Alternative Crassulacean Acid Metabolism Modes Provide Environment-Specific Water-Saving Benefits in a Leaf Metabolic Model

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Alternative Crassulacean acid metabolism (CAM) evolved in arid environments as a water-saving alternative to C3 photosynthesis. It is of utmost interest to engineer more drought-resistant crops by introducing CAM into C3 plants. However, it is unknown whether full CAM or alternative water-saving modes would be more productive in the environments typically experienced by C3 crops. To study the effect of temperature and relative humidity on plant metabolism in the context of water saving, we coupled a time-resolved diel (based on a 24-h day-night cycle) model of leaf metabolism to an environment-dependent gas-exchange model. This combined model allowed us to study the emergence of CAM as a trade-off between leaf productivity and water saving. We show that vacuolar storage capacity in the leaf is a major determinant of the extent of CAM. Moreover, our model identified an alternative CAM cycle involving mitochondrial isocitrate dehydrogenase as a potential contributor to initial carbon fixation at night. Simulations across a range of environmental conditions show that the water-saving potential of CAM strongly depends on the daytime weather conditions and that the additional water-saving effect of carbon fixation by isocitrate dehydrogenase can reach 11% total water saving for the conditions tested.

INTRODUCTION

Increasing aridity threatens agricultural productivity not only in hot and dry climates but also in temperate regions where extreme weather conditions are becoming more frequent (Olesen and Bindi, 2002; Gornall et al., 2010). Thus, the development of crop varieties that use water efficiently is of utmost importance to maintain food security (Borland et al., 2014). Several plant lineages living in arid environments have evolved CAM photosynthesis, a water-saving mode of carbon fixation in which CO2 uptake into the mesophyll cell and CO2 fixation by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) are temporally separated (Osmond, 1978). In plants performing CAM photosynthesis, the stomata open at night and CO2 is fixed and stored in the vacuole in the form of a carboxylic acid such as malate, citrate, or isocitrate (Maclennan et al., 1963; Lüttge, 1990; Gawnorska and Niewiadomska, 2015; Igamberdiev and Epritsev, 2016). During the hot, dry daytime hours, the stomata can remain closed to minimize water loss, and the stored CO2 is remobilized for fixation by Rubisco in the chloroplast, accompanied by the accumulation of storage carbohydrates. Although this cycle is energetically expensive, it conserves precious water and is an efficient alternative to direct daytime CO2 fixation by Rubisco as in C3 photosynthesis (Dodd et al., 2002; Garcia et al., 2014).

The implementation of CAM photosynthesis into a C3 crop plant is a promising engineering target for two reasons. First, all enzymes required for the CAM cycle are already present in C3 plants, although specific isoforms with different regulatory properties are required (Cushman and Bohnert, 1997). Second, some facultative CAM species, such as the ice plant (Mesembryanthemum crystallinum), can be induced to switch from C3 to CAM photosynthesis by a number of environmental factors such as drought or high salinity (Winter et al., 1978; Garcia et al., 2014). This suggests that it should be possible to engineer CAM into a C3 leaf. CAM photosynthesis is usually considered to be advantageous in hot and arid climates where water-use efficiency (WUE) is a strong determinant for plant growth and where the suppression of photorespiration through carbon concentration behind closed stomata becomes a considerable factor that balances the additional cost of running the energy-intensive CAM cycle (Cushman, 2001). In a previous study, we tested this hypothesis by investigating the energetics and productivity of CAM by employing a day-night flux balance analysis model simulating either CAM or C3 leaf metabolism. To
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**Background:** During photosynthesis, plants take in CO₂ from the environment and, with the help of sunlight, convert it into energy-rich sugars. CO₂ uptake is regulated via the opening and closing of small pores on the leaf known as stomata. However, when stomata are open, water is lost from the plant through transpiration. Therefore, a balance must be struck between water loss and CO₂ uptake. In C₃ photosynthesis, the stomata open during the day when sunlight is available. This is energetically efficient, but results in significant water loss in environments with high daytime temperatures and low humidity. As an alternative, some plants can perform Crassulacean acid metabolism (CAM) photosynthesis, in which they open their stomata and temporarily fix CO₂ at night when temperatures are lower and humidity is higher. They then release the fixed CO₂ and refix it for sugar synthesis during the day while keeping the stomata closed. CAM photosynthesis is water-saving but less efficient.

**Question:** Would full CAM or alternative water-saving modes be more productive in the environments typically experienced by C₃ crops? To answer this question, we coupled a day-night model of leaf metabolism and a gas-exchange model and performed simulations for a wide range of environments.

**Findings:** We found that, by running a partial CAM cycle, the plant could save more than 50% of its water while maintaining 80% of its maximum productivity in a temperate climate. Moreover, our model identified an alternative carbon cycle involving the mitochondrial enzyme isocitrate dehydrogenase (ICDH) as a potential contributor to initial carbon fixation at night. The additional water-saving effect of carbon fixation by ICDH can reach 11% of the total water saving for the conditions tested. We also found that the CO₂ storage capacity in the leaf vacuoles was a limiting factor on the extent of CAM and would need to be increased to establish a CAM cycle in C₃ crops.

**Next steps:** Our in silico predictions suggest engineering strategies for generating crop plants that are better equipped for current and future climates. Implementing these strategies and testing them in vivo will be required to establish the practicality of these strategies.

**RESULTS**

**Model Construction**

Light availability and gas exchange (CO₂ and water vapor) are major determinants of the metabolic behavior of a plant leaf. To model the interplay between leaf productivity and transpirational water loss, we extended a previous diel flux balance modeling framework (Shameer et al., 2018) in two ways. First, we increased the temporal resolution from a binary day-night scenario to modeling a 24-time-step diel cycle, in which each interval represents 1 h of the day. The time resolution in the models was achieved by coupling 24 copies of the model in series, each with a predefined list of metabolites (starch, sugars, amino acids, carboxylic acids, and nitrate; Supplemental Methods) that were allowed to accumulate in one model and then to be passed to the next in the time series. For each of these metabolites, we introduced linker reactions that transfer the accumulated metabolite from one time interval to the next. Upper bounds were placed on the quantity of carboxylic acids and other compounds that were allowed to accumulate in the vacuole based on vacuole size and leaf anatomy (leaf thickness and porosity) for average C₃ and CAM leaves.

In this work, we tested whether full CAM is necessarily the best solution for C₃ crops grown in temperate environments and attempted to identify alternative metabolic modes that best balance the trade-off between water loss and photosynthetic productivity under a range of environments. To this end, we constructed a time-resolved, large-scale metabolic leaf model and coupled it to a gas-exchange model that includes the two main determinants of water loss through the stomata: the temperature (T) and the relative humidity (RH). This environment-coupled model was used to investigate emergent metabolic flux modes when water-saving constraints are imposed while still requiring the leaf to be highly productive. We found that the leaf’s vacuolar storage capacity is a major determinant of the extent of CAM and that without engineering a higher vacuole-to-cytoplasm ratio it will be unlikely to succeed in introducing a full CAM cycle into a C₃ leaf. Moreover, mitochondrial isocitrate dehydrogenase (ICDH) might contribute to initial carbon fixation at night. Finally, simulations across a range of environmental conditions showed that the additional water-saving effect of carbon fixation by ICDH can reach 11% of the total water saving for the conditions tested.
detailed description of the model construction and the exchange
constraints is given in Methods. The resulting time-resolved,
environment-coupled model enabled us to simulate the effect of
the day changes in light, T, and RH on leaf metabolism and was
used to study the trade-offs between leaf productivity and WUE
(Figure 1).

In this study, we considered the metabolism of a mature
source leaf under field conditions (i.e., a metabolic system that
functions to assimilate C and N and to synthesize sugars and
amino acids at a defined composition for export to the phloem
[phloem output] while at the same time meeting costs for cell
maintenance). We assumed that mineral nutrients were not
limiting, as is likely in a fertilized high-intensity agricultural
system. Therefore, we started the simulations by using maxi-
mization of phloem output over the course of the day as the
primary objective. This optimality criterion led the metabolic
system to synthesize storage compounds in the light that were
then used to sustain nighttime metabolic processes such as
phloem output, maintenance, and nitrogen assimilation in an
overall optimal manner. In accordance with the metabolic mode
of the system, the model predicted changing CO₂ demand and,
depending on T and RH, water loss by transpiration over the
course of the day (see section Model Analysis and Biological
Implications below). Subsequently, we could also fix the mini-
mum required phloem output to a given value (thereby also
reducing the system’s demand for CO₂) and could use minimi-
zation of water loss as an optimality criterion to act on the
metabolic system. These constraints led to the prediction of
water-saving flux modes while maintaining high productivity.

Model Analysis and Biological Implications: A
Time-Resolved Diel Model Simulates the Dynamics of
C₃ Metabolism

To establish a reference model for a mature source leaf operating
under optimal conditions, we began our analysis without imposing
water-saving constraints. We simulated a typical summer day in
a temperate climate by using T and RH curves with a maximum T of
30°C and a minimum RH of 0.4, values that were based on
measurements from the IPK weather station in Gatersleben,
Germany (Supplemental Figure 1; Supplemental Methods, Sec-
tion 1.2.2). To simulate light intensities inside a canopy, we used
a normally distributed light curve that peaks at midday at
a moderate intensity of 250 mol m⁻² s⁻¹. The daylight was set
to 12 h (Figure 2A). The primary optimization objective was
maximization of phloem output; a second optimization criterion
was subsequently applied to minimize the metabolic flux sum
(Holzhütter, 2004). This objective was used as a proxy for the cost
of providing the enzymes for the active reactions. Applying it as
a second optimality criterion did not alter the primary objective
value, the phloem output, but chose the flux distribution with the
lowest sum of fluxes from a set of alternatives.

Using this setup, the model predicted a phloem output of
1.0 mol m⁻² d⁻¹. Daily total water loss was predicted to be 2.6
× 10⁶ mol m⁻² leaf and CO₂ uptake was 10.7 mol m⁻² leaf. This
resulted in 249.8 mol of water lost per mol of CO₂ fixed. For
more extreme conditions with a light intensity of 1200 µmol m⁻² s⁻¹,
a maximum T of 40°C, and a minimum RH of 0.1, this value
increased to 753.8 mol of water lost per mol of CO₂ fixed
(Supplemental Data Set; Supplemental Results, Section 2.3,
presents a sensitivity analysis of water loss to varying internal
CO₂ concentrations). Comparing this value with an average
value for C₃ plants, 900 to 1200 mol of water lost per mol of CO₂
fixed (Smil, 2003), we found that our model is in broad agreement
with experimental observations. This was reassuring, given the
simple nature of the gas-exchange model and the fact that water
loss was predicted from the model’s demand for CO₂ to maxi-
mize phloem output, thereby justifying the choice of the objective
function.

To get a better overview of the metabolic behavior over the
course of the diel cycle, we examined the CO₂ uptake, Rubisco
activity, and linker fluxes for starch and carboxylic acids
(Figure 2B). The magnitude of a linker flux corresponds to the
amount of the stored metabolite: a flux of 1 µmol m⁻² s⁻¹ means
that 3.6 mmol m⁻² is available (given that each time interval is 1 h)
for utilization in subsequent time intervals in the model. Both CO₂
uptake (gray line) and Rubisco flux (orange line) were predicted
to follow the light curve and peaked at midday, coinciding with light
availability. Carboxylic acid levels (magenta area) peaked before
midday, decreased until early afternoon, and slowly increased
from before sunset to dawn. Starch (green area) accumulated
during daytime hours and was subsequently degraded to sustain
metabolism at night. Overall, the predicted flux patterns are
characteristic of C₃ leaf metabolism. From this starting point, we
then asked the question: How will the metabolic fluxes change if
we alter the optimality criterion from maximizing phloem output to
that of minimizing water loss?

An Optimality Study Reveals Trade-Offs between
Productivity and WUE

Computationally, the question of how the behavior of a system
changes when operating between competing objectives can be
 tackled by performing a Pareto analysis: a decision-making
technique to identify trade-offs and the impact of increasing
one objective’s value on the other (Laundy and Steuer, 1988;
Farnsworth and Niklas, 1995; Kennedy, 2010; Cheung et al.,
2013). In our case, phloem output and water saving represented
two competing driving forces. We started the Pareto analysis from
the scenario described above: a mature leaf optimized for phloem
output (i.e., 100% phloem output, hereby termed Pareto step 1).
We then subsequently reduced the required phloem output in 5% steps
and used minimization of water loss as the primary optimi-
ization objective. Given this setup, we saw an almost linear
decline in water loss with decreasing phloem output, and thus
no significant water-saving mechanism that would increase the
ratio of phloem output to water loss (Figure 2C, top). Inspection of
the CO₂ uptake and Rubisco reaction flux in the model showed
that with decreasing phloem output, the model predicted a ces-
sion of CO₂ uptake (suggesting a closure of stomata) during the
warmest and driest hours of the day, a phenomenon known as
midday depression of photosynthesis (Figure 2D, left column),
which was accompanied by a minor peak of CO₂ uptake toward
the end of the night.

One possible explanation for the lack of water-saving metabolic
modes in the model was that the model was limited by the
A diel (24-h) leaf model was constructed by concatenating copies of a core model of plant metabolism (Shameer et al., 2018). The individual models were connected via linker reactions that allowed the transfer of storage compounds in the vacuole and the plastid between successive models. Light uptake was constrained by the diel light curve. The day:night ratios of phloem output and maintenance were set to 3:1 for each hour of the diel cycle, and N uptake was constrained to a ratio of 3:2 based on previous estimates (Cheung et al., 2014).

The effect of T and RH on stomatal water loss was modeled by a simplified gas-diffusion equation. T and RH data determined the relationship between CO2 uptake and water loss. The four stomata pores illustrate the water-saving mechanism of nocturnal CO2 uptake. While respiration occurs in all four scenarios, dominant carbon fixation leads to a net uptake of CO2 during the day in C3 plants and at night in CAM plants.

Combining metabolic and gas-exchange models allowed us to study the trade-off between productivity and water loss as competing objectives on a Pareto frontier (i.e., the line that denotes combinations of productivity and water-loss values where one objective cannot be improved without compromising the other) and revealed alternative water-saving carbon-fixation mechanisms.
Figure 2. Metabolic Fluxes and Water Loss for Different Modeling Scenarios.

(A) Example of the T and RH data used throughout the simulations.
(B) Metabolic flux profiles in a C₃ leaf optimized toward phloem output (100% phloem output). The diel light curve is indicated in yellow and peaks at a maximum intensity of 250 μmol m⁻² s⁻¹.
constraints we applied to mimic C3 leaf anatomy (e.g., total vacuolar volume per unit of leaf). To test this, we examined the differences between C3 and CAM leaf anatomy and adjusted the vacuolar storage constraints accordingly. Using morphological data for an average CAM leaf resulted in a 3.1-fold increase in vacuolar storage capacity per unit of leaf compared with a C3 leaf (Supplemental Methods, Section 1.2.4). When repeating the Pareto analysis using this CAM morphology, a nonlinear relationship between productivity and water loss emerged and the model predicted more than 50% water saving at 80% of the maximum phloem output. This was an increase of 19.8% in water saving with respect to C3 morphology (Figure 2C). It is worth noting that the upper limit for the vacuolar storage capacity had only a very minor impact on the maximum phloem output of the model. The output at Pareto step 1 for the C3 leaf model was 99.6% of the phloem output of the CAM leaf. Therefore, in subsequent analyses, we directly compared between the two sets of simulations.

What was causing the nonlinearity in the relationship between productivity and water loss? As in the C3 anatomy-constrained model, we observed reduced CO2 uptake and Rubisco activity during the hottest and driest hours of the day. However, in addition to these daytime changes to minimize water loss, we also observed a substantial peak of CO2 uptake at the end of the night that was accompanied by an accumulation of carboxylic acids in the vacuole and a greater amount of starch stored during the day and degraded at night (Figure 2D, middle column). These observations suggested that the model was performing a CAM or CAM-like cycle in which CO2 was initially fixed at night and stored in the form of carboxylic acids. During the day, when sufficient light energy was available, CO2 was released from its intermediate storage and refixed for triose phosphate synthesis during the day using Rubisco and the Calvin-Benson-Bassham (CBB) cycle. This was confirmed by inspection of the complete set of predicted fluxes in the model (Supplemental Results, Section 2.1). Note that the results described thus far are not sensitive to the time interval of the model; quantitatively similar results were obtained when the model was run again with 2-, 4-, or 6-h time intervals (Supplemental Results, Section 2.5).

When inspecting the Pareto frontier, we observed the steepest slope (i.e., the largest increase in water saving) between 95 and 100% of the maximum phloem output. At 95% maximum phloem output, the model had already switched to CAM-like behavior and predicted a partial cessation of CO2 uptake during the day and resumption for a short period at night. Comparing this with the almost linear Pareto frontier for the C3 leaf indicated that the additional effect of nighttime CO2 fixation contributed largely to the water saving.

**Vacuolar Storage Capacity Limits WUE and Influences the Extent of Phases II and IV of the CAM Cycle**

When investigating the CO2 uptake at different steps along the Pareto frontier, it became apparent that the model did not exhibit a full CAM cycle (Figure 2D, middle column). CO2 uptake ceased for only a few hours in the day, and it resumed only for a short period toward the end of the night. The CO2 uptake at nighttime coincided with the maximum RH and minimum T, which were reached just before sunrise. This can be explained by the anticipatory nature of our model, where the solution for time point t depends on the environmental parameters to be encountered at time point t + 1. In a real CAM plant, we would expect nighttime CO2 uptake to be more distributed across the cooler and more humid nighttime hours.

During the day, CO2 uptake continued during the early hours of the day and resumed in the evening hours before sunset. This behavior was exhibited for all Pareto steps with more than 30% of the maximum phloem output, meaning that nighttime CO2 fixation alone was not sufficient to sustain the required phloem output. The observed CO2 uptake pattern, which is consistent with opening and reopening of the stomata, during the day occurs in certain CAM species and is known as phases II and IV of the CAM cycle (Osmond, 1978). Nighttime stomata opening for CO2 uptake and daytime stomata closure are referred to as phases I and III (Table 1), respectively. Some CAM species show a remarkable plasticity with respect to these four phases, and the reasons for the occurrence and extent of these distinct patterns are still debated (Cockburn, 1985; de Mattos et al., 1999; Lüttge, 2004). Given the indication that vacuolar storage capacity had a major impact on the nighttime CO2 uptake pattern in the model and the fact that some CAM species exhibit a biphasic CAM cycle, we wondered whether we might have underestimated the vacuolar storage capacity of an average CAM leaf. We therefore repeated the Pareto analysis using the same model but without any vacuolar storage constraints. The results of this analysis are shown in Figure 2D, right column. In the absence of any limitation of the vacuolar storage capacity, the model performed a biphasic full CAM cycle (i.e., the CO2 uptake was limited to the night) without the appearance of phase II or IV of the CAM cycle. Therefore, the model suggested that continued CO2 uptake for at least a portion of the day was necessary to sustain a high productivity when vacuolar storage capacity is limiting.

**Nighttime Carbon Fixation by ICDH Contributes to WUE**

The occurrence of the four phases of CAM in the model raised the following question: How were metabolic fluxes distributed during these metabolically distinct phases? To analyze the underlying
flux modes in more detail, we focused the analysis on a model with the vacuolar storage capacity of a CAM leaf at 80% of maximum productivity (phloem output) optimized for water saving. We chose this value because a yield penalty of 20% would be an acceptable trade-off if water usage could be reduced by more than half. We followed the flux of CO₂ (including bicarbonate) from the stomata through the metabolic system by plotting time-resolved fluxes of all reactions that use CO₂ or bicarbonate as either a reactant or a product (Figure 3A, left). During the day, Rubisco fixed the majority of CO₂ available from gas exchange and released by metabolic processes. Cytosolic ICDH, Gly oxidation in the photorespiratory pathway (Gly decarboxylase), and NADP-malic enzyme in the cytosol were the main CO₂-releasing processes during the day.

To our surprise, we found that nighttime CO₂ fixation in the model was shared between two enzymes—phosphoenolpyruvate carboxylase (PEPC) in the cytosol and ICDH in the mitochondria. While the role of PEPC in CAM photosynthesis is well established, mitochondrial ICDH activity has not been previously linked to this metabolic cycle. In order for ICDH to be used for CO₂ fixation, it has to operate in the reverse of its conventional direction in the tricarboxylic acid (TCA) cycle. This is possible given an appropriate mass action ratio (e.g., due to a high 2-oxoglutarate [2OG] concentration), and indeed this reaction has been shown to operate in the reverse direction in several in vivo metabolic flux studies in developing rapeseed (Brassica napus) and soybean (Glycine max) embryos (Schwender et al., 2006; Allen et al., 2009; Allen and Young, 2013). ICDH has also been suggested as a kinetically

Table 1. Four Phases of the CAM Cycle

| Phase | Description |
|-------|-------------|
| I     | Stomata open during the dark period and nocturnal CO₂ assimilation by PEPC |
| II    | Transition phase between dark and light periods with a peak in CO₂ uptake and fixation of CO₂ by Rubisco |
| III   | Stomata closed during the light period and fixation of CO₂ that is released by decarboxylation of carboxylic acids |
| IV    | Transition phase between light and dark periods with direct Rubisco-mediated fixation of CO₂ |

Figure 3. Different Flux Distributions in a Water-Saving CAM Leaf at 80% Productivity with (Model ICDH_{rev}) and without (Model ICDH_{irrev}) Reversible Mitochondrial ICDH.

(A) CO₂ budget for the two models reveals different CO₂ turnover fluxes over the course of the day. Shown are all reactions with flux > 0.5 μmol m⁻² s⁻¹ for at least one time point. I to IV indicate the four phases of the CAM cycle. The values for the cumulative contribution are given next to the reaction name for either model ICDH_{rev} or both models (model ICDH_{rev} and model ICDH_{irrev}); c, cytosolic; m, mitochondrial; p, plastidial.

(B) Significant linker fluxes for both models. Model ICDH_{rev} accumulated (iso)citrate as carboxylic acid and additionally Pro and Asp. Model ICDH_{irrev} accumulated both malate and (iso)citrate but no amino acids. Starch levels in model ICDH_{irrev} were almost threefold higher than in model ICDH_{rev}.
acceptable option for synthetic carbon fixation pathways ($\Delta G = 21 \text{kJ mol}^{-1}$ at pH 7, ionic strength of 0.1 M, and reactant concentrations of 1 mM; Bar-Even et al., 2010, 2012). For convenience, we refer to this reaction as ICDH$_{\text{rev}}$.

Analysis of the linker fluxes revealed that citrate and/or isocitrate were the main carboxylic acids accumulating at night. Accumulation of either citrate or isocitrate or of both carboxylic acids resulted in the same phloem output and water saving. Additionally, two amino acids accumulated to high levels: Asn during the night and Pro during the day (Figure 3B, left). Additionally, Glu accumulated at lower levels at night. None of the other linker reactions in the vacuole carried a significant flux.

Closer inspection of the metabolic fluxes revealed an alternative CO$_2$ fixation pathway in which both PEPC and ICDH contributed to nighttime CO$_2$ fixation. An overview of the reactions involved is shown in Figure 4A. At night, PEPC catalyzed the fixation of CO$_2$ to PEP [marked as (I) in Figure 4A]. The resulting oxaloacetate (OAA) together with Glu was converted to 2OG and Asp by Asp aminotransferase (II). Asp was converted to Asn and stored in the vacuole (III). 2OG was translocated to the mitochondria as a substrate for ICDH$_{\text{rev}}$ to catalyze the carboxylation of 2OG to isocitrate (IV), which was either directly stored in the vacuole or further converted to citrate and then stored in the vacuole for daytime usage (V). Additionally, conversion of the vacuolar pool of Pro to 2OG supported the flux through ICDH in the mitochondria (VI). In total, this pathway—from PEP to the carboxylic acids stored in the vacuole—can fix 1 mol of CO$_2$ per mol of stored (iso)citrate or Asn. During the daytime, the (iso)citrate from the vacuole was converted to 2OG and CO$_2$ by cytosolic ICDH, and CO$_2$ was re-fixed in the CBB cycle (VII) and ultimately stored as starch to then support night-time metabolism. 2OG was converted to Pro and stored in the vacuole for the next nighttime period (VIII).

This flux mode raised the following questions: Does the shared nighttime carbon fixation between PEPC and ICDH represent an advantage? And, if so, to what extent is it more beneficial than using PEPC alone? To answer these questions, we set ICDH to be irreversible in the conventional forward direction (ICDH$_{\text{irrev}}$) and ran the simulations again. We found that the carboxylation activity of ICDH increased water saving by 1.7% (for this particular set of parameters). From these observations, we concluded that nighttime carbon fixation by ICDH$_{\text{irrev}}$ in combination with daytime storage of Pro as a precursor for 2OG might act as an additional water-saving mechanism by adding to the temporal separation of initial CO$_2$ fixation and the activity of the CBB cycle.

**PEPC Also Fixes CO$_2$ in the Early Hours of the Day When ICDH Is Irreversible**

How do the metabolic fluxes in our model differ when ICDH$_{\text{irrev}}$ is not available for carbon fixation, and how does it affect WUE? A first inspection of the metabolic fluxes for this scenario revealed that making ICDH irreversible had a major impact on the accumulation pattern of both carboxylic acids and starch (Figure 3B, right). While the overall diel pattern of carboxylic acid accumulation and degradation was similar in the two scenarios, the individual patterns for malate and (iso)citrate were different. When ICDH was irreversible, we observed low-level (iso)citrate accumulation at night and a sharp increase of malate around sunrise that was followed by a drop in the early morning together with a strong increase of (iso)citrate. Daytime starch levels were more than twice as high as in the scenario where ICDH was reversible, and the onset of starch accumulation was shifted toward the later hours of the morning. Pro and Asn did not accumulate. Closer inspection of the flux routes involved in the carbon-fixation cycle revealed differences between the two scenarios (Figure 4B). Nighttime CO$_2$ uptake only started at the end of the night. The (iso)citrate levels increased during the night due to the use of internally released CO$_2$. At the end of the night and the onset of sunrise, PEPC fixed CO$_2$ to PEP and formed OAA, which was mainly converted to malate and stored in the vacuole (l). Net, this pathway, from PEP to the carboxylic acid stored in the vacuole, can fix 1 mol of CO$_2$ per mol of stored malate.

During the day, the two models showed marked differences in the flux routes between phase II and phase III of the CAM cycle. While model ICDH$_{\text{rev}}$ predicted that CO$_2$ fixation through PEPC was mainly limited to the nighttime, model ICDH$_{\text{irrev}}$ showed additional PEPC activity during phase II in parallel with Rubisco activity (Figure 3A, right). This early-morning PEPC activity increased the amount of CO$_2$ that could be transiently stored in the vacuole until sufficient light energy was available for starch synthesis. PEPC used PEP delivered from the CBB cycle as a substrate (l) to generate OAA. At the same time, malate was released from the vacuole and converted to OAA by malate dehydrogenase in the peroxisome (lII). A part of the OAA pool and acetyl-CoA were substrates for citrate synthase in the peroxisome (lIV). The other part of the OAA pool, together with Glu, was used by an aminotransferase to generate 2OG and Asp in the cytosol (V). Asp was further metabolized, and the downstream product acetyl-CoA (see sequence of reactions in Supplemental Results, Section 2.1) acted as a precursor for the synthesis of citrate by citrate synthase and (iso)citrate replaced malate in the vacuole. PEPC is known to be active in phase II (Borland et al., 1993; Roberts et al., 1997); however, subsequent metabolic flux modes in the model were different from the canonical CAM cycle in which malate is decarboxylated to PEP or pyruvate by PEP carboxykinase or malic enzyme. Later during phase III, (iso)citrate was released from the vacuole and supplied CO$_2$ for the CBB cycle via a degradation route that involved the Gly decarboxylase system in the mitochondria (VI; see reaction sequence in Supplemental Results, Section 2.1). To test whether the observed malate-to-(iso)citrate exchange in the vacuole during phase II of the CAM cycle was indeed a water-saving advantage, we simulated a scenario in which (iso)citrate uptake into the vacuole was blocked during the day (termed model ICDH$_{\text{irrev, Cit,night}}$). This constraint increased water usage at 80% productivity but by only 0.5%.

**High Enzyme Costs Might Outweigh the Water-Saving Effect of Alternative Flux Routes**

The occurrence of specific metabolic patterns was determined not only by WUE but also by the cost for enzyme synthesis. An accurate description of these processes at a large scale is currently limited to microbial systems for which sufficient data are available and for which enzyme turnover rates can be neglected due to high doubling times (Goelzer et al., 2015; Lantier et al., 2015; Rügèn et al., 2015; Reimers et al., 2017; Bulović et al., 2019). An
Figure 4. Major Flux Routes Involved in the CAM-Like Temporally Separated Carbon-Fixation Mechanism in a Water-Saving CAM Leaf at 80% Productivity. Analysis with reversible mitochondrial ICDH (model ICDH_{rev}; [A]) and analysis without reversible mitochondrial ICDH (model ICDH_{irrev}; [B]) are shown. The two models used different pathways to fix and release CO_2. A-CoA, acetyl-CoA; CS, citrate synthase; (iso-)Cit, (iso)citrate; Mal, malate; P5C, 1-pyrroline-5-carboxylic acid. The gray area in (B) highlights those reactions that are active in phase II. Roman numerals indicate the sequences of reactions described in the text.
established alternative is the minimization of the sum of metabolic fluxes (Holzhütter, 2004; Lewis et al., 2010). In our analysis, the enzyme investment was considered by minimizing the metabolic flux sum after the leaf productivity and water loss had been determined. Therefore, flux minimization did not represent a competing objective on the Pareto frontier, and a slightly more water-efficient solution with a high enzymatic investment (high flux sum) would always be preferred over a slightly worse-performing mechanism with less enzyme investment. To account for this bias, we considered the metabolic flux sum for the three models: ICDH_{ev}, ICDH_{prev}, and ICDH_{rev, Cit_right}. The values were 9098, 9670, and 9126 μmol m^{-2} s^{-1}, respectively.

As a second indicator for metabolic efficiency, we considered the overall ATP budget (i.e., all ATP produced and consumed over the course of the day; Supplemental Figure 2). These values indicated the highest ATP turnover of 869 μmol m^{-2} s^{-1} for model ICDH_{prev} and lower values of 807 and 811 μmol m^{-2} s^{-1} for models ICDH_{ev} and ICDH_{rev, Cit_right}, respectively. From these observations, we conclude that the additional metabolic cost of exchanging malate for (iso)citrate in phase II of the CAM cycle would very likely outweigh the water-saving effect of this mechanism. On the other hand, the other two modeling scenarios, ICDH_{ev} and ICDH_{rev, Cit_right}, had similar metabolic flux sums as well as ATP budgets, indicating that the contribution of ICDH to CO₂ fixation is a feasible prediction with respect to enzyme cost.

**CAM WUE Depends on the Environment**

So far, we have focused our analysis on one particular environmental scenario. In the next step, we used the model to study the impact of different environments on the trade-off between productivity and WUE, focusing on two questions: In which environments is the introduction of a CAM cycle most beneficial? In which environments is the contribution of daytime CO₂ assimilation via ICDH to water saving the greatest? To systematically scan the space of possible environments, we chose four alternative light regimes representing cloudy to sunny days and/or the gradient of light intensity within a closed-canopy cropping system: low light = 100 μmol m^{-2} s^{-1}; normal light = 250 μmol m^{-2} s^{-1}; high light = 800 μmol m^{-2} s^{-1}; and very high light = 1600 μmol m^{-2} s^{-1}. We also used three different photoperiods representing the seasons from early spring/late autumn to summer: day:night (in h) = long days (16:8); medium days (12:12); and short days (8:16). For each of these scenarios, we analyzed combinations of RH_{min} and RH_{max} between 0.4 and 1.0 across a T regime between 10 and 48°C (Figure 5, top; Supplemental Data Set) for both models ICDH_{rev} and ICDH_{prev} at 80% of the maximum phloem output. We determined water saving with respect to C₃ metabolism (i.e., 100% productivity) and the water-saving contribution of running the isocitrate-citrate-Pro-2OG cycle with respect to the CAM cycle without carbon fixation by ICDH. An overview of all calculated parameters across all investigated conditions can be found in Supplemental Figures 3 and 4.

From these analyses, we made the following general observations. First, we found that, for the environmental conditions we investigated and using a productivity penalty of 20%, a CAM cycle with nighttime carbon fixation by ICDH can result in relative water saving between 25.4 and 92.4% with respect to a C₃ leaf. Under certain conditions, CAM with nighttime carbon fixation by ICDH could save up to 25% more water than CAM without nighttime carbon fixation by ICDH. This difference can make up to 10.6% of the total water saving. Second, we found that T_{max} and RH_{max}, which mainly determine daytime weather conditions, had much stronger effects on water saving than T_{min} and RH_{min}, which mainly determine nighttime conditions. This behavior can be explained by the different CO₂ uptake profiles of the C₃ and CAM scenarios and the gas-diffusion relationship between the system’s demand for CO₂ and the resulting water loss through transpiration. Whereas any closure of the stomata during the day results in high water saving, opening stomata at night causes much less water loss. Therefore, it is primarily the extent of daytime stomata closure that drives water saving. Third, both daylength and light intensity had impacts on water saving. Interestingly, while the highest absolute water saving was seen for long days and high T, the additional water-saving contribution of the isocitrate-citrate-Pro-2OG cycle was strongest for short days and lower T.

The interaction between daylength and light intensity on the contribution of nocturnal ICDH carbon fixation to water saving is illustrated for three growth scenarios in Figure 5, as follows: (A) a leaf on a sunny day at the top of the canopy with very high light intensities and long days (16:8); (B) a leaf on a cloudy day and/or in the middle of the canopy with normal light intensity on a 12:12 day:night cycle; and (C) a leaf on a cloudy day and/or at the bottom of the canopy with low light and short days (8:16). Figure 5, bottom, shows the absolute water saving for model ICDH_{rev} with respect to C₃ metabolism (orange), and the absolute (blue) and relative (green) water-saving contributions (i.e., model ICDH_{rev} − model ICDH_{rev}) of nighttime carbon fixation by ICDH for these three scenarios. Representative fluxes involved in carbon fixation for a selected T and RH combination are also shown for each of the three environments.

In scenario A under very high light and long days, we found water saving of up to 58.0 mmol m\(^{-2}\) s\(^{-1}\) (left column) or 43.3% with respect to C₃. Under these conditions, the contribution of ICDH was 0.52 mmol m\(^{-2}\) s\(^{-1}\) (middle column) or 0.4% with respect to C₃ (right column). In scenario B with a 12:12 day:night cycle and moderate light intensities, we found similar water saving of up to 56.8 mmol m\(^{-2}\) s\(^{-1}\), representing up to 57.9% of the C₃ scenario. The contribution of ICDH to total water saving reached up 1.5 mmol m\(^{-2}\) s\(^{-1}\) or 2.0% with respect to C₃. For the short-day and low-light scenario C, water saving could reach 33.3 mmol m\(^{-2}\) s\(^{-1}\) or 92.4% with respect to C₃, with carbon fixation by ICDH contributing up to 2.6 mmol m\(^{-2}\) s\(^{-1}\) or 10.6% with respect to C₃. Although the CAM cycle dramatically increased water-saving efficiency in scenario C, its absolute benefit was lower due to the low overall water loss. For all three conditions, the flux patterns of PEPC and ICDH were similar in shape and magnitude. This was driven primarily by the limited CO₂ uptake rate and the limited storage capacity of the vacuole. For the same scenarios with unlimited storage capacity, we observed prolonged nocturnal activity of up to 6 h for both PEPC and ICDH.

To summarize, we found that the introduction of a CAM or CAM-like cycle could be beneficial across a large range of conditions (normal light to very high light and normal days to long days) that are typically encountered by a crop plant in temperate and hot climates. The contribution of ICDH to water saving is greatest for
Figure 5. Water Saving of a Leaf with CAM-Like Nocturnal Carbon Fixation by ICDH at 80% Productivity for Different Environments.

(Top) Overview of the environmental conditions analyzed. The $T_{\text{max}}$-$RH_{\text{max}}$ space was analyzed for different combinations of light intensity and daylength. Conditions A, B, and C are shown below. Conditions with an x are shown in Supplemental Results, Section 2.3.

(Bottom) Shown are heat maps for the absolute water saving of model ICDH$_{\text{rev}}$ with respect to the C$_3$ scenario (orange), the absolute water-saving contribution of ICDH (i.e., the difference in water saving between model ICDH$_{\text{rev}}$ and model ICDH$_{\text{irrev}}$; blue), and the relative water-saving contribution of ICDH with respect to the C$_3$ scenario (green; note the different scaling of the color bar) for combinations of $T_{\text{max}}$ and RH$_{\text{max}}$. Also shown are representative fluxes involved in carbon fixation and the shared nocturnal carbon fixation by PEPC and ICDH for the different environments at $T_{\text{max}} = 30^\circ$C and RH$_{\text{min}} = 0.4$ (right column).
low-light and short-day conditions and can reach up to 10.6% of the total water saving; however, under these conditions, the absolute water-saving effect remains moderate due to overall reduced water loss.

**DISCUSSION**

Our time-resolved, environment-coupled model of leaf metabolism allowed us to study the trade-offs between productivity and water saving for different network configurations and across different environmental conditions in a systematic manner. The analysis led to three main conclusions. First, the vacuolar storage capacity of the leaf is a major determinant of the extent of the CAM cycle and, without engineering a higher vacuole-to-cytoplasm ratio, it is unlikely that a full CAM cycle can be engineered into a C3 leaf. Second, the reversibility of mitochondrial ICDH might contribute to initial carbon fixation at nighttime. This operational mode of the TCA cycle was previously demonstrated by metabolic flux analysis in rapeseed and soybean embryos (Schwender et al., 2006; Allen et al., 2009), but it is a novel prediction with respect to nocturnal CO2 assimilation. Third, the water-saving effect of CAM strongly depends on the environment, and the additional water-saving effect of carbon fixation by ICDH can reach up to 25.0% and make up to 10.6% of the total water saving for the environmental conditions tested here. The additional water-saving contribution is largest at lower light intensities and for broad ranges of T and RH—conditions typically encountered by C3 crops in temperate climates—and this makes introducing the isocitrate-citrate-Pro-2OG cycle a promising candidate for metabolic engineering.

**Reduced Photorespiration due to Daytime Stomata Closure Can Increase the Water-Saving Potential of CAM Leaves**

In previous work on CAM photosynthesis, we investigated the energetics and productivity of metabolic networks operating in C3 and CAM. We found that, depending on the rates of the carboxylase and oxygenase activities of Rubisco, the productivity of a CAM network could reach between 74 and 100% of the productivity of a C3 network (Shameer et al., 2018). In the analysis presented here, we focused on the water-saving potential of CAM without considering the potentially positive effect of carbon concentration behind closed stomata during the day. As we do not know how the carboxylation-to-oxygenation ratio changes as we move along the Pareto frontier and during the daytime, we used a constant value of 3:1 (Ma et al., 2014). Therefore, the implications of our analysis can be regarded as a conservative estimate. Due to the suppression of photorespiration in a leaf operating in CAM mode, the actual water-saving potential at the same productivity level is expected to be higher than calculated here.

**ICDH Might Play a Role in Facultative CAM Photosynthesis**

Diel cycles of Pro accumulation have been previously observed in ice plants exposed to CAM-inducing salt stress. Under this stress condition, Pro is known to act as an osmoprotectant. It has been reported that Pro accumulation proceeded in an oscillating manner in which high levels of Pro accumulated during the day (up to 16 μmol g\(^{-1}\) fresh weight), followed by a partial degradation at night that led to steadily increasing Pro levels during the CAM-induction phase (Sanada et al., 1995). The increase in Pro levels was accompanied by an increase in PEPC mRNA up to 10 d after stress exposure, a time when PEPC mRNA had reached a full CAM level. This oscillatory behavior led the authors to make the following statement: “Changes of proline in light and darkness suggested that proline plays an important role in addition to serving as an osmolyte.” However, they offered no further explanation of what this role could be. We suggest that, in addition to its function as an osmoprotectant during the day, Pro degradation at night might support carbon fixation by supplying the substrate 2OG for citrate synthesis through mitochondrial ICDH. Once PEPC capacity has been induced to the level required for full CAM, the initial CO2 fixation proceeds via this enzyme, as it is kinetically superior, catalyzing a thermodynamically favorable reaction compared with ICDH\(_{\text{rev}}\) (\(\Delta G = -40\) kJ mol\(^{-1}\) at pH 7, ionic strength of 0.1 M, and reactant concentration of 1 mM; Bar-Even et al., 2012).

This conclusion is further supported by another study in ice plants in which malate, citrate, and isocitrate levels and CAM-relevant enzyme activities were measured for the same CAM-inducing conditions (Gawronska and Niewiadomska, 2015). The authors reported malate and citrate levels of up to 27.5 and 29.4 mM, respectively. Isocitrate levels were -10% of citrate levels. These observations are within the range of our model’s predictions of carboxylic acid storage (see the comment in Supplemental Methods, Section 1.2.1). The phenomenon of (iso)citrate accumulation in CAM plants is well established, and a review by Lütge (1988) discussed its possible ecophysiological functions, its role in nocturnal CO2 storage, energetic considerations that favor citric acid accumulation, carbon recycling, and osmotic consequences.

To elucidate the specific role of citrate in the ice plant and to shed light on the underlying metabolic flux modes during the CAM induction period in facultative CAM, additional experiments are required in order to trace the fate of labeled CO2, particularly its incorporation into amino and carboxylic acids, as well as the levels of those metabolites and genetic manipulation of the (iso) citrate cycle.

**Implications for Engineering CAM into a C3 Species in Temperate Climates**

Strategies for introducing CAM into crop plants to make them more resilient to hotter and drier conditions have been largely discussed in the context of arid or marginal lands (Borland et al., 2009, 2014; Yang et al., 2015; Wai et al., 2019). Less attention has been given to the question of whether CAM could benefit the productivity of C3 species such as wheat (Triticum aestivum) or barley (Hordeum vulgare) that are typically grown in temperate climates, where hot and dry periods are becoming increasingly frequent (Lopez et al., 2018; Rasmijn et al., 2018). In this context, a flexible CAM (i.e., a C3 + CAM phenotype) could be beneficial, as it combines high productivity in the C3 mode with increased WUE in the CAM mode. In addition, partial or weak CAM, in which only a modest amount of nocturnal CO2 accumulation occurs and the
stomata are open for some of the day (Edwards, 2019), could be beneficial in temperate environments.

Naturally occurring CAM has two characteristics that make it a suitable target for engineering approaches for crops grown in temperate regions. First, CAM is extremely flexible. It has been shown that the contribution of CAM to diel CO₂ uptake patterns can range between 0 and 100%, particularly in plants with either an ontogenetically or an environmentally induced transition from C₃ to CAM (Winter, 2019). Second, CAM has evolved many times independently, and it is believed to be present in well over 5% of vascular plant species (Winter and Smith, 1996; Silvera et al., 2010). These observations can be attributed to the fact that CAM most likely evolved on a biochemistry-first, anatomy-second trajectory (Edwards, 2019) in which the C₃ + CAM phenotype is an evolutionarily accessible phenotype on the trajectory to strong CAM (Edwards and Donoghue, 2006; Bräutigam et al., 2017; Heyduk et al., 2018).

Our model demonstrates the water-saving potential of a partial CAM or CAM-like mode for plants grown under a broad range of conditions while maintaining a high net metabolic output; at 80% productivity, relative water saving is at least 25.4% across all conditions tested (up to 92.4%). It also shows that in terms of absolute water saving, the introduction of a classic CAM cycle is most beneficial for hot and dry conditions with high light and long days (scenario A), emphasizing the growth benefit of CAM plants in these climates. Much less intuitively, the model also revealed a significant water-saving potential for temperate climates and dense cropping environments in which lower light intensities prevail (scenarios B and C). Notably, under these conditions, the introduction of a CAM + isocitrate-citrate-Pro-2OG cycle would be most beneficial (contributing up to 10.6% of the total water saving) and therefore should be targeted at crops growing under such conditions. Despite the lower total water-saving potential in temperate climates, the high contribution of the isocitrate-citrate-Pro-2OG cycle to water saving could make a substantial contribution to water saving in environments where C₃ crops are typically grown.

**Strategies to Engineering an Isocitrate-Citrate-Pro-2OG Cycle**

In our previous work (Shameer et al., 2018), a number of engineering interventions beyond the core CAM cycle were identified, particularly concerning mitochondrial respiratory capacity. This work identified an additional isocitrate-citrate-Pro-2OG carbon-fixing cycle operating alongside the conventional CAM cycle. This additional cycle includes reactions in the mitochondria, cytosol, and vacuole and involves mitochondria-to-cytosol transport of Pro and (iso)citrate. The enzymatic reactions involved are the mitochondrial proline dehydrogenase (PDH), δ1-pyrroline-5-carboxylate dehydrogenase (PSCDH), glutamate dehydrogenase (GDH), as well as the TCA cycle enzymes IDH and aconitase (both in the mitochondria and cytosol), glutamate 5-kinase, δ1-pyrroline-5-carboxylate synthetase (PSCS), and pyrroline-5-carboxylate reductase. With the exception of IDH, these reactions already proceed in their favorable direction or operate close to equilibrium. Hence, a crucial aspect to engineering this flux in transgenic plants would be to ensure a high-enough 2OG level to drive IDH backward. Key to this would be generating sufficient nighttime Pro accumulation and degradation to Glu during the day. GDH activity would probably then be sufficient to generate 2OG in high-enough quantities to overcome the thermodynamic barrier of the reverse IDH reaction. In Nicotiana tabacum and potato (Solanum tuberosum), increased Pro production has been achieved by overexpression of PSCS (Kishor et al., 1995; Hmida-Sayaria et al., 2005). Moreover, as with any attempt to engineer CAM, transgene expression/enzyme activity would have to be diel-regulated, as reviewed elsewhere (Borland et al., 2014; De-Paoli et al., 2014; Yang et al., 2015) and here, including PDH and PSCDH. Replication of the precise temporal patterns of this cycle predicted by our model may not be necessary, but recent developments in synthetic gene regulatory systems make this increasingly feasible (Belcher et al., 2020).

**Increasing Midday Depression in C₃ Plants**

Another prediction of the model was the benefit of a cessation of CO₂ uptake (i.e., stomata closure) around midday in a leaf with C₃ anatomy. From a metabolic perspective, a potential strategy would be altering the tonoplast malate transporter (aluminum-activated malate transporter) in a way that malate is only released for decarboxylation at this time of the day. However, its regulation during day and night is still unknown. Therefore, generating a synthetic aluminum-activated malate transporter that could be switched on in a precise temporal fashion could be a key engineering target.

**Overcoming Vacuolar Storage Constraints by Increasing Cell Size**

Our study identified the vacuolar storage capacity as a limiting factor for introducing CAM into a C₃ plant. Comparison of models with different vacuolar storage capacities revealed that shifting from C₃ to CAM leaf anatomy (i.e., introducing a 3.1-fold increase in vacuolar storage capacity) increased water saving by 19.8% at 80% productivity. Given this observation, engineering leaf anatomical parameters of a C₃ leaf toward CAM architecture will be key to increasing water saving. How could this be achieved? Several anatomical traits, including cell size (as a proxy for vacuole size), percentage intercellular airspace (IAS), and tissue thickness, have been discussed in this context (Edwards, 2019). However, altering the latter two parameters could potentially limit photosynthesis in C₃ mode due to reduced CO₂ diffusion through the mesophyll and a lower internal CO₂ concentration (Evans and von Caemmerer, 1996) and therefore disadvantage flexible CAM. Indeed, facultative CAM plants when operating in the C₃ mode can have an internal CO₂ concentration as low as 110 μmol mol⁻¹ (Maxwell et al., 1997). Barrera Zambrano et al. (2014) proposed that Clusia species might overcome this problem by having a high percentage IAS in the spongy mesophyll for efficient CO₂ diffusion in the C₃ mode and large palisade cells for carboxylic acid storage in the CAM mode. They suggested this as a potential engineering strategy for transferring inducible CAM into a C₃ plant (Barrera Zambrano et al., 2014); however, engineering such tissue differentiation into a C₃ crop would be a major challenge. Of particular
relevance is a study that attempted to increase leaf cell size by overexpressing a grape berry (*Vitis vinifera*) transcription factor (VvCEB1\textsubscript{opt}) in Arabidopsis (*Arabidopsis thaliana*) and Nicotiana sylvestris (Lim et al., 2018). This approach increased cell size—but not number—in both species, with a 1.8- to 2.3-fold increase in the palisade mesophyll and a 2.0- to 2.5-fold increase in the spongy mesophyll in Arabidopsis. Assuming that the larger cell size is mainly driven by increased vacuolar volume, the reported increase would suffice to enable partial CAM with high water-saving potential. Besides increased cell size, the authors also reported a significant decrease in cell wall thickness, another feature typically observed in CAM plants (Yang et al., 2015). A follow-up study in Arabidopsis reported significant increases in cell size, succulence, and decreased IAS and WUE (Lim et al., 2020). Thus, overexpressing VvCEB1\textsubscript{opt} in the context of engineering CAM could indeed be a promising strategy to engineer more drought-resistant crops.

**Further Challenges To Be Considered**

Besides the challenge of engineering a CAM cycle into a C\textsubscript{3} crop, other factors such as the trade-off between improved WUE and potential constraints on leaf productivity due to anatomical changes or the response of CAM to increasing CO\textsubscript{2} levels need consideration (Winter, 2019). It is unclear not only how increased vacuolar size would affect the space available for enzymatic capacity in other intracellular compartments but also how it would affect mesophyll conductance. There is also the potential extra burden of increased structural costs (e.g., due to increased leaf thickness and altered water potential). Another challenge is that there may be other regulatory considerations that could affect the successful integration of CAM in C\textsubscript{3} crops. For example, highly succulent CAM plants such as cacti avoid water stress in their photosynthetic tissues due to a regulatory response involving root shrinkage and metering of stored water in the succulent tissues (Nobel and Cui, 1992). The extent to which CAM can operate when tissues are water stressed to the levels routinely experienced by C\textsubscript{3} leaves remains an open question. In conclusion, this study demonstrates the water-saving potential of introducing CAM-like metabolism into C\textsubscript{3} plants under a wide range of environmental conditions and suggests environment-specific engineering targets for improved drought resistance.

**METHODS**

**Developing a Time-Resolved Environment-Coupled Model of Leaf Metabolism**

The model-building process was divided into two parts: developing a time-resolved model of leaf metabolism and modeling gas exchange through the stomata. The time-resolved diel model is an extension of our previously published diel modeling framework (Cheung et al., 2014; Shameer et al., 2018). Starting from the latest version of a charge- and proton-balanced generic core model of plant metabolism (PlantCoreMetabolism_v1.2.3.xml), we concatenated 24 copies of the model (each representing 1 h of the day) by allowing a range of metabolites to accumulate and to be transferred from one time point to the other via so-called linker reactions. Starch was allowed to accumulate freely in the plastid. The sugars glucose, sucrose, and fructose, the carboxylic acids malate, citrate, and isocitrate (see comment on citrate and isocitrate accumulation in Supplemental Methods, Section 1.2.1), the proteinogenic amino acids, and nitrate were allowed to accumulate in the vacuole. The last time interval of the optimization routine was coupled to the first interval to form a closed diel cycle. A comparison of our previous and current model can be found in the Supplemental Table.

The overall vacuolar storage capacity was based on estimates for the vacuolar malate storage capacity and the vacuolar volume of an average C\textsubscript{3} plant (Supplemental Methods, Section 1.2.4). The specificity of the metabolic network at each hour of the day was achieved by setting the light input according to the diel light curve and by constraining the export of sugars and amino acids to the phloem (phloem output) to a day:night ratio of 3:1 and nitrogen uptake to a day:night ratio of 3:2, according to previous estimates (Cheung et al., 2014). Maintenance cost was modeled in a light-dependent manner, where the daytime cost depends on the average daytime light intensity (Supplemental Methods, Section 1.2.3). The day:night ratio was assumed to be 3:1, and the ratio of ATP maintenance cost to NADPH maintenance cost was assumed to be 3:1 (Cheung et al., 2013). Rubisco was only activated during daylight hours. Since the applied normally distributed light curve reaches zero only asymptotically, calculated light intensities below a light-compensation point of 30 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (measured for Kalanchoe daigremontiana; Adams et al., 1987) were set to 0 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and the respective time interval was considered as dark.

Due to the lack of knowledge about the Rubisco carboxylation:oxygenation ratio at different steps on the Pareto frontier and for different time points during the day, we used a value of 3:1 based on flux measurements in Arabidopsis (*Arabidopsis thaliana*; Ma et al., 2014). The uptake rate for CO\textsubscript{2} was limited to a value of 15 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) based on values for different C\textsubscript{3} and CAM species (Nobel, 2012). We assumed an average ratio of internal to atmospheric CO\textsubscript{2} concentration of 0.7 (Tan et al., 2017; Kumarathunge et al., 2019; Busch, 2020; Cai et al., 2020; see Supplemental Methods, Section 1.2.5, and Supplemental Results, Section 2.5, for more information and a sensitivity analysis of the predicted water loss as dependent on the internal CO\textsubscript{2} concentration). All other fluxes were unconstrained. To avoid any bias, we used phloem compositions of both C\textsubscript{3} plants (tomato [*Solanum lycopersicum*] and Arabidopsis) and a CAM species (prickly pear [*Opuntia ficus-indica*]) and we found no qualitative differences in our analysis. The phloem composition and output values for the Arabidopsis and prickly pear data-constrained model are listed in the Supplemental Data Set.

Gas exchange through the stomata was described by a linearized diffusion model that predicts the water loss depending on the metabolic model’s demand for CO\textsubscript{2} at particular T and RH values. The input values for T and RH for each time point were calculated by using a skewed sine-curve input according to the diel light curve and by constraining the export of water to atmospheric CO\textsubscript{2} concentration. The input values for T and RH for each time point were calculated by using a skewed sine-curve function (Supplemental Methods, Section 1.2.2), and this allowed us both to model the shape of actual T and RH curves and to systematically scan a multidimensional parameter space by adjusting the function parameters accordingly.

The model equations for optimizing phloem output and water saving were solved as a linear optimization problem (L\textsubscript{1} norm). The subsequent minimization of the metabolic flux sum was solved as a quadratic optimization problem (L\textsubscript{2} norm) to select, from a possible set of multiple solutions, the one with the least variation in fluxes between time points. To exemplify this, consider a three-time-step model with the flux sequence [1, 1, 1], [2, 0, 1], and [3, 0, 0]. When applying the L\textsubscript{1} measure, all three cases will be weighted with 3, although in the second and third cases more enzyme needs to be synthesized and degraded and therefore would be costlier. The L\textsubscript{2} distance yields values of 3, 5, and 9 and would therefore prefer the flux distribution in which fluxes are equally split between the three phases. The code for constructing and solving the full 24-phase model takes around 2 min to run on a standard desktop computer, and the Pareto scan takes ~20 min. A derivation of the gas-water exchange relationship through the stomata, any further modeling assumptions, parameter derivations, and auxiliary calculations are detailed in the Supplemental Methods, Section 1.
1. The effect of varying T and RH profiles and of different time intervals is summarised in Supplemental Figures 5 and 6.

Data Availability
All modeling-relevant code is available at https://github.com/nadinetoepfer/Toepfer_et_al_CAM_2020.

Supplemental Data
Supplemental Figure 1. Weather data on a typical summer day in 2019 in Gatersleben, Germany.

Supplemental Figure 2. Cumulative ATP turnover for the three models considered.

Supplemental Figure 3. Overview of the environmental conditions analyzed.

Supplemental Figure 4. Heat maps illustrating water saving for different combinations of light intensity and daylengths and different combinations of RH_{min} and T_{max}.

Supplemental Figure 5. Effect of varying T and RH profiles on the occurrence of the four phases of the CAM cycle and the main flux routes.

Supplemental Figure 6. Effect of different time intervals on the overall model behavior.

Supplemental Table. Comparison of our previous and current large-scale metabolic model of CAM.

Supplemental Methods.

Supplemental Results.

Supplemental Data Set. Environmental parameters, leaf parameters for different C_{3} and CAM species, phloem sap compositions, and modeling results.

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AUTHOR CONTRIBUTIONS
N.T. and L.J.S. designed the research; N.T. and T.B. performed research; N.T., T.B., and S.S. contributed new computational tools; N.T., R.G.R., and L.J.S. analyzed data; N.T., R.G.R., and L.J.S. wrote the article; N.T. created the figures.

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