes associated with various signal transduction mechanisms that regulate stomatal movement in the constitutive CAM species *Agave americana* relative to C_3 *Arabidopsis* (Abraham et al. 2016). These reprogrammed genes included the CO_2-sensing *HIGH LEAF TEMPERATURE 1* together with several redox-related genes; genes demonstrated to play important roles in abscisic acid signaling in *Arabidopsis thaliana*, and potassium, calcium and chloride channels known to

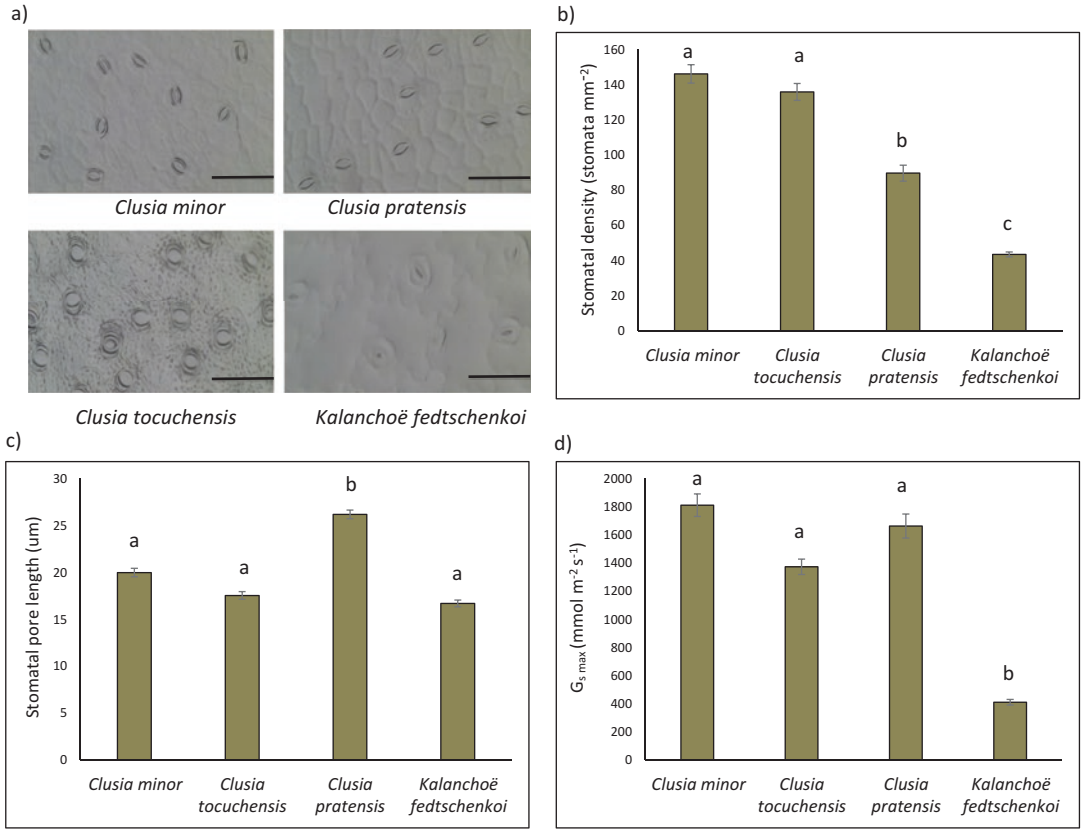

Fig. 10.6. Stomatal patterning characteristics in plants with different modes of photosynthesis. (**a**) Stomatal imprints were obtained using clear nail varnish applied to the lower surface of the leaf for 2 facultative CAM species, *Clusia minor* and *C. pratensis*, a constitutive C_3 species, *C. tocuchensis*, and the constitutive CAM species *Kalanchoe fedtschenkoi*. The scale bar is 100 μm, (**b**) stomatal densities for the 4 species (note stomata are located only on the abaxial leaf surface in *Clusia* and are amphistomatic in *Kalanchoë*), (**c**) stomatal pore length for the 4 species and (**d**) the calculated anatomical G_{smax} for all 4 species. All measurements are shown as the mean of 60 measurements taken from 4 biological replicates ± standard error. Statistical analysis of data was performed using ANOVA and for each graph, different letters above the bars indicate significant difference where $p < 0.05$

be key players in determining stomatal movement. Many of these shifts in transcript abundance were confirmed at the level of protein abundance (Abraham et al. 2016), suggesting a concerted re-programming of the temporal regulation of key components in the core signalling mechanism responsible for inverse stomatal activity in CAM plants. The complex and dynamic nature of diel stomatal regulation which underpins the 4 phases of CAM remains to be fully resolved (Males and Griffiths 2017).

VII. Engineering Anatomical Traits That Are Conducive to CAM

Major research efforts are underway to harness the inherently high WUE of CAM by engineering this pathway into existing food, feed, and bioenergy crops (Borland et al. 2014; Yang et al. 2015). The engineering of CAM into non-CAM crops offers the potential to sustain plant productivity in the hotter and drier climates that are predicted over the next 10–20 years (Dai 2013; Cook et al.

2014). This grand challenge will require further elucidation of the genomic features and regulatory mechanisms that underpin CAM in order to achieve the day/night separation of carboxylation processes catalysed via PPC and Rubisco as well as a temporal reprogramming of stomatal conductance (Yang et al. 2015; Abraham et al. 2016). In addition, leaf anatomical traits will be an important determinant of the mode and level of CAM that is engineered in a non-CAM species. As discussed above (Sect. 3.1), strong constitutive CAM requires adequate vacuolar storage capacity for malate, a trait that is associated with more succulent cells. Engineering succulent cells in a host species for bioengineered CAM could result in less intercellular air space (IAS) between mesophyll cells and a reduction in the length of mesophyll exposed to intercellular air spaces (L_{mes}/area), traits that reduce internal conductance to CO_2. Thus, succulence presents a 'trade-off' between the optimal leaf anatomy for CAM and the internal structure ideal for C_3 photosynthesis. For many productive non-CAM crops, the mode of engineered CAM used to improve WUE should not compromise productivity when water is in plentiful supply. Thus, the option to engage in CAM for only limited periods of time when water is in low supply (i.e., exploit CAM to maintain viability during periods of drought) would appear to be the best model configuration for engineering CAM in many crop species (Borland et al. 2015; Yang et al. 2015).

Studying the functional leaf anatomy of facultative CAM plants, where CAM can be reversibly induced in response to water limitation, should provide valuable pointers towards the optimal leaf anatomy that would accommodate the bioengineering of inducible CAM without incurring detrimental consequences for direct C_3-mediated photosynthesis. Across the different photosynthetic types of the dicotyledonous genus *Clusia*, it has been suggested that the relatively well-aerated spongy mesophyll of the facultative species helps to optimize direct C_3-mediated CO_2 fixation, whereas the enlarged and densely packed palisade mesophyll cells accommodate the potential for C_4 carboxylation and nocturnal storage of organic acids (Barrera-Zambrano et al. 2014). Thus, in principle, differentiated leaves that contain distinct layers of palisade and spongy mesophyll present further options for accommodating both direct Rubisco-mediated daytime uptake of atmospheric CO_2 and the nocturnal uptake of CO_2 that defines CAM. A differential distribution of PPC and Rubisco proteins between palisade and spongy mesophyll cells (with relatively more PPC localized to palisade versus spongy mesophyll cells) could further enhance the efficacy of engineered CAM within leaves made up of these cell layers (Barrera-Zambrano et al. 2014; Sect. 3.3 above). Selecting genotypes with increased levels of ploidy as potential hosts for bioengineered CAM should, in principle, provide increased cell size and biomass productivity due to the positive correlation that exists between ploidy and cell size (De Veylder et al. 2011). In addition to finding species with enlarged cells across the whole leaf, species with a well-developed and tightly packed palisade mesophyll layer should enhance the level of engineered CAM (Barrera-Zambrano et al. 2014). Enhanced development of palisade mesophyll tissue is commonly found in thicker-leaved C_3 species and is hypothesized to improve the harvesting of light, thereby helping to offset the increased investment of biomass in thicker leaves (Smith and Hughes 2009). Overexpression of CBF/DREB transcription factors has been shown to result in thicker leaves with more chlorophyll and higher rates of photosynthesis (Savitch et al. 2005) and this could be one possible strategy for genetically modifying the leaf anatomy of a C_3 host for optimal operation of engineered CAM.

In terms of which stomatal patterning traits might be preferred for bioengineered CAM,

similar values for G_{smax} (anatomical stomatal conductance) that exist between *Clusia* species with different modes of photosynthesis imply that endogenous control over stomatal conductance will be the key factor determining potential water loss from engineered CAM plants (Barrera-Zambrano et al. 2014; Fig. 10.6d). It is generally believed that stomatal conductance in CAM plants is regulated via the substantial diel changes in leaf internal partial pressure of CO_2 (pCO_2) that result from the day/night turnover of malate (Borland et al. 2014; Males and Griffiths 2017). Genotypic variation in stomatal responsiveness to pCO_2 has been reported within the genus *Populus* (AM Borland, unpublished observation), fast-growing bioenergy trees that have been targeted for CAM bioenegineering (Borland et al. 2014, 2015). Thus, it can be argued that an appropriate C_3 host for bioengineered CAM will possess stomata that open in response to low C_i (internal CO_2 concentration) at night and close completely in response to high C_i during the day.

Further detailed and comparative analyses of physiological and morphological characteristics across other lineages that contain C_3, CAM, and intermediate C_3/CAM species are needed to highlight the anatomical traits that are vital for nocturnal CO_2 uptake. A recent study that investigated biochemical, physiological and anatomical traits for the hybrid offspring of C_3 and CAM parents belonging to the genus *Yucca* (Asparagaceae) indicated that leaf anatomical traits seem to be segregating (i.e., show phenotypic variation) among individuals of the hybrid (Heyduk et al. 2016a, b). The *Yucca* hybrid system shows future promise for elucidating the genetic architecture of morphological and CAM related traits within a monocotyledonous genus. In turn, this system should help inform the bioengineering of CAM into economically important non-CAM monocots as a means of improving crop WUE.

VIII. Conclusions

Despite the convergence of CAM across taxonomically diverse groups of plants, common anatomical traits are found in the photosynthetic organs which have a profound influence on physiological strategies for photosynthetic CO_2 assimilation and water-use. Future research should seek to apply advances in the study of leaf and stem hydraulics alongside improved phylogenies and knowledge of geographical distribution in order to aid our understanding of environmental and physiological constraints that have shaped the evolution of CAM. As more CAM genomes become publically available, it should also be possible to identify key regulatory factors involved in the induction and development of leaf and/or stem succulence within the context of photosynthetic pathway divergence. Collectively, such approaches will be crucial for supporting the successful exploitation of engineered versions of constitutive or facultative CAM as a means of improving the water-use efficiency of non-CAM crops grown for food, feed, fibre and bioenergy.

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