On the Evolutionary Origin of CAM Photosynthesis

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Evolution of carbon concentrating mechanisms appears complex. In the case of C₄ photosynthesis, an enabling mutation is thought to have formed an initial C₄ 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 C₃ 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 CO₂ supply for photosynthesis. Two different evolutionary solutions to this dilemma are known in land plants: CAM and C₄ 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 C₃ photosynthesis. CAM is not limited to the Crassulaceae but prevalent in many taxa (Silvera et al., 2010); C₄ photosynthesis is named for the first labeled product of carbon fixation, a C₄ 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). C₄ photosynthesis is prevalent among productive crop plants, including maize, sugarcane, sorghum, and millet (Hibberd et al., 2008). Therefore, both CAM and C₄ photosynthesis have been considered for engineering C₃ crop plants to withstand adverse conditions while maintaining high yield (Hibberd et al., 2008; Borland et al., 2014).

CAM plants fix CO₂ via phosphoenolpyruvate 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 (Knaut and Arditti, 1969; Lüttge, 1988), are stored in the central vacuole and decarboxylated during the day to provide CO₂ 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 CO₂ fixation efficiency with some species only cycling, i.e. reflexing nightly respiratory CO₂, and others reaching high fixation rates under ideal conditions (Silvera et al., 2010).

Both C₄ photosynthesis and CAM have evolved independently multiple times from C₃ ancestors. C₄ 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 C₄—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 C₄ EVOLUTION

C₄ and CAM photosynthesis are similar in the sense that initial CO₂ fixation and Rubisco reaction are separated, spatially in the case of C₄ and temporally in the case of CAM. The enzymes for the carbon concentrating mechanism are similar, too, occasionally down to the isomorph recruited to either pathway (Christin et al., 2014). We use the concepts emerging from the recent progress in the study of C₄ 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 C₄ 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 C₂ photosynthesis, as an essential initial event of C₄ evolution. It initiates the division of labor between the mesophyll and bundle sheath cells and the establishment of metabolic fluxes typical for C₂ photosynthesis. However, the models could not explain the molecular events leading from photorespiratory shuttle to C₄ photosynthesis. Modeling the metabolism of C₂-C₄ 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 C₄ 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 C₄ 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 C₃ AND CAM PLANTS

We thus set out to identify whether metabolite fluxes similar to CAM fluxes are already in existence in C₃ plants or generated by a simple mutation. The core features are daytime release of the CO₂ 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. This model is obsolete for many C₃ species due to the results of flux analyses. In many C₃ plants, the use of organic acids is based on organic acids produced and stored during the night according to flux analyses. 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.

Figure 1. Daytime metabolism of organic acids in C₃ and CAM plants; arrow thickness denotes flux. A, Organic acids are directly derived from photosynthesis during the day. This model is obsolete for many C₃ species due to the results of flux analyses. B, In many C₃ 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; CHO₃, carbohydrates; OAA, oxaloacetate; Pyr, pyruvate; TP, triosephosphate.

Using ¹³CO₂, ¹³C-pyruvate, and ¹³C-Glc labeling have questioned this model for several C₃ 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 ¹³CO₂ 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 ¹³CO₂ pulse (Gauthier et al., 2010). Feeding with precursors such as ¹³C-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).

**Organic Acid Storage during the Night**

Flux analysis has established that C$_3$ and C$_4$ 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, phosphoenolpyruvate, 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 TCA cycle (Fig. 2A). The CO$_2$ used during phosphoenolpyruvate carboxylation stems from pyruvate decarboxylation, from the TCA cycle, 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).
2014). Although architectural adaptations were not considered in the analysis, a rather small storage vacuole for organic acids is in place already in C₃ plants and may come under selection if increased storage capacity is required.

This evolutionary scenario explains several previous observations about CAM and C₄ plants. In many CAM species, citric acid coaccumulates with malic acid (Knauft and Arditti, 1969; Lützge, 1988). The evolutionary scenario presented here explains citrate accumulation as an atavism of the evolutionary origin. The CAM trait can revert to C₃ metabolism over evolutionary time (Crayn et al., 2004; Silvera et al., 2009). Unlike C₄ 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 C₃ possible and likely. Indeed, many if not all CAM species retain the ability to photosynthesize in C₃ mode (Winter and Holtum, 2014). In consequence, mutation of CAM genes in CAM plants is not lethal (Dever et al., 2015), while in C₄ species, the C₄ 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 C₄ 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 C₃ to CAM explains why seamless induction and recovery are possible in so-called facultative CAM plants but is unknown in C₄.

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 C₄ species (amino acid metabolism in C₃, 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 CO₂ 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; Szewcowka et al., 2013). We thus argue that CAM evolution, unlike C₄, is a true continuum from C₃ to CAM. This bodes well for the engineering of CAM into C₃ crops.

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LITERATURE CITED


