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Engineering of Crassulacean Acid Metabolism

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Abstract

Crassulacean acid metabolism (CAM) has evolved from a C_3 ground state to increase water use efficiency of photosynthesis. During CAM evolution, selective pressures altered the abundance and expression patterns of C_3 genes and their regulators to enable the trait. The circadian pattern of CO_2 fixation and the stomatal opening pattern observed in CAM can be explained largely with a regulatory architecture already present in C_3 plants. The metabolic CAM cycle relies on enzymes and transporters that exist in C_3 plants and requires tight regulatory control to avoid futile cycles between carboxylation and decarboxylation. Ecological observations and modeling point to mesophyll conductance as a major factor during CAM evolution. The present state of knowledge enables suggestions for genes for a minimal CAM cycle for proof-of-concept engineering, assuming altered regulation of starch synthesis and degradation are not critical elements of CAM photosynthesis and sufficient malic acid export from the vacuole is possible.

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1. INTRODUCTION TO CAM

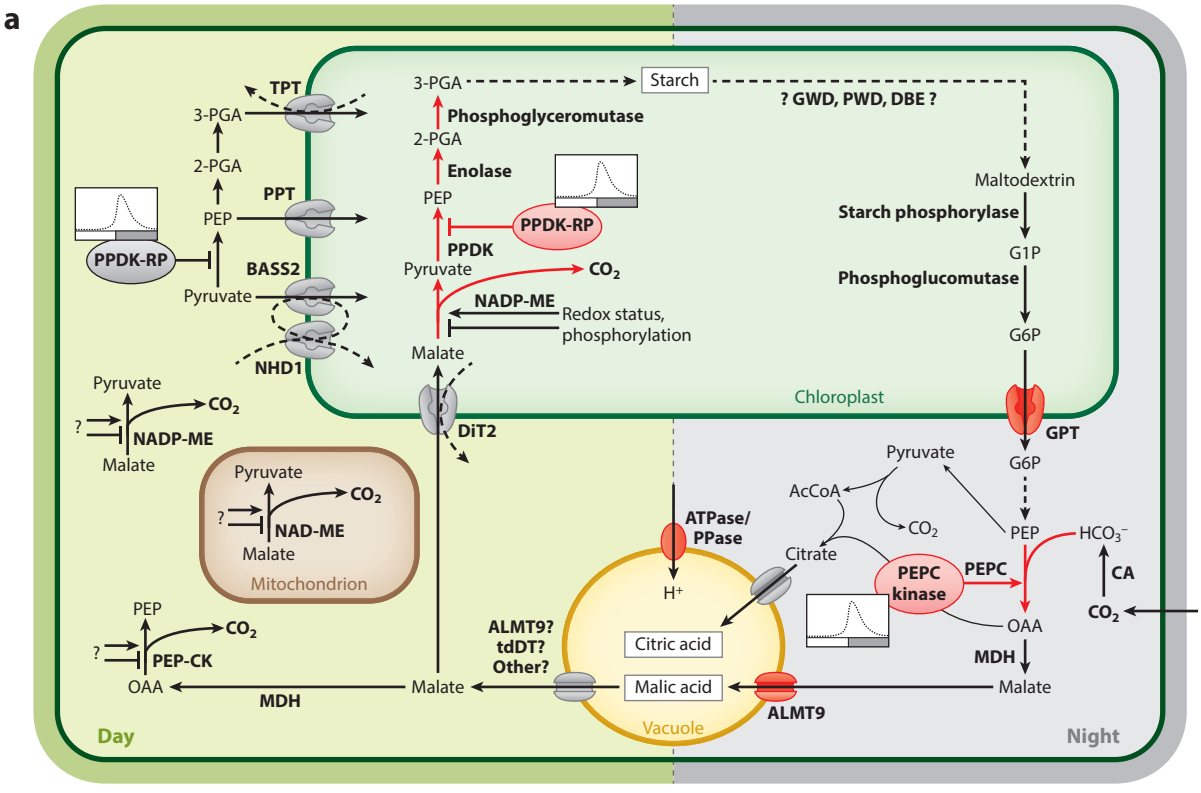
Crassulacean acid metabolism (CAM) is an add-on pathway to classic C_3 photosynthesis and primarily increases water use efficiency (176). It is an attractive engineering target in a world where the climate is shifting toward warmer temperatures and more weather extremes (15). Similar to C_4 photosynthesis, CAM holds promise for the study of complex trait evolution because it evolved independently in many branches of the plant tree of life. When the pathway is operating, plants fix CO_2 at night using phospho*enol*pyruvate carboxylase (PEPC), which leads to two signature characteristics: acidification of the plant tissue by L-malic acid storage for CO_2 (12) and net CO_2 uptake during the night (5). Compared with daytime photosynthesis, CO_2 fixation at night leads to water savings, as temperatures are cooler and transpirational water loss is reduced (71). All CAM species rely on CO_2 fixation at night by PEPC, malic acid storage in the vacuole, and CO_2 release during the day by a decarboxylation enzyme followed by refixation in the Calvin-Benson Bassham cycle (**Figure 1*a***). CAM photosynthesis is not a binary trait; it can be plastic with plant age (80) and external conditions such as water availability (24) and salinity (33, 179). Successful engineering of the trait will rely on integrating its subtraits into C_3 plant tissue and cell architecture, regulatory processes, and metabolic networks.

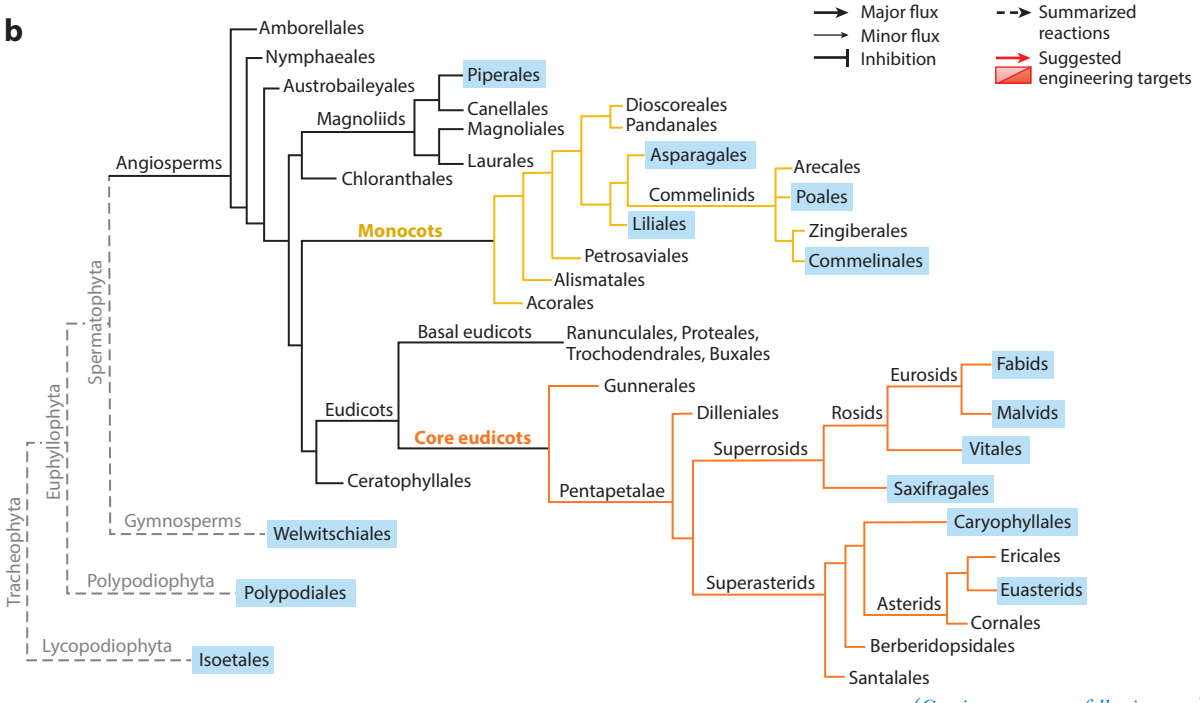
Strong CAM: carbon uptake occurs at night, dawn, and dusk; stomata are closed during the day, preventing water loss

$\delta^{13}C$: the carbon isotope discrimination value is an indicator of the photosynthetic mode

Weak CAM: pattern similar to that of strong CAM but with less nightly acidification and stronger contribution of daytime direct CO_2 fixation by Rubisco

In its classic form, which is presently called strong CAM, the trait enables plants to open their stomata for CO_2 fixation at night, when it is comparatively cool, and, if conditions are favorable, at dawn and dusk. These plants are characterized by strong shifts of $\delta^{13}C$ toward less negative values (145), as most CO_2 is fixed by PEPC, which discriminates less between CO_2 isotopes. As the carbon acceptor, a C_3 carbon compound, phospho*enol*pyruvate (PEP), is generated from carbon storage (e.g., starch or stored sugars) (7, 32, 82). The affixed CO_2 is stored as malic acid in the vacuole and released again internally during the day for fixation by Rubisco (**Figure 1*a***). During the day, sufficient carbohydrate reserves, from which the CO_2 acceptor can be generated for nightly CO_2 fixation, must be stored (**Figure 1*a***). This cycle manifests in diurnal changes in acidity and inverse stomatal opening, which were recognized as early as the nineteenth century (summarized in 128). The pathway itself was recognized in the 1940s (128). Although CAM is primarily a water-saving measure, strong CAM plants are capable of direct CO_2 fixation in the evening and in the morning, when water loss is lower than in midday (5). The carbon fixed during that time is directly fixed by Rubisco. In addition to the strong CAM photosynthetic type, there is weak CAM, which refers to less nightly acidification but similar patterns (145). Some plants





(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

(*a*) Metabolism and (*b*) the tree of life for CAM plants. (*a*) The CAM cycle during the day and night. Timing of expression for engineering is represented by small graph icons. (*b*) CAM photosynthesis evolved independently in many phylogenetic groups in plants, including monocots, dicots, gymnosperms, and non-seed plants. Groups that evolved CAM are indicated with blue boxes. Abbreviations: AcCoA, acetyl coenzyme A; ALMT9, aluminum-activated malate transporter 9; BASS2, bile acid sodium symporter 2; CA, carbonic anhydrase; CAM, crassulacean acid metabolism; DBE, debranching enzyme; DiT2, dicarboxylate translocator 2; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; GPT, glucose phosphate translocator; GWD, glucan, water dikinase; MDH, malate dehydrogenase; NAD(P)-ME, NAD(P)-dependent malic enzyme; NHD1, sodium hydrogen antiporter 1; OAA, oxaloacetate; PEP, phospho*enol*pyruvate; PEP-CK, PEP carboxykinase; PEPC, phospho*enol*pyruvate carboxylase; PGA, phosphoglycerate; PPDK, pyruvate phosphate dikinase; PPDK-RP, PPDK regulatory protein; PPT, phospho*enol*pyruvate phosphate translocator; PWD, phosphoglucan water dikinase; pyr, pyruvate; TPT, triosephosphate/phosphate translocator.

demonstrate CAM photosynthesis only if induced by adverse conditions such as drought (177) or salt stress (179), and this subtype is called inducible CAM. Other species use CAM fixation of CO_2 generated by respiration at night (143) but keep their stomata closed at all times as an extreme survival measure, which is called CAM idling.

CAM photosynthesis is present across the Tracheophyta in multiple independent origins and has probably evolved more often than C_4 photosynthesis (134) (**Figure 1*b***). Because CAM does not absolutely require specialized cell architecture, fossilized plants cannot be associated with the trait. The extant monocots, Bromeliaceae (Poales) and Orchidaceae, are CAM-species-rich families (35, 145). In addition, CAM evolved in the Agavaceae (see, e.g., 116, 122). Among the dicots, plants in the rosids, the asterids, and the Caryophyllales have evolved CAM (**Figure 1*b***). Examples in the rosids include a tree that performs CAM photosynthesis, *Clusia minor* (98), and species in the family Crassulaceae, for which the trait is named (128). CAM is also present in *Welwitschia mirabilis* (165), ferns in the Pteridophyta (181), and *Isoetes* in the lycophytes (16) (**Figure 1*b***). Two reference organisms frequently studied for inducible CAM are *Talinum triangulare*, a more recent target of research (24, 72), and the traditional model, *Mesembryanthemum crystallinum*, for which 361 manuscripts contain the species name in the title (ISI Web of Science, 2/2021). Model species with sequenced genomes are *Ananas comosus* (pineapple), for its commercial value (111); *Kalanchoë fedtschenkoi*, for its use by molecular tools (42, 183); and *Phalaenopsis equestris*, as a representative of the orchids and the first CAM plant sequenced (26). The widespread occurrence and the plasticity of the CAM trait suggest that there is no general barrier to trait evolution and therefore likely no general barriers to trait engineering. To assess engineering possibilities, we examine CAM traits from the point of view of C_3 plants.

Inducible CAM: plants perform C_3 photosynthesis until induced by, e.g., drought or salt stress; when induced, plants switch to CAM

CAM idling: extreme survival mode in which stomata are closed at all times; respired carbon is refixed without additional carbon uptake

Circadian rhythm: endogenous rhythm with a period of ~24 h that persists in the absence of light or temperature cues

2. SUBTRAITS OF CAM: CIRCADIAN AND DIURNAL RHYTHMS

One of the hallmark traits of (strong) CAM is an inversed CO_2 fixation pattern, with CO_2 fixation during the night, which continues if diurnal cues such as light are abolished (174). Free-running rhythms with a period of approximately 24 h are called circadian rhythms (107). The CO_2 fixation of CAM photosynthesis is apparently tied to a circadian clock (64, 174). A circadian clock allows organisms to anticipate daily changes. It consists of a central oscillator, input pathways that entrain the clock, and output pathways that transmit the clock status to other signaling pathways. The central oscillator in higher plants consists of the central genes, *TIMING OF CAB EXPRESSION 1* (*TOC1*), which peaks in the evening, and *CIRCADIAN CLOCK ASSOCIATED 1/LATE ELONGATED HYPOCOTYL 1* (*CCA1/LHY1*), which peaks in the morning (3, 64). Interlocked feedback loops keep the time. In *Arabidopsis thaliana*, several additional genes are required for proper time keeping. *PSEUDO-RESPONSE REGULATOR5* (*PRR5*), *PRR7*, and *PRR9*, which are similar to *TOC1* and the genes *GIGANTEA* (*GI*), *LUX ARRHYTHMO* (*LUX*),

EARLY FLOWERING 3 (*ELF3*), *ELF4*, and *ZEITLUPE* (*ZTL*), are central components of the clock (summarized in 107). Time is kept through transcriptional and posttranscriptional regulation. In natural conditions, light and temperature cues entrain the clock and maintain a precise 24-h rhythm (108). When plants are transferred to constant light or constant darkness with no temperature cues, the circadian clock maintains oscillatory behavior, although the amplitude of the output diminishes over time (see, e.g., 174). The clock is also influenced by signals derived internally from photosynthesis, notably the presence of sugar (52, 68). Under natural conditions, circadian and diurnal changes cannot be distinguished. In contrast to circadian changes, which continue in free-running conditions, diurnal changes are controlled by environmental changes during day and night cycles and cannot be detected in free-running conditions. The circadian rhythm itself accounts for at least 10% (135) and perhaps up to 35% (110) of gene expression changes during the day. In many plant species, a one-third to one-half of the genes display changes at the transcript level during a day-night cycle (14) and are thus either circadian or diurnal.

Rhythms in the proteome are attenuated compared with those in the transcriptome in *A. thaliana* (10). Moreover, studies of protein turnover in *A. thaliana* and *Hordeum vulgare* (barley) revealed that only a small proportion of the measurable proteome turned over with a dissociation constant (K_d) greater than 1, namely the proteins D1 (core of photosystem II) and THIAZOLE REQUIRING 1 (THI1), which is involved in thiamine metabolism (93, 117). Of those proteins that are abundant enough to measure via mass spectrometry, such as proteins involved in metabolism, virtually none turns over fast enough to be regulated by protein synthesis and degradation over short times (93, 117). Because proteomic measurements require a certain protein level to detect and quantify a protein, low-abundance proteins, such those with regulatory roles, which are barely detectable by mass spectrometry, may turn over much faster. This may explain why proteomics approaches detect a fairly stable proteome (10, 93, 117), although phenotypic analyses have demonstrated clear phenotypes of circadian aberrations. If the internal period set by the clock does not match the external period set by the growth chambers, plants suffer a fitness penalty (45). Precise anticipation of dusk and dawn enhances growth and biomass production. In addition to its contribution to overall fitness, the circadian clock also gates plant responses to stresses. In other words, plants are capable of an adequate response only at a certain time of day. In the model C_3 plant *A. thaliana*, these gated responses include responses to biotic stressors such as bacteria (13) and herbivores (55) and responses to abiotic stressors such as cold (50) and heat (60). The gated responses make intuitive sense; for example, bacteria need open stomata and hence can attack only during the day, as heat stress is most severe during the day, so one intuits an advantage if their occurrence can be anticipated.

The strong, altered rhythmicity of CO_2 fixation has prompted many investigations into diurnal transcriptional rhythms in CAM plants, such as *Ananas comosus* and *Agave deserti* (agave) (2, 111). Robust rhythmic expression of central oscillator genes in *M. crystallinum* was also shown (18). Rhythmic transcript abundance for the CAM cycle genes could be detected in some genes in pineapple and agave, but the phase only occasionally matched peak enzyme activity. Proteomic analyses of *Agave americana* showed that PEPC protein abundance peaked with maximal activity, but the protein was present throughout the day and night (2). A proteomics analysis of *K. fedtschenkoi* mesophyll cells also revealed that rhythmic abundance changes for all proteins involved in CAM photosynthesis did not match their peak activity (1), but as in *A. americana*, PEPC also peaked at night and was present throughout the day and night (1). Continued presence may be due to slow turnover of most metabolic proteins (93, 117). Hence, control of CAM photosynthesis and CO_2 fixation is likely only partially managed through circadian or diurnal abundance changes of CAM transcripts and proteins but critically influenced by regulatory proteins. Rhythmicity of CO_2 fixation in CAM is likely mediated by diurnal expression of PEPC (19) and of a labile kinase

that posttranslationally modifies PEPC and therefore changes its activity (27). Kinase activity appears at the beginning of the night and coincides with insensitivity of its target enzyme, PEPC, to malate and hence activation (27, 65). The corresponding gene was cloned and demonstrated the expected high expression during the night (65, 154). Kinase-based regulation of PEPC activity is not specific to CAM photosynthetic plants. In the C_3 plant *Flaveria pringlei*, phosphorylation of PEPC was fairly even during the day and night, whereas in the C_4 plant *Flaveria trinervia*, phosphorylation peaked during the day, when PEPC needs to be active in the C_4 cycle (4). The circadian output observed in CAM plants, rhythmic CO_2 fixation in constant darkness, is likely a clock output mediated by the PEPC kinase. In CAM plants, evolution may have optimized additional clock outputs. Whereas cold and heat stress patterns remain the same for CAM and C_3 plants, stomatal opening is inverted to a nightly opening; hence, the abundance of bacterial defense genes may have shifted to accommodate the new timing of the threat. The importance of shifting defense gene expression could be analyzed in published diurnal CAM transcriptomes and proteomes (1, 2, 111) to assess the amount of changes required. For initial engineering of a CAM pathway, the circadian clock may remain untouched while some outputs (e.g., PEPC and PEPC kinase expression) need to be connected.

3. SUBTRAITS OF CAM: STOMATAL REGULATION

The inversed CO_2 fixation pattern in strong CAM plants is accompanied by an inversed stomatal opening pattern. In C_3 plants, stomata typically open in the morning and close in the evening, whereas in strong CAM plants, they open at night and, conditions permitting, at dawn and dusk (**Figure 2*a***). For a detailed description of stomatal function at the physiological and molecular levels, please see reviews on the physiology of stomata (92) and the molecular function of stomata (83). Briefly, at the molecular level, stomata are hydraulically driven and opening is counterintuitively mediated by swelling (summarized in 83). The guard cells swell when H^+ ATPase is activated, causing K^+ uptake through inward-rectifying channels followed by anions (8, 90) (**Figure 2*b***). Sucrose and malate fluxes also positively influence stomatal opening. Stomatal closing requires the S-type anion channel SLAC1 (162). Stomatal aperture is modulated by light, CO_2, and abscisic acid (ABA) signals, which are integrated to decide whether stomata will open or close (138) (**Figure 2*b***). The blue light receptor phototropin mediates the blue light response, which initiates stomatal opening (84). Downstream of this initial response is a phosphorylation event of H^+ ATPase, which activates the ATPase (85). Ca^{2+} may be involved in modulating this response (summarized in 132). Photosynthetically active radiation also mediates stomatal opening likely via intercellular CO_2 concentration (131). CO_2 sensing requires carbonic anhydrases, because a double mutant of carbonic anhydrases in *A. thaliana* abolishes CO_2-dependent stomatal responses independent of cytosolic pH changes (75). Expression of a mammalian carbonic anhydrase in guard cells restores the response, indicating that the guard cells themselves are the site of signal recognition (75). The CO_2 signal is transmitted by the protein kinase HT1 (66), and changes in Ca^{2+} concentrations and the signal ultimately act on the anion channel SLAC1 (138) (**Figure 2*b***). The ABA signal is generated in the plant and transmitted to the guard cells, where it is sensed by the ABA receptor family PYL/PYR/RCAR (101, 123). Via PP2C and the kinases OST1 and SnRK2, the signal is transferred directly to SLAC1 and to the internal Ca^{2+} pool (56, 113, 114, 124). ABA also inhibits H^+ ATPase (142). Integration of the ABA, CO_2, and light signals thus converges on SLAC1 on the one hand and on the internal Ca^{2+} pools on the other hand. In nondrought conditions in C_3 plants, the combined action of blue light and lowered internal CO_2 leads to stomatal opening in the morning and stomatal closing at night. When the water status is limiting, ABA signaling interferes with the stomatal opening signals and induces stomatal closing to prevent water loss.

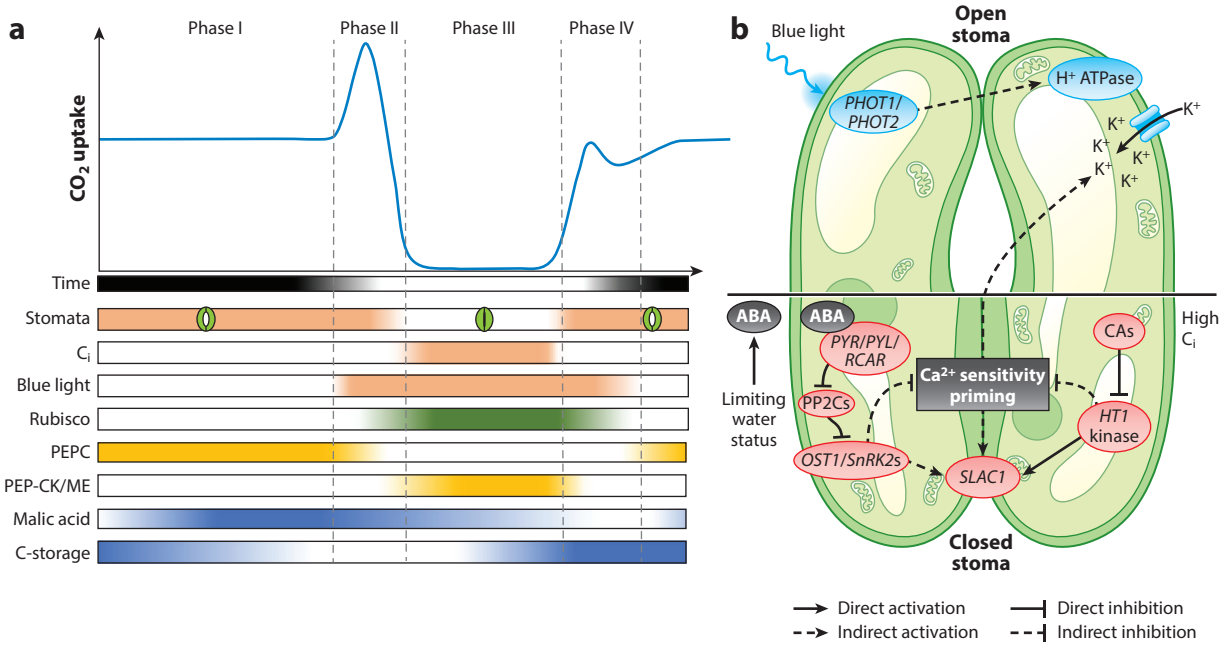


Figure 2

(*a*) The opening and closing signals for stomata change throughout the day. In phase I, PEPC activity lowers C_i concentrations and enables CO_2 uptake. In phase II, dawn light allows a brief burst of C_3 photosynthesis before the onset of malate decarboxylation, rise of C_i, and stomatal closing in phase III. Depleted malate storage reduces C_i at dusk and enables another burst of C_3 photosynthesis in phase IV. (*b*) The molecular players involved in stomatal signaling are the blue light receptors *PHOT1/PHOT2*, ABA, the ABA receptor *PYR/PYL/RCAR*, PP2Cs, the kinase *OST1/SnRK2*, the S-type anion channel *SLAC1*, the kinase *HT1*, and CAs. Panel *b* adapted from an image created with BioRender.com. Abbreviations: ABA, abscisic acid; CA, carbonic anhydrase; C_i, internal CO_2; *HT1*, *HIGH TEMPERATURE 1*; ME, malic enzyme; PEPC, phospho*enol*pyruvate carboxylase; *OST1*, *OPEN STOMATA 1*; PEP-CK, PEP carboxykinase; *PHOT1/PHOT2*, *PHOTOTROPIN 1* and *2*; PP2C, *HIGHLY ABA-INDUCED PP2C GENE 2*; *SLAC1*, *SLOW ANION CHANNEL-ASSOCIATED 1*; *SnRK2*, SNF1-related protein kinase 2 family kinase.

The key question for engineering a basic CAM pathway is whether stomatal signaling is rewired in CAM plants. There are indications that signal transduction toward stomatal opening and closing is modulated (79, 152), but the basic principles known from C_3 plants are sufficient to explain the stomatal opening patterns observed (**Figure 2*a***). In C_3 plants, intercellular CO_2 concentrations are comparatively high at night because no CO_2 fixation is taking place and plants respire. High CO_2 concentrations induce stomatal closure; absence of blue light means no opening signal. In CAM plants, PEPC actively sequesters at night CO_2 into oxaloacetate, which is reduced to malate, and intercellular CO_2 is lowered. Hence, there is no closing signal for the stomata and they can remain open during the night. In the morning, conflicting signals occur: Blue light sends an opening signal (but see 152), whereas beginning decarboxylation increases the CO_2 concentration and hence signals for stomatal closing (**Figure 2*a***). If plants are water limited, ABA will also signal for stomatal closing. If the CO_2 or ABA signals are stronger than the light signaling, the integrated response of a CAM plant will be stomatal closing during the daytime. Therefore, the most parsimonious explanation for the apparent inverse regulation of stomatal closing during the day and stomatal opening during the night is CO_2 signaling (**Figure 2*a***). If no changes are assumed, the four phasic CO_2 assimilation curves of CAM plants can be explained with the existing regulatory infrastructure (**Figure 2*a***). At night, during phase I, internal CO_2 is low; hence,

stomata are open and CO_2 is fixed. In the morning, during phase II, blue light sends a stomatal opening signal and C_3 photosynthesis commences. Once decarboxylation activity starts and internal CO_2 rises, a stomatal closing signal is sent that may be exacerbated by water limitation signals based on high temperatures during the day (phase III). In the evening, organic acid storage is exhausted and no more internal CO_2 can be produced. If not precluded by a stomatal closing signal through water status [ABA and vapor pressure deficit (VPD)], stomata can reopen for another phase of C_3 photosynthesis before night commences. This model posits that the signals generated by the unique biochemistry of CAM (i.e., CO_2 concentration changes mediated by PEPC and the decarboxylation of organic acids) cause the unique stomatal opening pattern (164, 182). A PEPC mutant that (*a*) fails to lower CO_2 at night and (*b*) precludes organic acid accumulation and thus internal release of CO_2 during the day should fail to display CAM stomatal opening and closing patterns. The PEPC mutant of *K. fedtschenkoi* confirms said model predictions, because it reverts to C_3 photosynthesis with classical gas-exchange timing (19). Engineering may not require changes to stomatal regulation for initial proof-of-concept experiments. However, it is likely that stomatal opening and closing will ultimately require fine-tuning to balance the CAM cycle and C_3 photosynthesis in phase II and phase IV with the environment.

4. SUBTRAITS OF CAM: BIOCHEMISTRY

Changes to leaf biochemistry are at the heart of CAM and are present to some degree in all species that are defined as CAM plants. When the CAM cycle is operating, it needs to be tightly controlled to avoid a futile cycle of malate production and decarboxylation. Regulation is particularly critical for the enzymes that catalyze exergonic and therefore virtually irreversible reactions. Two stored metabolites, malic acid for nightly storage of CO_2 and carbohydrates for daily storage of the nighttime CO_2 acceptor, are the cornerstones of CAM photosynthesis (**Figure 1*a***; **Table 1**). An isocitrate-based cycle may contribute to carbon storage (159).

4.1. Carbon Storage and Breakdown

The stored carbohydrate can be starch in the chloroplasts (32), sugars such as glucose and fructose in the vacuole (7, 32, 82), or galactomannan (32). The storage metabolite for carbohydrates varies even between closely related CAM species and is not correlated with the decarboxylation enzyme in the CAM cycle (32). In C_3 plants, starch accumulates during the day to support nighttime supply of carbon to the sink tissues and to supply cellular metabolism at night. All enzymes required for starch synthesis and turnover in CAM plants are present in the ancestral C_3 state. In the chloroplasts, starch is synthesized from triosephosphates, which themselves are produced in the Calvin-Benson Bassham cycle. Triosephosphate is condensed to fructose-1,6-bisphosphate, dephosphorylated to fructose-6-phosphate, and converted to glucose-6-phosphate and to glucose-1-phosphate, from which ADP-glucose is formed (125). Starch synthesis proceeds via soluble and granule-bound starch synthase and starch structure is remodeled with branching and debranching enzymes (125). Formation of ADP-glucose produces pyrophosphate, which is split by pyrophosphatase, making the reaction essentially irreversible (61). The reaction is thought to be the committed and regulatory step of starch synthesis in C_3 plants. ADP-glucose pyrophosphorylase (AGPase) is allosterically activated by 3-phosphoglycerate, is inhibited by inorganic phosphate (146), and is sensitive to redox regulation (158). At the end of the day, 15% of chloroplast volume is occupied by starch (36), based on 30–50% of carbon that was routed into this storage metabolite (149). C_3 plants can route nearly all fixed carbon through starch, as shown in a mutant defective in triosephosphate/phosphate translocator (TPT), and can use multiple degradation

Table 1 List of *Arabidopsis* homologs to CAM transcripts

Protein	*Arabidopsis* gene identifier	Block description	Enzyme Commission no.
Carbon storage and breakdown			
SS	AT1G32900 AT1G11720 AT3G01180 AT4G18240 AT5G24300	(Granule-bound) starch synthase	(2.4.1.242) 2.4.1.21
SBE	AT5G03650 AT2G36390	Starch branching enzyme	2.4.1.18
AGPase	AT2G21590 AT1G27680 AT5G19220 AT5G48300 AT1G05610	Glucose-1-phosphate adenylyl transferase/ ADP glucose pyrophosphorylase	2.7.7.27
TPT	AT5G46110	Triosephosphate/phosphate translocator	NA
GWD	AT1G10760 AT4G24450 AT5G26570	Glucan, water dikinase	2.7.9.4
BAM	AT3G23920 AT4G17090	β-Amylase	3.2.1.2
SEX4/LSF2	AT1G10760 AT3G52180	Phosphoglucan phosphatase	2.7.9.4
DPE	AT2G40840 AT5G64860	Disproportionating enzyme	2.4.1.25
PHS	AT3G29320 AT3G46970	Starch phosphorylase	2.4.1.1
AMY	AT1G69830	α-Amylase	3.2.1.1
GPT	AT5G54800 AT1G61800	Glucose-6-phosphate/phosphate translocator	NA
LSF1	AT3G01510	Potential regulator	NA
BAM4	AT5g55700	Potential regulator	NA
Carboxylation by PEP carboxylase			
PEPC	AT1G53310 AT1G68750 AT2G42600 AT3G14940	Phospho*enol*pyruvate carboxylase	4.1.1.31
PPCK	AT1G08650	PEPC kinase	2.7.11.1
CA	AT1G23730 AT1G58180 AT1G70410 AT3G01500 AT4G33580 AT5G14740	Cytosolic carbonic anhydrase	4.2.1.1
Vacuolar storage			
V-ATPase	Multiple subunit system	Vacuolar H+-ATPase	NA
PPase	AT1G15690	Vacuolar H^+-pyrophosphatase	7.1.3.1
tDT	AT5G47560	Tonoplast dicarboxylate transporter	NA
ALMT9	AT3G18440	Aluminum-activated malate transporter 9	NA

(*Continued*)

Table 1 (*Continued*)

Protein	*Arabidopsis* gene identifier	Block description	Enzyme Commission no.
Malate release and decarboxylation			
NAD-ME	AT4G00570 AT2G13560	NAD-dependent malic enzyme	1.1.1.39
NADP-ME	AT1G79750	NADP-dependent malic enzyme (chloroplastidic)	1.1.1.40
	AT2G19900 AT5G11670 AT5G25880	NADP-dependent malic enzyme (cytoplasmic)	
PEP-CK	AT4G37870 AT5G65690	Phospho*enol*pyruvate carboxykinase	4.1.1.49
DIC	AT2G22500 AT2G22500 AT4G24570 AT5G09470	Dicarboxylate carrier	NA
DiT2	AT5G64290 AT5G64280	Glutamate/malate translocator, the putative malate importer (for dicarboxylate translocator 2)	NA
Gluconeogenesis			
PPDK	AT4G15530	Pyruvate orthophosphate dikinase, dual targeted	2.7.9.1
PPDK-RP	AT4G21210	PPDK regulatory protein (chloroplastidic)	2.7.11.32
	AT3G01200	PPDK regulatory protein (cytoplasmic)	
ENO	AT2G36530	Enolase (plastidic)	4.2.1.11
	AT2G29560	Enolase (cytosolic)	
PGlyM	AT1G09780 AT3G08590	Phosphoglyceromutase	5.4.2.2
PGK	AT1G79550 AT1G79550	Phosphoglycerate kinase	2.7.2.3
PPT	AT5G33320 AT3G01550	PEP/phosphate translocator	NA
BASS2	AT2G26900	Pyruvate transporter (for BILE-ACID SODIUM SYMPORTER 2)	NA

Abbreviation: NA, not applicable.

routes if necessary (137). Presently, minor changes in transcript abundances for starch synthesis in CAM plants have been reported (24), although starch synthesis during the day needs to support not only nightly export and cellular metabolism but also nightly carbon fixation. Starch synthesis is required for CAM photosynthesis (38). In C_3 plants, starch storage and starch export are approximately equal (149). In CAM plants, some nightly storage carbohydrate feeds the sink tissues of the plant at night. In addition, each CO_2 molecule fixed during the day by Rubisco requires three carbon equivalents in starch. CAM starch synthesis needs to exceed C_3 starch synthesis by a factor of 7 if the same overall photosynthetic rates are to be achieved. Given that the chloroplasts are of limited volume, very high storage capacity for malic acid must be accompanied by sufficient storage capacity for carbohydrates.

The transitory starch stored during the day in C_3 plants is degraded at night. In C_3 plants, the degradation rate is tuned to allow starch availability until the new day dawns and requires

an intact circadian clock (57). Two pathways are presently known: phosphorolytic degradation, which preserves the energy of the glucose-glucose bonds, and hydrolytic degradation (171). Hydrolytic starch degradation produces maltose and glucose. Initially, glucan, water dikinase (GWD) sparsely introduces phosphate esters (130). β-amylases and debranching enzymes produce smaller moieties (136), which are dephosphorylated by a starch phosphatase (88) (**Table 1**). Disproportioning enzyme is required to process maltotriose. The model plant *A. thaliana* virtually exclusively relies on hydrolytic starch breakdown, with phosphorolytic breakdown contributing only a small share (150). Phosphorolytic starch breakdown requires starch phosphorylase (185) potentially supported by α-amylase (150) and disproportioning enzyme. ABA induces α-amylase in the model plant *A. thaliana* (157) and starch phosphorylase displays an abiotic-stress-induced phenotype (185), linking phosphorolytic starch breakdown to stress responses in C_3 plants. Photorespiration induces intermediates of phosphorolytic starch breakdown (170). In contrast to that in C_3 plants, the major starch breakdown route in CAM plants is phosphorolytic (**Figure 1*a***). *M. crystallinum* switches the breakdown mechanism from hydrolytic to phosphorolytic when switching from C_3 to CAM mode (39, 120). The export products of chloroplasts change from mostly maltose and glucose to mostly glucose-6-phosphate. The transporter required for export of hexose phosphates, glucose phosphate translocator (GPT), increases in expression as does starch phosphatase (39). *T. triangulare* also increases transcript abundance of GPT and α-amylase when CAM is induced (24). The phosphorolytic breakdown products are converted to PEP for carbon fixation at night by glycolysis. Because glycolytic flux is controlled at least in part by PEP (127), PEP consumption by PEPC may increase glycolytic flux. It is assumed that all CAM plants use phosphorolytic starch breakdown with a small, if any, contribution from hydrolytic breakdown (73). Some CAM species store osmotically active sugars, in addition to starch (7, 32), which are exported from the vacuole before conversion to PEP. Several monosaccharide and disaccharide transporters in C_3 plants (summarized in 70) may be used in CAM plants. The breakdown products of starch (or the stored sugars) are processed glycolytically to PEP in an ATP-neutral pathway (if from glucose) or in an ATP-producing pathway (if from glucose-6-phosphate) in the cytosol. For engineering CAM, it will be critical to understand how starch degradation is steered toward hydrolytic or phosphorolytic breakdown to be able to manipulate the direction.

4.2. Carboxylation by Phospho*enol*pyruvate Carboxylase

PEP, the nighttime carbon acceptor, is carboxylated by PEPC using HCO_3^- in a highly exergonic and therefore irreversible reaction. PEPC is a well-studied enzyme (78). In *A. thaliana*, PEPC is critical for efficient ammonia assimilation and organic acid production (140) and is regulated via phosphorylation (58). In C_4 plants, the C_4 PEPC has a hallmark serine at position 780 (31) (numbering based on *Zea mays* PEPC), which reduces sensitivity to malate and is thought to increase PEPC activity in C_4 plants. Notably, this residue is the alanine typical for C_3 PEPCs in CAM PEPCs. Because its reaction is irreversible, PEPC needs to be a highly regulated enzyme. Its transcript and protein are diel, but because there is always protein present, its activity is regulated by phosphorylation, which ensures it is active only during the night (27, 65, 154). The oxaloacetate produced by PEPC is reduced to malate. Because glycolysis toward PEP produces reducing equivalents in the glyceraldehyde 3-phosphate dehydrogenase (GAP-DH) step, the reduction is quantitatively covered by glycolysis. Malic acid now needs to be stored for daytime decarboxylation and refixation of the CO_2 by Rubisco.

4.3. Vacuolar Storage

In C_3 plants, the storage vacuoles occupy 70–80% of the cell volume (175) and are involved in nitrogen storage, salinity tolerance, heavy metal homeostasis, calcium signaling, guard cell

movements, and cellular pH homeostasis (106). They are a slightly acidic compartment (106). Vacuoles in plant cells play numerous roles: They are required to form large cells with optimal distribution of chloroplasts, they may temporarily store nutrients and maintain homeostatic conditions in the cytosol, and they may accumulate toxic material (i.e., as a herbivory deterrent) (106). Their membrane, the tonoplast, is energized by proton-pumping ATPase and PPase (H^+-pyrophosphatase) (69), which create an electrochemical gradient across the vacuolar membrane (106). The V-ATPase can be controlled at the transcriptional level and also posttranscriptionally via redox status (153) and phosphorylation (74). The proton gradient and the electrical gradient energize transport processes. Because the cost of the electrochemical gradient varies with the acidity of the vacuole, the precise cost for a transport process cannot be calculated. In yeast, the tonoplast membrane disintegrates at pH 4 or lower (141), which shows that the tonoplast limits acidification. Five classes of sugar transporters enable sugar storage and release in C_3 vacuoles (70). Malate can be transported via the tDT (tonoplast dicarboxylate transporter) antiporter (48) or malic acid can be transported via the ALMT9 (aluminum-activated malate transporter 9) channel (89) across the tonoplast; citrate is also taken up into the vacuole (129). ALMT9 is required for stomatal opening (40) in C_3 plants and it may be regulated by cytosolic nucleotides (187). For *A. thaliana*, phosphorylation has been reported in two independent experiments (30, 133) as visualized by Cruz et al. (37); hence, there is potential for regulation by posttranslational modification. C_3 vacuoles can also take up and store metal cations, phosphate and sulfate, amino acids, and xenobiotics (106).

In CAM plants, vacuoles occupy up to 95% of the cellular volume (147). Malic acid is taken up into the vacuole following the proton gradient established by ATPase or PPase (summarized in 106). The gradient is dependent on the availability of ATP or pyrophosphate in the cytosol. In *M. crystallinum*, induction of CAM is accompanied by induction of V-ATPase but not PPase activity (23). In *Kalanchoë daigremontiana*, PPase activity at the tonoplast is high (105). Thus, in principle, both enzymes are suitable to acidify the vacuolar lumen. Phosphorolytic degradation of starch provides ATP, which can be used to energize the transport into the storage compartment (139). The total storage capacity for malic acid is limited by the pH gradient across the vacuolar membrane, the buffering capacity of the vacuole, and the vacuole size. Unlike yeast vacuoles, CAM vacuoles tolerate pH as low as 2.9 (51). The buffering capacity of the vacuole depends on the presence of metabolites (i.e., citrate) with a high buffering capacity (98, 112) and potentially proteins with buffering capacity. Citrate storage itself is CO_2 neutral (**Figure 1*a***); in other words, during the night, no external CO_2 is stored in citrate (97). Many strong CAM species with large vacuoles do not co-accumulate citrate. No nightly storage metabolite besides malic acid is presently known, which means malic acid cycling predicts photosynthetic capacity in CAM species. Electrophysiological measurement in *K. daigremontiana* identifies a strongly rectifying malic acid–selective anion channel in the tonoplast of CAM plants (62). ALMT9 is the molecular candidate for the vacuolar malic acid uptake channel (89), because it increases in expression during the transition from C_3 to CAM (24) (**Figure 1*a***). Channels can operate at or near the speed of diffusion, and small changes in abundance lead to large changes in absolute carrying capacity and only small changes in transcript and protein abundance are expected. The actual malic acid flux through the channel will likely depend on the electrochemical gradient across the tonoplast established by the vacuolar ATPase or PPase (23, 105). A genetic approach is probably required to ultimately define the CAM tonoplast malic transporter. The malic acid stored during the night requires export from the vacuole during the day. The strongly rectifying channel that imports malic acid (62) is a poor candidate for export unless one assumes a reverse electrochemical gradient. Alternatively, posttranslational modifications may alter its transport characteristics. The malic acid exporter from the vacuole is thus presently unknown.

4.4. Malic Acid Release and Malate Decarboxylation

In plants, malate can be decarboxylated directly via a malic enzyme in the chloroplast [NADP-dependent malic enzyme (NADP-ME)], in the cytosol (NADP-ME) (173), or in the mitochondria [NAD-dependent malic enzyme (NAD-ME)] (160) or via oxaloacetate by cytosolic PEP carboxykinase (PEP-CK) (47) (**Figure 1*a***). For two enzymes, malate needs to be imported into the organelles. The malate importer for chloroplasts remains unknown; it may be dicarboxylate translocator 2 (DiT2), also known as dicarboxylate transport (DCT) in monocots (172). The malate importer for mitochondria is likely DIC, a mitochondrial dicarboxylate carrier family protein that may import malate into the mitochondria in exchange for phosphate or sulfate (126). Once imported, malate is decarboxylated to CO_2 and pyruvate. In *A. thaliana*, a NAD-ME knockout displays changes in steady-state monosaccharide and disaccharide levels at the end of the day and changes in steady-state levels of amino acids, especially those derived from the tricarboxylic acid (TCA) cycle and some TCA cycle intermediates, at the end of the night (160), whereas NADP-ME is hypothesized to play specialized roles and the mutants show no observable phenotype under standard greenhouse conditions (173). PEP-CK mutants are affected in seed germination because gluconeogenesis is impaired (47). In the CAM plant *K. fedtschenkoi*, downregulation of NAD-ME via RNA interference abolishes nighttime CO_2 fixation, indicating that *K. fedtschenkoi* uses mainly NAD-ME for decarboxylation (42, 43). CAM plants also use NADP-ME and PEP-CK for decarboxylation (32, 44), with mixed types using both NADP-ME and NAD-ME (32).

Malate and oxaloacetate decarboxylation require tight regulation to circumvent a futile cycle between carboxylation and decarboxylation. During the night, when PEPC synthesizes oxaloacetate, which is then converted to malate, no decarboxylation may take place. PEP-CK activity is modulated by phosphorylation (167, 168) and phosphorylated at night, which makes the enzyme less active (166, 168). Engineering of CAM using PEP-CK as the decarboxylation enzyme likely requires engineering this regulatory subsystem as well. The plastidic NADP-ME of C_4 plants is redox regulated and is active in its reduced form (6, 46). The cysteine residues implicated in redox regulation (6) are conserved in plastidic NADP-MEs. Activation of NADP-ME is thus tied to a reduced plastid stroma and linked to light intensity by photosynthetic reduction of the plastid stroma. For cytosolic NADP-ME and mitochondrial NAD-ME, many posttranslational modifications in *A. thaliana* have been identified but not yet functionally validated. Both NAD-ME isoforms and the cytosolic NADP-ME2 and NADP-ME3 isoforms are acetylated (95, as visualized in 37). For the major cytosolic isoforms NADP-ME1 and NADP-ME2, phosphorylation and nitrosylation sites in *A. thaliana* have been detected (76, 95, 115, 161, 186, as visualized in 37). Although enzymatic control via acetylation in plants has not yet been demonstrated, PEP-CK in mice toggles between carboxylation and decarboxylation based on acetylation (91), demonstrating the regulatory potential of acetylation. All malic enzymes show potential for posttranslational modification, which may control their activity.

4.5. Gluconeogenesis

The carbon contained in PEP and pyruvate is returned to storage as sugar or starch to be available for carboxylation the following night. In *A. thaliana*, gluconeogenesis from pyruvate is required during seedling establishment and uses pyruvate phosphate dikinase (PPDK) (47). The enzyme produces PEP from pyruvate, ultimately using two ATP molecules for each reaction. The conversion of PEP to triosephosphates via enolase, phosphoglycerate mutase, and phosphoglycerate kinase is followed by reduction via GAP-DH. In typical C_3 chloroplasts, enolase or phosphoglycerate mutase is absent (11, 148). Either the cytosolic enzymes substitute, which necessitates additional transport processes [i.e., through PEP/phosphate translocator (PPT)] (87, 163), or the

plastidic isoforms are expressed in CAM leaves. Once triosephosphates are produced, they can enter starch synthesis or be exported to the sink tissues via sucrose. PPDK is made an irreversible enzyme by the presence of pyrophosphatase, which immediately removes the pyrophosphate generated by its reaction and hence needs to be controlled. A dual-function kinase/phosphatase PPDK regulatory protein (PPDK-RP) regulates PPDK activity (9, 25), with the phosphorylated form being inactive in the dark. PPDK is required for efficient CAM photosynthesis (42). There is indirect evidence for its control via phosphorylation in CAM plants (42).

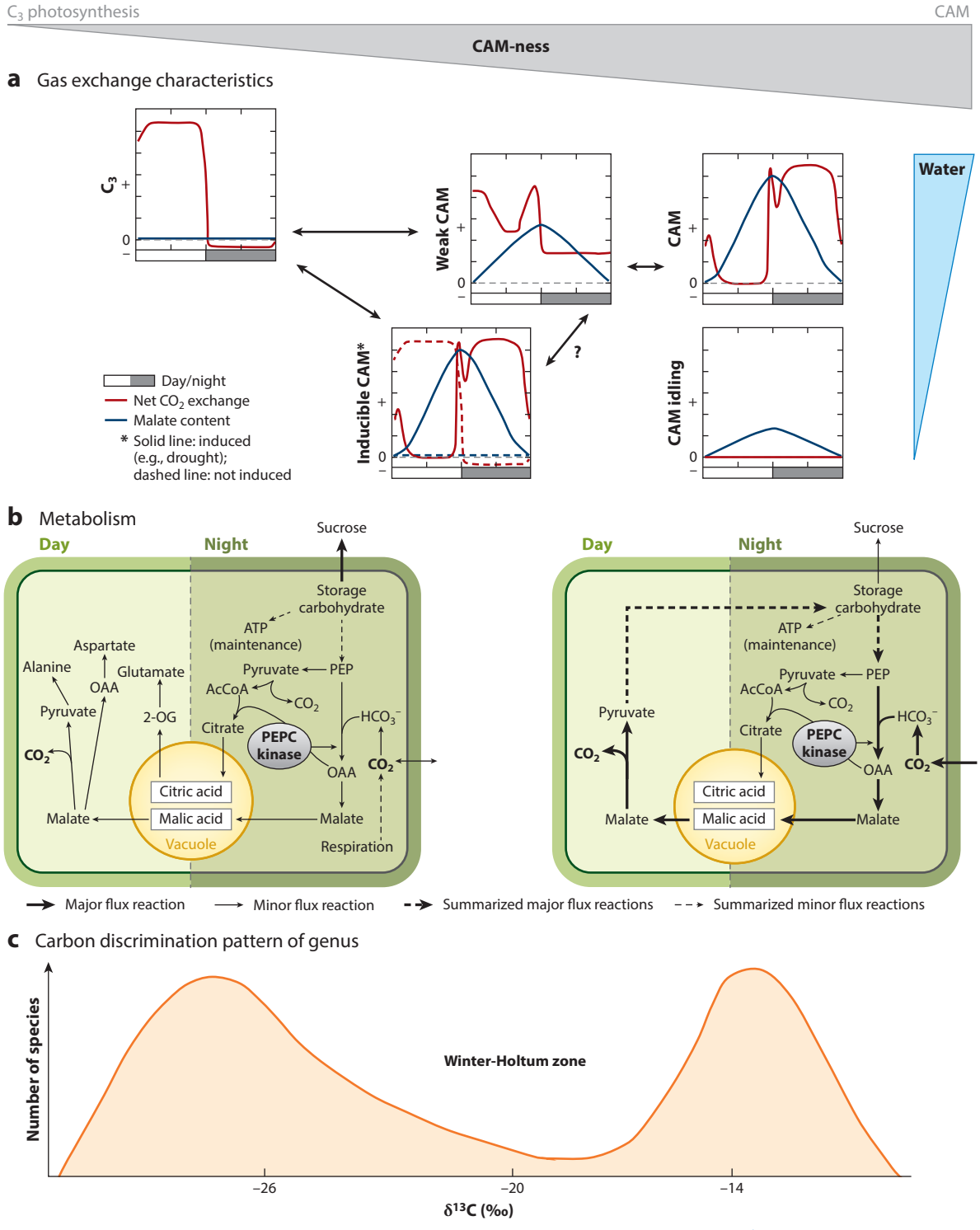
CAM cycling: daytime carbon uptake comparable to C_3; respired carbon is refixed at night without additional nighttime carbon uptake

5. EVOLUTION AND CONSTRAINTS OF THE CAM PATHWAY

Mutations in the C_4 pathway are lethal unless plants are grown in high CO_2 (41). In contrast, knockdowns in the CAM pathway in obligate CAM plants are well tolerated (17, 19, 42). Evolution of C_4 photosynthesis appears to be a one-way street (20), whereas CAM photosynthetic plants likely have reverted back to C_3 photosynthesis over evolutionary time given a suitable set of selective pressures (144). C_4 photosynthesis is not environmentally plastic—plants are C_4 or they are not. CAM photosynthesis can be induced in some species (24, 39, 102, 179, 184). All available evidence points to CAM photosynthesis being a true add-on to C_3 metabolism that leaves the underlying C_3 photosynthetic pathways intact. In contrast, C_4 photosynthesis requires the loss of Rubisco in one cell type, and subsequently, high expression of photorespiratory genes is lost, making it a one-way street (20). Unlike C_4 photosynthesis, CAM photosynthesis appears as a continuum from C_3 photosynthesis to CAM (**Figure 3**). If CAM does not require changes to the underlying C_3 photosynthesis, as evidenced by phases II and IV in CAM plants and the phenotype of *Kalanchoë* mutants, selective pressures toward and away from CAM may slide species along the scale during evolutionary time (144) (**Figure 3*a***).

At the C_3 end, the CAM continuum starts with nightly storage of malate or organic acids (22), continues over CAM cycling and weak CAM, and reaches strong CAM photosynthesis at the CAM end (**Figure 3*a***). Drought- and salt-inducible CAM (24, 179) may be an intermediate stage or an alternative stage (**Figure 3*a***). The evolution of CAM does not require de novo enzyme or transporter evolution, because all required enzymes and transporters are present in C_3 plants, albeit used in different pathways (22, 28) (**Table 1**). For selection to act on a pathway and increase flux through the pathway, this pathway needs to exist with at least minimal flux. There is indirect evidence for a CAM-like pathway in C_3 plants (22) (**Figure 3*b***). In some species, amino acids derived from C_4 and C_6 acids are synthesized from carbon fixed nightly by PEPC (54, 151, 155, 156). This pathway represents a minimal flux pathway for storage of organic acids or malate, and selection could have acted on it toward CAM (**Figure 3*b***). Similarly, there is low expression of decarboxylation enzymes also in leaves in C_3 plants (from 86) and there is evidence of a pyruvate pool derived from malate decarboxylation in the C_3 plant *A. thaliana* (151). Evolution of CAM requires flux changes but not de novo gene evolution (**Figure 3*b***). Modeling suggests the CAM pathway confers a small advantage in a C_3 leaf but requires succulence to decouple water savings from reductions in productivity (159).

To understand the limitations of nonsucculent architecture and its potential for CAM engineering, we calculated theoretical photosynthetic rates in C_3 architecture. For comparison, a typical crop C_3 plant achieves photosynthetic rates of 20 μmol CO_2 m^{-2} s^{-1}. Different CAM plants can store different amounts of malic acid, where each malic acid molecule essentially represents one CO_2 molecule available for carbon fixation. For example, *Agave deserti* stores malic acid at a concentration of 217 mM (169), *Cereus validus* at 88 mM (99), and *K. daigremontiana* at 146 mM (100). If barley was made a CAM plant and stored malic acid at a concentration similar to that of *A. deserti* in its nonsucculent blade-like leaf of 0.5 mm thickness, its CAM photosynthesis



(Caption appears on following page)

Figure 3 (*Figure appears on preceding page*)

The complex trait CAM exists on a sliding scale (C_3, *left*; CAM, *right*). (*a*) Gas exchange characteristics of different CAM types, with increasing degree of nightly malate storage shown from left to right. Different CAM types may evolve from or be induced by each other depending on water availability. (*b*) The metabolic flux scheme of fluxes in C_3 and CAM using typical CAM cycle enzymes. Minor fluxes in C_3 plants evolve to carry major fluxes in CAM plants. (*c*) The distribution of $^{13}C/^{12}C$ carbon ratios in a genus with CAM plants (schematic representation of observed values in Bromeliaceae and Orchidaceae). Abbreviations: 2-OG, 2-oxoglutarate; AcCoA, acetyl coenzyme A; CAM, crassulacean acid metabolism; OAA, oxaloacetate; PEP, phospho*enol*pyruvate.

would be equivalent to 2.5 μmol CO_2 m^{-2} s^{-1}. A concentration of 217 mM malic acid equates to 217 M m^{-3} malic acid. With a leaf thickness of 0.0005 m, we get 108.5 mmol m^{-2} malic acid stored and therefore CO_2 fixed. For ease of comparison, we rescale to daytime photosynthesis by assuming a 12-h day and get 2.5 μmol CO_2 m^{-2} s^{-1}. Similar calculations for *C. validus* and *K. daigremontiana* yield 1 and 1.7 μmol CO_2 m^{-2} s^{-1}, respectively. These numbers assume that the leaf architecture of grass leaves is similar to that of CAM plants with regard to cells per leaf volume. If we apply the above calculation to a leaf weight rather than to volume bases, 14 different species (97) yield predicted equivalent photosynthetic rates of less than 1 in barley leaves. These calculations and modeling echo earlier work in which CAM was associated with succulence (59, 180), although there is some debate about how succulence is defined (103).

Comparison of C3, C_4, weak CAM, and strong CAM plants has identified intercellular air space as a functional CAM trait (118, 119). Typically, C_3 plants have more air space with fewer tightly packed cells than CAM plants do (118, 119, 184). Mesophyll conductance is determined by the path from the substomatal cavity to the site of initial CO_2 fixation and depends on air space and spaces filled by aqueous fluid. Because diffusion speed of CO_2 is more limited in water than in air, a tightly packed leaf with only small air space limits mesophyll conductance, a trait that has not yet been incorporated into recent C_3–CAM transition models (28, 139, 159). During CAM mode, PEPC as the initial fixation enzyme alleviates some of the limitation imposed by limited air space, because its K_m value for CO_2 is lower than that of Rubisco (78) and hence a larger gradient is available. Plants that use appreciable amounts of C_3 photosynthesis to sustain survival and growth must allow for sufficient air space in order to avoid mesophyll conductance from becoming limiting in conditions where stomata can largely be kept open (184). It has been suggested that the architecture of *Clusia* species represents a suitable compromise, with tightly packed palisade parenchymal cells and a wide, airy, spongy parenchyma (184). The balance between efficient CAM photosynthesis and efficient C_3 photosynthesis, with their trade-offs between storage capacity and mesophyll conductance, may be an explanation for the Winter-Holtum zone (104, 121) (**Figure 3*c***).

Intercellular air space: air-filled space between cells with important functions in gas exchange

Winter-Holtum zone: apparent drop in number of species within a family (e.g., Bromeliaceae, Orchidaceae) representing intermediacy between CAM and C_3

In both the Bromeliaceae (35, 178) and the Orchidaceae (143, 145), carbon discrimination values indicate that plants are either CAM or C_3, with only very few species in between. This zone is where inducible CAM species and weak CAM species with a strong contribution of daytime direct CO_2 fixation by Rubisco should reside. From an evolutionary standpoint, intermediacy is apparently selected against at least in some genera (178). The Winter-Holtum zone in between C_3 and strong CAM modes apparently represents a fitness valley occupied only by very few species (34, 145) (**Figure 3*c***). Ecological analyses of the CAM plants occupying this zone may reveal the conditions under which these plants are favored and may allow more models of evolution toward strong CAM to be built. Extending the most recent CAM model (159) to include mesophyll conductance on the basis of leaf architecture may reveal whether the observed compromise in *Clusia* (184) can be replicated. Models and ecological analyses may bring about an understanding of how succulence, reduced intercellular air space, and increased storage during CAM are balanced with sufficient CO_2 conductance through the mesophyll. Engineering of typical C_3 leaves toward

increased succulence has recently been achieved for *A. thaliana* (94). Moreover, increasing and decreasing mesophyll porosity are dependent on stomata distribution and are amenable to genetic manipulation (96). More investigation into the genetic manipulation of cellular architecture, cell packing, and mesophyll porosity, especially in the spongy parenchyma, will be required in order to fine-tune the leaf architecture for particular CAM types and environments.

6. CAM ENGINEERING

On the basis of the presently available knowledge about C_3 and CAM plants, a design schematic of CAM engineering can be developed. Circadian and diurnal rhythms in CAM plants are likely similar to those in C_3 plants and stomatal responses may only require fine-tuning (see above). So the key challenges are posited by the biochemistry of CAM. Engineering the trait in a C_3 architecture will critically depend on co-implementing the known regulatory processes to avoid costly futile cycling (**Figure 1*a***). A potential starting point is PEPC, because a mild increase in PEPC activity is not detrimental to plant growth (66, 81). Because futile cycles are to be avoided, the corresponding PEPC kinase with an expression pattern matching that of PEPC activity at night (27, 65) should be transferred alongside to limit PEPC activity to nighttime. Linking PEPC gene expression with the circadian clock, potentially via evening elements (63), will support PEPC control. PEPC-overexpressing plants accumulate malate (67), so presumably, the existing metabolic infrastructure in C_3 plants supports at least some degree of malate accumulation if not malic acid accumulation. It can be assumed that depleted PEP caused by PEPC activity will activate glycolysis because PEP itself acts a glycolytic regulator (127). Overexpression of GPT should provide ample glucose-6-phosphate as a substrate for glycolysis (24) (**Figure 1*a***). It is conceivable that depletion of glucose-6-phosphate and overoxidation of the plastid will increase phosphorolytic starch breakdown (170). If this assumption is not true, successful engineering of CAM will require more knowledge about the regulation of phosphorolytic starch breakdown before CAM reconstruction can be achieved. The minimal complement required to enable the nightly CAM reactions is thus PEPC, PEPC kinase, GPT, and potentially ALMT9 to ensure the vacuole can import adequate malic acid (**Figure 1*a***). Increased expression of ALMT9 will increase flux capacity but not necessarily flux, so engineering efforts will have to include engineering the electrochemical gradient built by proton ATPases or PPases (69, 106) (**Figure 1*a***).

For daytime release of CO_2, malic acid export and a decarboxylation enzyme are required. Malic acid export remains a knowledge gap that may preclude successful engineering. Knowledge about the regulation of decarboxylation is also incomplete (**Figure 1*a***). For engineering, it will be critical to shut off the decarboxylation pathway during the night. Plastidic NADP-ME is a good engineering candidate given that the enzyme is regulated by redox status of the chloroplast (6, 46) and therefore light itself. Overexpression of NADP-ME in *A. thaliana* had no macroscopic phenotype under standard conditions but plants had lower malate levels (49). The chloroplast importer for malate may have been DiT2 if the results from *Zea mays* analyses for malate import can be transferred (172), and it is present in C_3 chloroplast envelopes.

Once malate is decarboxylated, the resulting pyruvate needs to be rerouted toward starch biosynthesis. Expression of PPDK and the corresponding PPDK-RP will enable daytime phosphorylation of pyruvate to PEP. If the phosphorylation occurs in the chloroplast, either PPT will have to be expressed for PEP export (87, 163) and converted to 3-phosphoglyceric acid (3-PGA) in the cytosol or plastidic enolase and plastidic phosphoglyceromutase (PGlyM) will need to be coexpressed to allow conversion of PEP to 3-PGA in the chloroplast itself (11, 148) (**Figure 1**). If phosphorylation of PEP occurs in the cytosol, gluconeogenesis can proceed toward 3-PGA. Enolase and PGlyM are reversible enzymes and may be driven by substrate concentrations,

possibly circumventing the need for additional regulation. The carbon in 3-PGA produced from PEP needs to be stored in starch rather than exported to the sink organs. No starch thermostat controlling overall starch levels by synthesis or degradation has presently been described, which may posit another barrier to successful engineering. The use of alternative decarboxylation pathways through PEP-CK, mitochondrial NAD-ME, or cytosolic NADP-ME will require knowledge about their posttranslational regulation to safeguard against enzyme activity during the night. For PEP-CK, engineering may require expression of a mitochondrial ATP exporter to ensure sufficient ATP levels in the cytosol (21). For NAD-ME, malate uptake into mitochondria may require DIC (21); for cytosolic NADP-ME, uptake of pyruvate into the plastids may require expression of BILE-ACID SODIUM SYMPORTER 2 (BASS2) and SODIUM:HYDROGEN ANTIPORTER 1 (NHD1) (53).

Taken together, a minimal set of enzymes and transporters for CAM establishment would be PEPC (and PEPC kinase), GPT, ALMT9 and its energization system, plastidic NADP-ME, PPDK (and PPDK-RP), and plastidic enolase and PGlyM (**Figure 1**). The potential wild cards are the two storage steps, storage of carbon and storage of malic acid. Control of starch synthesis and degradation may be essential because changes to starch turnover enzymes were detected during CAM induction in *T. triangulare* (24). Likewise, control of storage and release of malic acid are not well understood. In addition to these hurdles, defining promoters for the expression of the regulatory proteins at the right time and at sufficient levels will be a challenge. To ensure survival of the C_3 host plant, one could engineer an inducible CAM system controlled by ABA-responsive elements (29) and attempt a mimic of *T. triangulare* or *M. crystallinum* with regard to the CAM cycle. Even if controlled by ABA-responsive elements, expression of at least the regulators and ideally also the metabolic enzymes would have to be linked to the circadian clock or to light/dark cues. Although evening elements (65, 109), the CCA1-binding site (109), and the G-box (77) mediate expression patterns, the construction of synthetic plant promoters with predictable expression patterns remains difficult.

Once proof-of-concept engineering has been achieved and the potential knowledge gaps closed, two alternative scenarios can be envisioned for successful CAM engineering in crops. Inducible, reversible CAM as realized in *T. triangulare* may be a survival strategy for crop plants if drought conditions are expected for only part of the life cycle. CAM photosynthesis together with classic drought responses would enable short-term survival until conditions improve. *T. triangulare* is only somewhat succulent, so its tissue and cell architecture still resembles C_3 architecture. Alternatively, designer crops for marginal lands may be engineered with tailor-made strong CAM solutions. Engineering constitutively strong CAM with adaptations to extended drought would require knowledge about leaf and cellular development control; knowledge about control of efficient drought defense structures such as stomatal density, stomatal positioning, epicuticular evaporation barriers, and potential water capture mechanisms; and more knowledge about mechanistic control of the metabolic flux. Given the potential of safely growing crops in a warming world with more unpredictable weather, both approaches are well worth the necessary research investment.

SUMMARY POINTS

1. Crassulacean acid metabolism (CAM) photosynthesis evolved from an ancestral C_3 state and largely retains the underlying architecture of regulation and metabolism of C_3 plants.
2. Explanations for circadian patterns and stomatal regulation may be based largely on a regulatory architecture known in C_3 plants.

3. Successful metabolic engineering depends on recreating some or all of the regulatory controls over the CAM cycle genes and proteins that are present in CAM plants.
4. Balancing storage capacity with sufficient mesophyll conductance in CAM may be the critical factor for fine-tuning by engineering efforts.
5. Proof-of-concept engineering is within reach if regulation of starch metabolism is not critical and malic acid storage and export can be achieved.

FUTURE ISSUES

1. The molecular identity of the vacuolar malic acid exporter remains unknown for all plants.
2. The molecular entity that controls starch synthesis and starch degradation is presently unknown for all plants.
3. Posttranslational modifications of many genes co-opted into the CAM cycle in C_3 plants have been demonstrated but their potential regulatory roles for CAM photosynthesis are unexplored.
4. Modeling of CAM evolution and of the disadvantages and advantages of CAM does not yet encompass mesophyll conductance, which, based on ecological observations, may be a major factor.
5. Engineering of CAM in C_3 plants may help us understand which, if any, processes are limiting the engineering effort.

DISCLOSURE STATEMENT

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