EVOLUTIONARY ASPECTS OF CRASSULACEAN ACID METABOLISM

Fernanda Reinert[1]* & Robert E. Blankenship[2]

[1] Universidade Federal do Rio de Janeiro (UFRJ), Instituto de Biologia, Departamento de Botânica, Ilha do Fundão, Rio de Janeiro, Brasil. CEP: 21951-290.

[2] Washington University in St. Louis, Departments of Biology and Chemistry, St. Louis, MO 63130, USA.

E-mails: freinert@biologia.ufrj.br, blankenship@wustl.edu

ABSTRACT

Crassulacean acid metabolism (CAM) is a carbon concentrating metabolism present in more than 30 botanical families. This metabolism is often associated with water stressed environments although it can be found among aquatic plants. CAM's main feature is nighttime stomatal opening and acid accumulation due to CO_2 fixation into a four carbon organic acid, often malate. CAM is present among pteridophytes, gymnosperms and angiosperms, in monocotyledonous and dicotyledonous species. Within some families, such as Crassulaceae, Orchidaceae and Bromeliaceae, CAM is widely represented. The genus Clusia has both facultative and constitutive CAM species. Regulation of the diel cycle of CAM hinges on the activation status of phosphoenolpyruvate carboxylase, responsible for fixation of CO_2 at night. This enzyme is in turn regulated by phosphoenolpyruvate carboxykinase, a dedicated phosphorylating enzyme which is under circadian control. As CAM is widely distributed among botanical families, its origin is believed to be polyphyletic; however, the evolutionary mechanisms which allowed reappearance of this complex metabolism are not yet understood. Perhaps the answer relies on viewing CAM as a network that evolved by gene duplication from the pre-existing non photosynthetic C_4 cycle.

Keywords: Carbon concentrating mechanisms; photosynthesis; carbon isotope; PEPC; CAM in angiosperms.

RESUMO

ASPECTOS EVOLUTIVOS DO METABOLISMO ÁCIDO DAS CRASSULACEAS. O metabolismo ácido das crassuláceas (CAM) é um metabolismo fotossintético de concentração de CO_2 presente em mais de 30 famílias botânicas. Está frequentemente associado à economia de água, embora esteja presente também em espécies aquáticas. CAM é caracterizado pela abertura dos estômatos à noite com acúmulo de ácidos em função da fixação de carbono em compostos de quatro carbonos, em geral, o malato. CAM está presente em pteridófitas, gimnospermas e angiospermas, tanto mono quanto dicotiledôneas. Em algumas famílias, como Crassulaceae, Orquidaceae e Bromeliaceae, o metabolismo ácido das crassuláceas está fortemente representado. No gênero Clusia, CAM pode estar presente nas espécies de forma facultativa ou constitutiva. A regulação do ciclo diurno do metabolismo ácido das crassuláceas é fortemente determinada pela regulação da enzima fosfoenolpiruvato carboxilase, responsável pela fixação do CO_2 durante a noite. Por sua vez, sua regulação é feita através da fosforilação pela enzima fosfoenolpiruvato carboxiquinase que apresenta ritmo circadiano. Como a distribuição de CAM é descontínua acredita-se que sua origem seja polifilética, contudo, não estão claros os mecanismos evolutivos que tornaram o reaparecimento desse complexo metabolismo possível. Talvez CAM possa ser visto como um complexo que evoluiu por duplicação de genes do pré-existente não fotossintético ciclo C_4.

Palavras-chave: Mecanismos de concentração de carbono; fotossíntese; isótopo de carbono; PEPC; CAM em angiospermas.

RESUMEN

ASPECTOS EVOLUTIVOS DEL METABOLISMO ACIDO DE LAS CRASSULACEAS. El metabolismo ácido de las Crassuláceas (CAM) es un metabolismo fotosintético de concentración de CO_2 presente en más de 30 familias botánicas. Está frecuentemente asociado a la economía de agua, a pesar de estar presente también en especies acuáticas. El CAM se caracteriza por la abertura de los estomas en la noche, y con la acumulación de ácidos en función de la fijación de carbono en compuestos de cuatro carbonos, en general, o malato. El

CAM está presente en Pteridófitas, Gimnospermas e angiospermas, tanto mono como dicotiledóneas. En algunas familias, como Crassulaceae, Orquidaceae y Bromeliaceae, el metabolismo ácido de las Crassuláceas está fuertemente representado. En el género *Clusia*, el CAM puede estar presente en las especies de forma facultativa o constitutiva. La regulación del ciclo diurno del metabolismo ácido de las Crassuláceas es fuertemente determinada pela regulación de la enzima fosfoenolpiruvato carboxilasa, responsable por la fijación de CO_2 durante la noche. A su vez, la regulación es realizada a través de la fosforilación que ejecuta la enzima fosfoenolpiruvato carboxiquinasa que presenta ritmo circadiano. Como la distribución de CAM es discontinua se cree que su origen sea polifilético, sin embargo, no están claros los mecanismos evolutivos que tornaron el reapareciendo de este complejo metabolismo posible. Tal vez CAM posa ser visto como un complejo que evoluciono por duplicación de genes del pré-existente no fotosintético ciclo C_4.

Palabras clave: Mecanismos de concentración de carbono; fotosíntesis; isótopo de carbono; PEPC; CAM en Angiospermas.

INTRODUCTION

Crassulacean Acid Metabolism, CAM, is a carbon concentrating mechanism present in approximately 5 to 10% of vascular plants, comprising approximately 35 botanical families (Table 1). CAM species have been reported among pteridophytes (Keeley 1998, Holtum and Winter 1999, Rut *et al*. 2008), gymnosperms (Ting 1985, Vovides *et al*. 2002) but mainly in angiosperms, both monocotyledonous and dicotyledonous species (Ting 1989, Sayed 2001). CAM has been well reviewed from a number of different perspectives and there is considerable information available (e.g. Dodd *et al*. 2002, Black & Osmond 2003, Luttge 2004, Cushman 2005, Cushman *et al*. 2008). The aim of this review is to highlight some of the intriguing aspects of the evolution of CAM and raise a possible mechanism behind its multiple reappearances.

The main characteristic of CAM is nocturnal uptake of carbon via the enzyme phosphoenolpyruvate carboxylase (PEPC) to form organic acid (malic acid) which accumulates in the cellular vacuole until the following morning. There is considerable variation in the expression of CAM, which may be triggered by environmental conditions, stage of development or be constitutively present in a particular species (Ting 1985, Dodd *et al*. 2002, Cushman *et al*. 2008, Motomura et al. 2008).

Current thinking is that the first appearance of CAM might date back 250-300 million years ago with the evolution of aquatic ferns (Keely & Rundell 2003) and early gymnosperms (Vovides *et al*. 2002). This is in contrast to C_4 metabolism, which first arose in grasses 24-35 million years ago (Sage 2004). Several studies demonstrate the evolutionary flexibility of CAM, given by different patterns of evolution across plant families, including polyphyletic origins and reversal events (Reinert *et al*. 2003, Crayn *et al*. 2004, Motomura *et al*. 2008, Silveira *et al*. 2009). Multiple independent evolution of CAM is likely to be facilitated by the fact that there are no unique enzymes or metabolic reactions specifically required for CAM, although CAM-specific PEPC-isoforms have evolved (Ermolova *et al*. 2003, Gerhrig *et al*. 2005, Vaasen *et al*. 2006).

Table 1. List of botanical families with at least one CAM species reported. * yet to be confirmed.
*Tabela 1. Lista de famílias botânicas com, pelo menos, uma espécie CAM. * ainda por ser confirmado.*

Pteridophytes	Isoetaceae
	Polypodiaceae
	Vittariaceae
Gimnosperm	Cycadaceae
	Welwitschiaceae
Angiosperm Dicotyledons	Aizoaceae
	Crassulaceae
	Asclepiadaceae
	Asteraceae
	Cactaceae
	Clusiaceae
	Crassulaceae
	Cucurbitaceae
	Didieraceae
	Euphorbiaceae
	Geraniaceae
	Gesneriaceae
	Lamiaceae
	Oxalidaceae
	Plantaginaceae
	Piperaceae
	Portulacaceae
	Rubiaceae
	Salvadoraceae
	Vitaceae
Angiosperm Monocotyledons	Agavaceae
	Aloaceae
	Amaryllidaceae
	Araceae
	Bromeliaceae
	Commelinaceae
	Dracaenaceae
	Liliaceae
	Orchidaceae
	Rapataceae*

CAM confers higher water use efficiency and is intrinsically associated with water-limited environments (Cushman 2001), including epiphytic habitats, under both sun and shade conditions (Lüttge 2002). Water availability for epiphytic plants is irregular and plants tend to endure drought stress between precipitation events. It is estimated that CAM plants fix 10 to 40g of carbon for every 1,000g of water transpired. C_3 and C_4 plants fix 1-3g and 2-5g, respectively for the same amount of transpiration (Nobel 2009). Additionally, in the aquatic environment, CAM offers the advantage of a concentrating mechanism optimizing carbon uptake during the night period, because in several natural water habitats photosynthetic rate can be limited by reduced CO_2 availability (Robe & Griffiths 2000, Rut *et al.* 2008).

CAM DIEL CYCLE

In what has been described as a typical CAM plant (Figure 1), nighttime accumulation of organic acids is designated phase I, whereas daytime CO_2 recovery (via decarboxylation of accumulated organic acids) and assimilation (via the Calvin-Benson cycle) comprises phase III. These two phases are separated by transitional periods: phase II is the early morning burst of net CO_2 uptake, and phase IV is characterized by afternoon stomatal opening (Osmond 1978). During phases II and IV both Rubisco and PEPC are active in carbon uptake (Griffiths *et al.* 1990, Reinert & Griffiths 1999).

PEPC catalyzes the irreversible β-carboxylation of phosphoenolpyruvate (PEP) using bicarbonate, yielding oxaloacetate (OAA) and inorganic phosphate (Chollet *et al.* 1996). OAA is then reduced by NADPH yielding malate. The 3-carbon substrate, PEP, is produced by glycolysis of storage starch or soluble sugars formed in the previous day. The final 4C product, malic acid, moves through the tonoplast via specific channels, following active transport of protons via the H^+-ATPase pump (Lüttge 1987, Lüttge *et al.* 2000) and malate is stored in the vacuole. The end of the night period marks the end of phase I.

Daytime carboxylation via the Calvin-Benson cycle, phase III, is preceded by decarboxylation of stored malate, which can be mediated by one or a combination of three enzymes: NAD-dependent malic enzymes (NAD-ME), NADP-dependent malic

enzymes (NADP-ME) and phosphoenolpyruvate carboxykinase (PEPcK), depending on the species, yielding pyruvate and/or PEP and CO_2 (Christopher & Holtum 1996). The rate-limiting step during phase III may be malate efflux from the vacuole, malate decarboxylation in the cytoplasm, or assimilation of CO_2 via Rubisco in the chloroplasts. Daytime carbon assimilation via Rubisco is strictly light dependent while stomata are closed leading to high internal partial pressure of CO_2. The balance between the two carboxylation enzymes during the CAM diel cycle in living tissue can be studied using gas exchange techniques associated with carbon isotope analyses, the so called "on-line" carbon discrimination (Griffiths *et al.* 1990).

CARBON ISOTOPES

The use of carbon isotope signatures in plants is central to the study of CAM (and C_4) because the distribution of the three photosynthetic modes can be assessed using the ratio of stable carbon isotopes (Smith 1972, Ting 1985). The chemical properties of the carbon isotopes ^{12}C and ^{13}C, differ slightly, which in turn determines their relative proportion in organic and inorganic matter (Smith 1972). Carbon isotope fractionation can be thought of as the variation in the stable isotopes molar ratio ($^{13}C/^{12}C$) expressed during the formation/destruction of chemical bonds involving carbon atoms, and is expressed with the $^{\delta 13}C$ notation as "units per mille" (‰).

The observed difference in the carbon isotope signature of plant material is due to fractionation of ^{12}C and ^{13}C in photosynthetic carbon fixation (O'Leary 1988). One of the main fractionation points in vascular plants is during diffusion through the stomata, which favours ^{12}C. The other major fractionation point is the carboxylation step, which can be mediated by Rubisco or PEPC. Rubisco discriminates against ^{13}C and PEPC, which does not utilize CO_2, but rather, the hydrated form of inorganic carbon, bicarbonate (HCO_3^-), favors ^{13}C. The isotopic signature of C_3, C_4 and CAM plants is thus a result of the primary carboxylating enzyme that dominates CO_2 uptake. C_4 plants are less depleted in ^{13}C than C_3 plants, and in CAM plants, because atmospheric CO_2 is fixed by both PEPC (mainly at night) and Rubisco (daytime), the isotope signature falls somewhere in between that of C_3 and C_4 plants, depending on the contribution of

phase I to overall carbon fixation. Although reported limits vary because some plants utilize intermediate photosynthetic modes, the isotopic signature of plants generally falls between -7 and -35‰, with C_4 plants having values between -7 and -15‰, CAM often between -10 and -22‰, although it can go to -29‰, and C_3 between -20 and -35‰ (Allaway *et al*. 1974). Medina *et al*. (1977) and O'Leary (1988) proposed and Winter and Holtum (2002) confirmed for a number of species that the relationship between the carbon isotope ratio and the proportion of dark and light CO_2 fixation in CAM plants is linear.

In "on-line" discrimination, activation and deactivation of the two carboxylation enzymes present in CAM cycle, Rubisco and PEPC can be inferred. As the enzymes discrimination values differ (27‰, for assimilation via C_3 pathway and 5.7‰ for the C_4) the determination of the carbon isotope ratio of the air passing a leaf reveals the isotope discrimination happening during photosynthesis (Fig 1) (Griffiths *et al*. 1990, Reinert & Griffiths 1999).

ANCIENT CAM

As presently understood, CAM is limited to vascular plants, and the initial appearance of this photosynthetic metabolism is associated with pteridophytes. CAM has been described in epiphytic and aquatic pteridophytes, such as *Dictymia* (Drynariaceae), *Isoetes* (Isoetaceae), *Plantycerium* (Polypodiaceae), *Pyrrosia* (Polypodiaceae) and *Vittaria* (Vittariaceae) (Keeley 1998, Robe & Griffiths 2000, Martin *et al*. 2005, Rut *et al*. 2008). Estimates of the evolution of Isoetes date from 230-250 million years ago, the Triassic period (DiMichele & Bateman 1996, Rascio 2002), a period of relatively low atmospheric partial pressure of CO_2 (Berner 1994, Laws *et al*. 2002). CAM submerged macrophytes are all secondarily aquatic plants (plants that have returned to aquatic life after having spent some of their evolutionary history on land as terrestrial plants(Rascio 2002). However, due to non-specific anatomical characteristics of CAM plants and the difficulties in discerning CAM through δ13C, the timing of appearance of CAM among secondary aquatic macrophytes is still not determined (Rascio 2002).

Among other ancient CAM plants, a weak form of CAM, CAM-cycling, was reported in the cycad *Dioon edule* Lindl. (Vovides *et al*. 2002) This genus

was considered the basal-most clade in the suborder Zamiineae (Chaw *et al*. 2005). Cycads (Cycadaceae) also appear to have a Paleozoic origin (Crane 1988, Vovides *et al*. 2002) and the presence of CAM might have conferred an additional advantage during the drying climates which marked the end of the Paleozoic Era (Vovides *et al*. 2002). CAM is also found among another gymnosperm, *Welwitschia* (von Willert 2005), one of the three genera of Gnetales. This group was originally seen as the sister-group of angiosperms, because of their morphological similarities such as flower-like reproductive structures and double fecundation. However, more recent analysis based on different gene data sets showed conifers as the closest relatives of Gnetales (Chaw *et al*. 1998, Braukmann *et al*. 2009).

PEPC

PEPC is a cytoplasmic enzyme involved in both anapleurotic and photosynthetic metabolism. PEPC catalyzes the irreversible carboxylation of PEP using HCO_3^- in the presence of Mg_2^+ (or Mn_2^+) and yields oxaloacetate and Pi. Among the functions of PEPC is regulation of cellular osmolarity and pH, and replenishment of Krebs cycle intermediates, thus providing carbon skeletons for nitrogen assimilation and amino acid biosynthesis (Nimmo 2006, Xu *et al*. 2006). Provision of Krebs cycle intermediates is especially important for plants during intensive growth and/or activity of certain tissues. For example, expression of PEPC in legume root nodules is particularly enhanced in order to provide enough energy and carbon skeletons for nitrogen fixation (Vance 2008). Stylet chloroplasts of *Nicotiana tabacum* L. are devoid of Rubisco and the light-driven photosynthetic flux is functionally coupled to PEPC mediated formation of malate. This reaction enables re-utilization of respiratory CO_2 during pollen tube elongation (Jansen *et al*. 1992). In stomatal opening, the malate anion functions as a solute, decreasing water potential of the guard cells favoring water movement into these cells (Tarczynski 1993).

PEPC REGULATION

PEPC is widely distributed from bacterial and archaeal to vascular plant species (including

nonphotosynthetic bacteria and protozoa), but is absent from fungi and animals (Lepiniec *et al.* 1994, Izui *et al.* 2004). Several PEPC isoforms have been detected immunologically, and it is well recognized that PEPC arises from a multigene family (Lepiniec *et al.* 1994, Nimmo 2006, Xu *et al.* 2006). Plant PEPC is an allosteric enzyme often found as a tetramer of identical ~95–110-kDa subunits, although exceptions exist in the Archaea and green microalgae (Xu *et al.* 2006). The work of Xu *et al.* (2006) with *Sorghum* showed that the strictly conserved, C-terminal glycine residue in PEPC is crucial for overall catalysis because even a modest, neutral alteration of the PEPC C-terminal hydrogen atom side chain is detrimental to enzyme function.

Vascular plant PEPC is activated by reversible phosphorylation of a specific serine residue near the amino terminus, mediated by a dedicated Ser/Thr kinase, PEPC-Kinase, and an opposing heterotrimeric protein phosphatase 2A. Post-translational regulation results in both improved catalytic activity and increased tolerance to malate. PEPC-Kinase is the smallest protein kinase (~31-kDa), believed to be under control of a circadian clock and synthesized de novo every night. Originally, PEPC-kinase was considered to be a major controlling point of CAM (Nimmo 2000), however more recent work (Taybi *et al.* 2004) comparing PEPC and PEPC-Kinase mRNA expression with day/night patters of gas exchange, organic acid and soluble sugars in Clusia species revealed that the diel regulation of PEPC-Kinase is a consequence of the circadian rhythm of CAM metabolite cycling rather than a major controlling factor for the temporal separation of carboxylation phases of CAM. Sullivan *et al.* (2004) working with *Glycine max* L. reported the expression patterns of PEPC and PEPC-Kinase gene family (GmPpc and GmPpcK), including one encoding a bacterial-type Ppc. The PEPC expression patterns do not match those of any of the PEPC-K, arguing against the existence of specific PEPC and PPCK gene expression partners. Additionally, Xu *et al.* (2007), also working with *G. max*, proposed that the expression of GmPpcK2 and GmPpcK3 homologs and GmPpc7 are the key molecular steps in the regulatory phosphorylation system within mature nodules.

Most plant PEPC genes (from C3, CAM or C_4) are formed by 10 exons and show a highly conserved structure (Chollet *et al.* 1996). C_4-PEPC is only expressed in the mesophyll cell, whereas non-photosynthetic PEPC may be expressed in different tissues or organs, providing evidence for new expression patterns for the expression of C_4 photosynthesis (Svensson *et al.* 2003). CAM-PEPC, on the other hand, is temporally regulated to function mostly at night. C_4-PEPC and, especially, CAM-PEPC function in a different metabolic context with substantially higher substrate and product concentrations, which probably called for altered kinetics and/or regulation of the enzyme. C_4-PEPC shows a rapid (few seconds) turnover in the order of magnitude of 1–10 mM of ^{4}C acids, and CAM-PEPC turns over 100–500 mM pools of ^{4}C acids in 24h cycles. A comprehensive study of a PEPC gene family was carried out for *Kalanchoe pinnata* (Lam.) Pers. (Crassulaceae) (Gehrig *et al.* 2005). The authors found and sequenced fragments from seven isoenzymes, of which only one was suggested to be responsible for the photosynthetic functions of CAM.

The wide distribution of PEPC and the many different roles of this enzyme suggest that PEPC genes can provide powerful markers not only for the molecular evolution of the enzyme itself but for the metabolic pathways where the enzyme is involved and the organisms that show such pathways.

CAM EVOLUTION

Unraveling the evolution of both CAM and C_4 pathways is contributing to the elucidation of the molecular evolution of PEPC (Hermans & Westhoff 1990, Lepeniec *et al.* 1993, 1994, Slocombe *et al.* 1993, Toh *et al.* 1994, Nakagawa *et al.* 2003, Sanchez & Cejudo 2003). Nevertheless, it is important to note that although PEPC is a key enzyme for both photosynthetic pathways, and certainly crucial to the development of CAM and C_4, PEPC evolution does not necessarily reflect that of the photosynthetic pathway. In the last decade it has been proposed that one of the primary events in the evolution of CAM and C4 plants may have been the development of a regulatory mechanism that generates novel patterns of gene expression of PEPC and of other enzymes involved in temporal and spatial expression patterns of CAM and C_4 (Hermans & Westhoff

1990, Lepeniec *et al*. 1993, Svensson *et al*. 2003). Lepeniec *et al*. (1994) suggested that, following gene duplication and divergence, an isoform with a specific mode of regulation, at least at the level of gene expression, could have been conserved in the plant families containing C_4 species. Further, more recent modifications in the kinetic or regulatory properties of the C_4-PEPC would have taken place differently in the various species after the enzyme had acquired its function in C_4 photosynthesis.

At this point we would like to re-address a basic point regarding the debate on the evolution of CAM: the question as to whether the multiple aspects involved in this metabolism are parts of a whole, with an identity of its own, or if these aspects represent independent phylogenetic units with independent evolutionary histories, functioning together and producing a trait (CAM). However, to properly address this question beyond rhetoric, it would be necessary to provide empirical evidence of the mechanism of convergent evolution that made it possible for CAM to have such broad distribution among plant species. The lack of a framework for the investigation of the evolution of CAM, other than the evolution of its best studied enzyme, PEPC, has puzzled at least another author. Monson (2003) pointed out our limitation to explain why or how C_4 (and we would include CAM in a similar scenario) photosynthesis evolved so many times independently and why there are certain patterns in its taxonomic distribution. Monson (2003) noted that C_4 is absent in canopy-forming forest tree species and the paucity
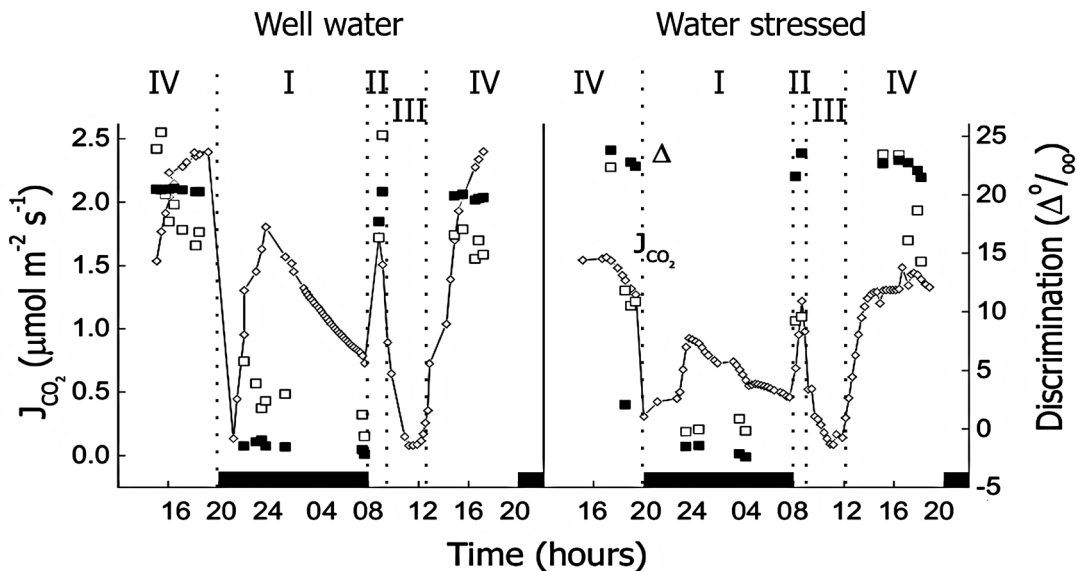

Figure 1. Gas exchange (∘), showing the four phases of CAM (roman numbers) and on-line carbon isotope discrimination in *Neoregelia cruenta* (R. Graham) L.B. Smith (Bromeliaceae) with regular water supply (left) and under water stress (right). Close squares represent calculated values according to the model proposed by Farquhar *et al*. (1989), where $\Delta = a + (b\text{-}a)p_i/p_a$; *a* refers to the theoretical carbon isotopic fractionation due to the diffusion in air (4.4‰), *b* to the fractionation occurring during carboxylation (27‰, for assimilation via C_3 pathway and 5.7‰ for the C_4) and p_i/p_a refers to the partial pressure of CO_2 inside and outside the stomata chamber. Open squares represent measured values of the carbon isotope ratio of the reference and analysis air stream following gas exchange measurements. Black bars represent the dark period. Gas exchange was measured using a Walz mini-cuvette system (CMS 400) with an external IRGA (Binos 100 Walz, Effeltrich, Germany). Leaf cuvette day/night temperature of 22°C and incident PFD of 300 μmol photon m^2s^{-1}. During phase II, isotope ratio signal shifts from C_4 to C_3 carboxylation as Δ values increase and during phase IV from C_3 to C_4 carboxylation as Δ values decrease, showing that both enzymes (Rubisco and PEPC) are active. During phase I (night period), Δ approach theoretical values of carbon assimilation via PEPC. During phase III no Δ values are presented as stomata are closed (Reinert 1995).

Figura 1. Trocas gasosas (∘), mostrando as quatro fases de CAM (algarismos romanos) e descriminação isotópica on-line em Neoregelia cruenta (R. Graham) L.B. Smith (Bromeliaceae) com água (esquerda) e sem água (direita). Quadrados fechados representam valores calculados a partir do modelo proposto por Farquhar et al. (1989), onde $\Delta = a + (b\text{-}a)p_i/p_a$; a refere-se ao fracionamento teórico do isótopo de carbono em função da difusão no ar (4.4‰), b refere-se ao fracionamento teórico do isótopo de carbono em função da carboxilação (27‰, para assimilação via rota C_3 e 5.7‰ via rota C_4) e p_i/p_a refere-se à pressão parcial de CO_2 dentro e fora da câmara sub-estomática. Quadrados abertos representam valores de razão isotópica do gás referência e análise após medidas de trocas gasosas. A barra em preto representa o período de escuro. Trocas gasosas foram medidas pelo Walz mini-cuvette system (CMS 400) com IRGA externo (Binos 100 Walz, Effeltrich, Germany). A temperatura da câmara foliar foi de 22°C e luz incidente de 300 μmol photon m^2s^{-1}. Durante a fase II, o sinal da razão isotópica (Δ) aumenta à medida que a carboxilação muda de C_4 para C_3; e durante a fase IV Δ diminui à medida que a carboxilação muda de C_3 para C_4, mostrando que ambas enzimas (Rubisco e PEPC) estão ativas. Durante a fase I (período noturno), Δ aproxima-se dos valores teóricos de assimilação de carbono via PEPC. Durante a fase III não há medidas de Δ porque os estômatos ficam fechados (Reinert 1995).

of C_4 species within eudicots compared to monocots. Similarly, among CAM species the genus Clusia is the only example of a CAM tree, although among CAM plants there is no clear predominance of monocots over dicot species. Monson (2003) argued that the evolution of C_4 photosynthesis is largely a story of gene duplication while plants are still in the ancestral, C_3 state. In his own words: "Once a reservoir of key, duplicated, and preserved C_3 genes is present, a small amount of subsequent modification within gene promoter regions is all that is necessary to transform certain C_3 patterns of gene expression to C_4 patterns."

Corroborating the gene duplication hypothesis, the work of Amoutzias *et al.* (2004) with bHLH proteins and nuclear receptor proteins combining genomic data, domain architecture and protein–protein interactions, provided the first empirical evidence that scale-free protein networks can emerge through single-gene duplications. bHLH (basic helix-loop-helix) comprises an ancient class of eukaryotic transcription factors that are found in fungi, plants and animals. It is presently believed that gene duplication event(s), both large-scale and single-gene duplication, are the predominant mechanism for the generation of complex networks, such as new members of a protein family, and so is central to the evolution of complex traits. Studies on the evolution of the CAM pathway would certainly benefit from a similar approach and should be pursued. In conclusion CAM (and C_4) evolution should be investigated as a sub-network that evolved by gene duplication from a pre-existing network such as the non photosynthetic C_4 cycle.

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