On the Evolutionary Origin of CAM Photosynthesis[OPEN]

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Evolution of carbon concentrating mechanisms appears complex. In the case of C_4 photosynthesis, an enabling mutation is thought to have formed an initial C_4 cycle, which is then selected for flux, and, finally, high expression of photorespiratory genes is lost (for summary, see Bräutigam and Gowik, 2016). However, in the case of Crassulacean acid metabolism (CAM) photosynthesis, we suggest that evolution directly acts on a low flux pathway already in place for amino acid metabolism. We thus propose a true continuum from C_3 to CAM plants.

CAM PHOTOSYNTHESIS

Photosynthesis in arid and/or hot and/or high light conditions faces unique challenges as water loss limits stomatal opening and thus limits CO_2 supply for photosynthesis. Two different evolutionary solutions to this dilemma are known in land plants: CAM and C_4 photosynthesis (Lüttge, 1988; Sage et al., 2012; Borland et al., 2014; Hartwell et al., 2016). Both traits are add-ons to the classic photosynthetic pathways, which are frequently termed C_3 photosynthesis. CAM is not limited to the Crassulaceae but prevalent in many taxa (Silvera et al., 2010); C_4 photosynthesis is named for the first labeled product of carbon fixation, a C_4 acid. Many crop plants growing in challenging environments carry either adaptation: the CAM plants *Agave tequilana* and pineapple are productive in challenging climates (Borland et al., 2014). C_4 photosynthesis is prevalent among productive crop plants, including maize, sugarcane, sorghum, and millet (Hibberd et al., 2008). Therefore, both CAM and C_4 photosynthesis have been considered for engineering C_3 crop plants to withstand adverse conditions while maintaining high yield (Hibberd et al., 2008; Borland et al., 2014).

CAM plants fix CO_2 via phospho*enol*pyruvate carboxylase (PEPC) during the night when it is cooler and less water is lost. The resulting organic acids, canonically malic acid but also citric acid (Knauft and Arditti, 1969; Lüttge, 1988), are stored in the central vacuole and decarboxylated during the day to provide CO_2 to Rubisco and the Calvin-Benson-Bassham cycle (Silvera et al., 2010). During the night, stored carbohydrates are partially exported and partially used for organic acid synthesis (Borland and Dodd, 2002), which leads to a large proportion of storage carbohydrate cycling. CAM photosynthesis may be facultative, that is, induced environmentally and reversibly (i.e. *Talinum triangulare* [Brilhaus et al., 2016], *Mesembryanthemum* [Winter and Holtum, 2014]), or obligatory (Silvera et al., 2010). CAM plants also display great variation in their CO_2 fixation efficiency with some species only cycling, i.e. refixing nightly respiratory CO_2, and others reaching high fixation rates under ideal conditions (Silvera et al., 2010).

Both C_4 photosynthesis and CAM have evolved independently multiple times from C_3 ancestors. C_4 species represent about 3% of flowering plant species (Sage et al., 2012), while CAM species represent about 6% (Silvera et al., 2010). The evolutionary path and the fact that it has been traversed multiple times independently are somewhat puzzling given that both pathways represent complex traits, which require multiple genes to change simultaneously. They require architectural adaptations—large storage vacuoles in obligatory CAM, Kranz anatomy, or highly specialized cell anatomy in C_4—and biochemical adaptations with at least a dozen gene products altered in abundance and regulation.

As the basis for the evolution of CAM metabolism, a priori changes in abundance of multiple transcripts, especially with regard to their circadian patterns, were proposed (Silvera et al., 2010).

LESSONS FROM C_4 EVOLUTION

C_4 and CAM photosynthesis are similar in the sense that initial CO_2 fixation and Rubisco reaction are separated, spatially in the case of C_4 and temporally in the case of CAM. The enzymes for the carbon concentrating mechanism are similar, too, occasionally down to the isoform recruited to either pathway (Christin et al., 2014). We use the concepts emerging from the recent progress in the study of C_4 photosynthesis evolution

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(Heckmann et al., 2013; Williams et al., 2013; Mallmann et al., 2014) and apply them to CAM evolution.

Kinetic modeling revealed that the evolutionary path to Kranz anatomy based C_4 is smooth with a Mount Fuji pattern, which has neither troughs nor steps (Heckmann et al., 2013), and that the path recapitulates the succession of earlier conceptual, stepwise evolutionary models (Monson et al., 1984; Rawsthorne et al., 1988; Sage et al., 2012). All of these models consider the establishment of a photorespiratory Gly shuttle via spatial expression of a key photorespiratory gene, also termed C_2 photosynthesis, as an essential initial event of C_4 evolution. It initiates the division of labor between the mesophyll and bundle sheath cells and the establishment of metabolic fluxes typical for C_2 photosynthesis. However, the models could not explain the molecular events leading from photorespiratory shuttle to C_4 photosynthesis. Modeling the metabolism of C_3-C_4 intermediates with a combination of a kinetic model and flux balance analysis indicated that the introduction of the photorespiratory Gly shuttle already predicts the immediate existence of a C_4 cycle with low flux, to balance the nitrogen metabolism of mesophyll and bundle sheath cells (Mallmann et al., 2014). From this enabling mutation onward, selective pressure for higher expression of C_4 cycle genes rests with the limiting enzyme or transporter capacity until high expression for all is reached (Mallmann et al., 2014; Bräutigam and Gowik, 2016). In summary, an enabling mutation transforms the trait from complex (i.e. multiple genes change at once) to additive (i.e. each change in gene expression increases fitness).

For CAM photosynthetic evolution, a model passing through intermediates with ever increasing cycle capacity has been proposed (Silvera et al., 2010). However, the possible starting point, the initial CAM event, has not been identified so far. To establish an efficient CAM cycle via natural selection on a limiting enzyme, transporter, or architectural adaptation, a basal "CAM cycle" has to be in place.

CARBON FLUXES IN C_3 AND CAM PLANTS

We thus set out to identify whether metabolite fluxes similar to CAM fluxes are already in existence in C_3 plants or generated by a simple mutation. The core features are daytime release of the CO_2 for fixation by Rubisco and nightly production and storage of organic acids produced by PEPC.

Use of Stored Organic Acids during the Day

Based on textbooks and depictions in repositories (i.e. http://www.plantcyc.org/), it is frequently assumed that amino acids are directly derived from photosynthesis during the day. In this concept, triosephosphates from the Calvin-Benson-Bassham cycle are converted to organic acids via cytosolic reactions and the TCA cycle (TCAc) and then transaminated to amino acids and derived products (Fig. 1A). However, flux analyses

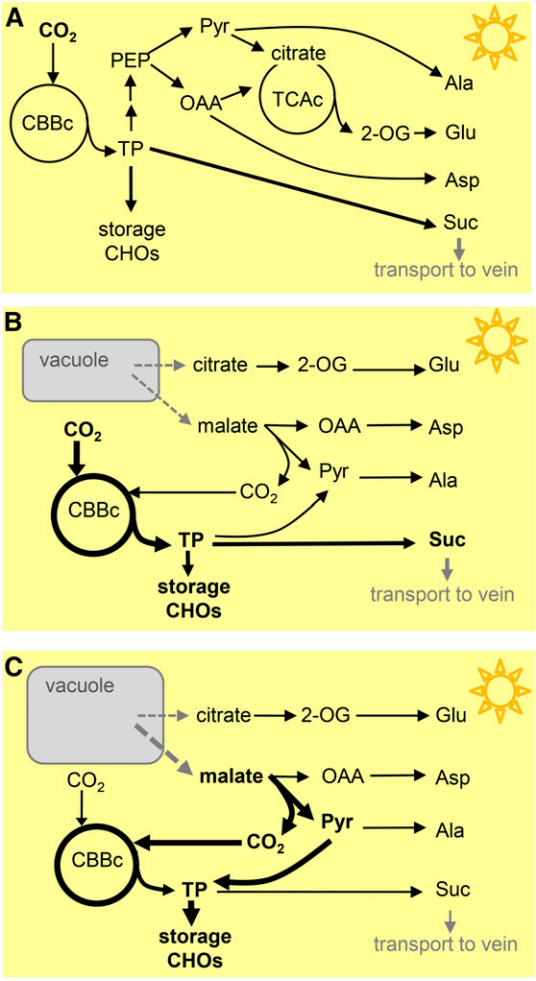

Figure 1. Daytime metabolism of organic acids in C_3 and CAM plants; arrow thickness denotes flux. A, Organic acids are directly derived from photosynthesis during the day. This model is obsolete for many C_3 species due to the results of flux analyses. B, In many C_3 plants, the use of organic acids is based on organic acids produced and stored during the night according to flux analyses. C, Daytime metabolism of organic acids in CAM plants. 2-OG, Oxoglutarate; CBBc, Calvin-Benson-Bassham cycle; CHOs, carbohydrates; OAA, oxaloacetate; Pyr, pyruvate; TP, triosephosphate.

using $^{13}CO_2$, ^{13}C-pyruvate, and ^{13}C-Glc labeling have questioned this model for several C_3 species from distant eudicot groups (rosids with Fabales and Brassicales, asterids with Asteraceae; Tcherkez et al., 2005, 2009, 2012; Gauthier et al., 2010; Szecowka et al., 2013).

Daytime pulse labeling with $^{13}CO_2$ indicates that the organic acids malate, citrate, isocitrate, and fumarate remain largely unlabeled as do the derived amino acids (Szecowka et al., 2013). Similarly, Glu and Gln, both derived from the organic acid 2-oxoglutarate, are not labeled during a daytime $^{13}CO_2$ pulse (Gauthier et al., 2010). Feeding with precursors such as ^{13}C-pyruvate demonstrated that pyruvate entry into the TCAc is very low (Tcherkez et al., 2005, 2009). Taken together,

these observations refute the model depicted as Figure 1A (Tcherkez et al., 2012).

Observing label distribution in the night and the following day after giving a label pulse of $^{13}CO_2$ on the previous day showed that, during the night and the following day, ^{13}C is transferred to Glu and Gln (Gauthier et al., 2010). Under the current model (Tcherkez et al., 2012), daytime use of organic and amino acids is based on organic acids produced and stored during the night before and used during the following day (Fig. 1B). So there is no immediate connection of their production to the present daytime photosynthetic metabolism (Fig. 1B). Unlike, the pools of C_4, C_5, and C_6 organic acids and derived molecules, the pyruvate pool labels to about 50% (Szecowka et al., 2013). That is, half the pool is produced from photosynthetic products and half from reserves (Fig. 1B). In summary, C_3 plants store organic acids at night to fuel daytime amino acid synthesis. Part of the stored organic acids is decarboxylated to pyruvate during the day.

If one considers the evolutionary changes required for daytime CAM photosynthesis, it becomes apparent that the framework of the CAM cycle actually is already in place in C_3 species and that it carries flux. It is not a question of rewiring metabolism but of selecting for increased flux (Fig. 1C).

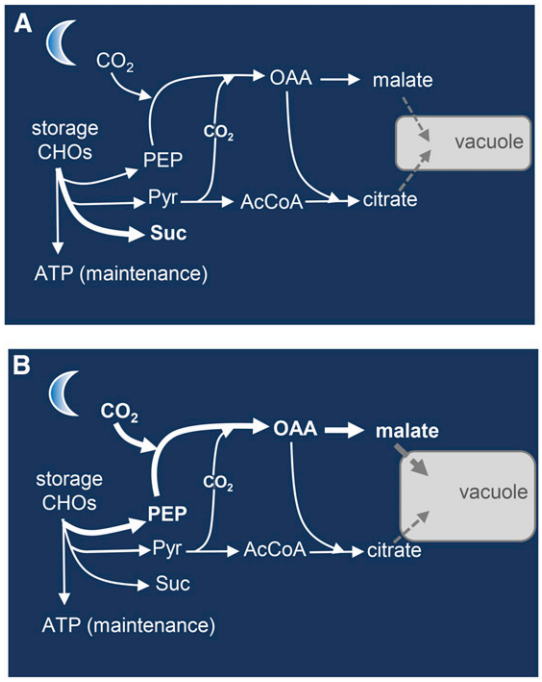

Figure 2. Nighttime metabolism of organic acids in C_3 (A) and CAM (B) plants. AcCoA, Acetyl coenzyme A; CHOs, carbohydrates; OAA, oxaloacetate; PEP, phospho*enol*pyruvate; Pyr, pyruvate.

Organic Acid Storage during the Night

Flux analysis has established that C_4 and C_5 organic acids used during the day are largely drawn from a store produced during the night (Gauthier et al., 2010; Szecowka et al., 2013), which in turn is dependent on storage carbohydrates synthesized on the previous day (Gauthier et al., 2010). PEPC for C_4 acid synthesis and beyond requires activation by a kinase. In a C_3 plant, a PEPC activating kinase is preferentially expressed during the night and PEPC phosphorylation indeed persists during the night (Aldous et al., 2014). The substrate of PEPC, phospho*enol*pyruvate, is produced from stored carbohydrates (Gauthier et al., 2010). The resulting oxaloacetate can be reduced to malate and stored, or converted to citrate in the TCAc (Fig. 2A). The CO_2 used during phospho*enol*pyruvate carboxylation stems from pyruvate decarboxylation, from the TCAc, or from outside. If from interior sources, the resulting oxaloacetate would have an isotopic composition in the C_3 range at the C_1 position. If the CO_2 stems from the outside, its isotopic composition at the C_1 carbon would be in the C_4 range. Analysis of Asp contained in proteins in *Nicotiana tabacum* demonstrated that half of the Asp C_1 carbon is in the C_4 range and thus derived from outside CO_2 and not from CO_2 prefixed by Rubisco (Melzer and O'Leary, 1987). Thus, nightly organic acid metabolism of C_3 plants likely involves atmospheric CO_2 fixation (Fig. 2A).

If one considers the evolutionary changes required for nighttime CAM photosynthesis, it becomes apparent that the framework of the CAM cycle actually is already in place in C_3 species. Nighttime CAM metabolism does not require de novo fluxes but an increase in existing fluxes, including increased flux of CO_2 from the outside (Fig. 2B).

EARLY EVOLUTIONARY EVENTS OF CAM PHOTOSYNTHESIS

In summary, the temporally separated CAM cycle including the fixation of outside CO_2 during the night, synthesis and storage of organic acids during the night, and use of organic acids including malate decarboxylation (Szecowka et al., 2013) during the day is already in place in C_3 plants, but has never been called a CAM cycle (Tcherkez et al., 2012). A properly constrained diel stoichiometric C_3 model is capable of predicting CAM photosynthesis (Cheung et al., 2014), underscoring that evolution of efficient CAM does not require rewiring or temporally changing flux capacity but only increasing existing flux capacity (Figs. 1C and 2B).

While stomatal opening patterns are completely reversed in strong CAM species (Abraham et al., 2016), initial evolution of weak CAM likely only requires incrementally increased flux and therefore incrementally increased stomatal opening during the night. At least in some CAM species, daytime stomata closure may simply be caused by water limitation (Winter and Holtum, 2014). A single mutation induces nightly stomatal opening while leaving daytime closure intact (Costa et al., 2015). Some known CAM plants remain capable of daytime stomatal opening if water is available to the transpiration stream (Winter and Holtum,

2014). Although architectural adaptations were not considered in the analysis, a rather small storage vacuole for organic acids is in place already in C_3 plants and may come under selection if increased storage capacity is required.

This evolutionary scenario explains several previous observations about CAM and C_4 plants. In many CAM species, citric acid coaccumulates with malic acid (Knauft and Arditti, 1969; Lüttge, 1988). The evolutionary scenario presented here explains citrate accumulation as an atavism of the evolutionary origin. The CAM trait can revert to C_3 metabolism over evolutionary time (Crayn et al., 2004; Silvera et al., 2009). Unlike C_4 evolution, which includes the loss of expression for multiple genes (Bräutigam and Gowik, 2016), weak CAM likely requires expression gain and no changes in temporal expression (Figs. 1B and 2A), which makes sliding back along the gradient of CAM toward C_3 possible and likely. Indeed, many if not all CAM species retain the ability to photosynthesize in C_3 mode (Winter and Holtum, 2014). In consequence, mutation of CAM genes in CAM plants is not lethal (Dever et al., 2015), while in C_4 species, the C_4 cycle is obligatory (Dever et al., 1995). CAM has a higher incidence in plant species (Silvera et al., 2010). The evolutionary scenario shows that, unlike during evolution of C_4 photosynthesis, which requires the loss of expression of photorespiratory genes in a certain cell type, the pathway on which selective pressure for CAM can act does not require an enabling mutation to be present. CAM metabolism can be induced and shut off multiple times during a plant's life cycle (Taisma and Herrera, 1998). The continuum from C_3 to CAM explains why seamless induction and recovery are possible in so-called facultative CAM plants but is unknown in C_4.

This evolutionary scenario predicts testable CAM features including but not limited to gene duplication are probably not required. CAM genes are orthologs to genes involved in amino acid assimilation, and selective pressures selected for higher expression rather than change of pattern. The evolutionary scenario also predicts that plants that do not possess nighttime organic acid storage (i.e. Leport et al., 1996) are unlikely to evolve toward CAM.

We propose to extend the currently accepted continuum of CAM evolution (cycling, weak, idling, strong; Silvera et al., 2010) to C_3 species (amino acid metabolism in C_3, cycling, weak, idling, strong). The requirements for CAM listed by Silvera et al. (2010) are all present at the right time of the diurnal cycle and only need enhancement: nocturnal CO_2 uptake, diel fluctuations of organic acids, associated transport activities, (enhanced) PEPC and malic enzyme expression, (enhanced) flow through glycolytic and gluconeogenic pathways, and a storage vacuole (Figs. 1B and Fig. 2A; derived from Tcherkez et al., 2005, 2009; Gauthier et al., 2010; Szecowka et al., 2013). We thus argue that CAM evolution, unlike C_4, is a true continuum from C_3 to CAM. This bodes well for the engineering of CAM into C_3 crops.

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