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Crassulacean acid metabolism species differ in the contribution of C_3 and C_4 carboxylation to end of day CO_2 fixation

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Abstract

Crassulacean acid metabolism (CAM) is a photosynthetic pathway that temporally separates the nocturnal CO_2 uptake, via phosphoenolpyruvate carboxylase (PEPC, C_4 carboxylation), from the diurnal refixation by Rubisco (C_3 carboxylation). At the end of the day (CAM-Phase IV), when nocturnally stored CO_2 has depleted, stomata reopen and allow additional CO_2 uptake, which can be fixed by Rubisco or by PEPC. This work examined the CO_2 uptake via C_3 and C_4 carboxylation in phase IV in the CAM species *Phalaenopsis* "Sacramento" and *Kalanchoe blossfeldiana* "Saja." Short blackout periods during phase IV caused a sharp drop in CO_2 uptake in *K. blossfeldiana* but not in *Phalaenopsis*, indicating strong Rubisco activity only in *K. blossfeldiana*. Chlorophyll fluorescence revealed a progressive decrease in ΦPSII in *Phalaenopsis*, implying decreasing Rubisco activity, while ΦPSII remained constant in phase IV in *K. blossfeldiana*. However, short switching to 2% O_2 indicated the presence of photorespiration and thus Rubisco activity in both species throughout phase IV. Lastly, in *Phalaenopsis*, accumulation of starch in phase IV occurred. These results indicate that in *Phalaenopsis*, PEPC was the main carboxylase in phase IV, although Rubisco remained active throughout the whole phase. This will lead to double carboxylation (futile cycling) but may help to avoid photoinhibition.

1 | INTRODUCTION

Crassulacean acid metabolism (CAM) is a specialized photosynthetic pathway with an inverse day/night pattern of stomatal opening. CAM plants temporally separate CO_2 fixation during the night from refixation via the Calvin-Benson cycle behind closed stomata during the daytime. Osmond (1978) introduced a framework to distinguish the different processes taking place over a diel cycle in a CAM plant. The nocturnal period in which stomata are open is referred to as phase I. During this phase, phosphoenolpyruvate carboxylase (PEPC) catalyzes atmospheric or respiratory CO_2 (as HCO_3^-) to bind with phosphoenolpyruvate (PEP), which is stored in the vacuole as a C_4 acid (Lüttge, 2001). The refixation period during the day, when stomata are closed, is referred to as phase III. In between these two phases with distinct carboxylation processes, transitional phases (II and IV) may occur under environmentally favorable conditions (Dodd et al., 2002). During phase II at dawn, the transition from CO_2 carboxylation via PEPC to carboxylation via Rubisco occurs. PEPC carboxylation may continue at dawn while Rubisco is activated to avoid photoinhibition (Roberts et al., 1997). Phase II is defined to last until stomata close. At the end of phase III, C_i decreases due to malate depletion, causing stomata to reopen. This marks the start of phase IV, in which CO_2 uptake takes place again (Males & Griffiths, 2017; Winter & Smith, 1996). Phase IV lasts until dark.

Abbreviations: ΦPSII, photosynthetic operating efficiency; C_a, C_i, ambient internal CO_2 concentration; CAM, crassulacean acid metabolism; ETR, electron transport rate; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PPFD, photosynthetic photon flux density.

Diel regulation of the two carboxylases is essential for the functioning of CAM. Carbon isotope discrimination data confirmed that PEPC and Rubisco can be simultaneously active in both phases II and IV (Ritz et al., 1986; Roberts et al., 1997). Gas exchange measurements on CO_2 uptake and stomatal conductance are most commonly used to determine diel patterns and identify CAM-phases. To determine the contribution of different carboxylation pathways on CO_2 uptake, Winter and Tenhunen (1982) used a combination of ambient and low CO_2 and O_2 concentrations while measuring CO_2 gas exchange and found that both PEPC and Rubisco are active during phase II. Also, chlorophyll fluorescence measurements can be used to distinguish between carboxylases. Griffiths et al. (2008) showed that there is a direct positive correlation between Rubisco activity, Rubisco activation and the electron transport rate (ETR). Carboxylation via PEPC in the light can also be considered an indirect electron sink, but not a strong one (Maxwell et al., 1999). PEPC carboxylation activity is associated with low ETR and high non-photochemical quenching (NPQ) (Griffiths et al., 2008). NPQ mechanisms safely dissipate excess excitation energy that cannot be used for photochemistry (Demmig-Adams & Adams, 2006). Low ETR and high NPQ typically occur in CAM plants at the beginning and the end of the day under natural conditions, when PEPC may be active. Gas exchange measurements at different CO_2 and O_2 concentrations combined with chlorophyll fluorescence can be used as a non-invasive and non-destructive measurement to determine which carboxylation pathway is likely to be active.

Previously it was assumed that the transitional phases II and IV do not contribute much to carbon gain (Borland & Griffiths, 1996). Later on, in particular phase IV was recognized for contributing substantially to the total carbon uptake under well-watered conditions (Dodd et al., 2002), which could help increase productivity. While CAM has the potential of high yields, it is typically not seen as a trait that is favorable for crops in agriculture (Davis et al., 2019). CAM has a higher energetic cost per fixed CO_2 due to temporally separating two CO_2 fixation steps. While this might seem significant, the higher energetic cost results in a trivial productivity penalty compared to C_3 plants (Nobel, 1991; Shameer et al., 2018). The limited penalty on productivity is considered to be mainly due to the suppression of photorespiration, which can increase the cost of CO_2 fixation in C_3 plants by 25% (Nobel, 1991). On the other hand, direct CO_2 fixation via Rubisco in CAM plants in phases II and IV may go hand in hand with significant photorespiration (Borland et al., 2000). The succulent structure of CAM leaves results in reduced intercellular space (Nelson & Sage, 2008), which reduces the internal conductance for CO_2. Maxwell et al. (1997) showed for *Kalanchoe daigremontiana* that CO_2 concentration at the carboxylation site for Rubisco can be as low as 110 ppm during phase IV. For that reason, Rubisco would be less effective for CO_2 fixation compared to PEPC, which has a lower compensation point (Ceusters & Borland, 2010). If uptake by PEPC in phase IV is supplementary to nocturnal uptake, it could bypass photorespiration and result in additional CO_2 uptake. It is generally believed that, in phase IV, CO_2 is mainly fixed via Rubisco (Ceusters et al., 2010; Cushman, 2017; Osmond & Holtum, 1981). Also, the carbon that is fixed during this phase is largely partitioned for growth (e.g. Dodd et al., 2002; Maxwell et al., 1999; Nobel, 1996), which could boost the productivity of CAM plants. More recently, Ceusters et al. (2010) already questioned these statements by showing that, for the CAM bromeliad *Aechmea*, the CO_2 uptake in phase IV can be dominated by PEPC, and that phase IV activity is not explanatory for plant growth rates and biomass accumulation.

The main aim of this study was to investigate the extent to which C_3 and C_4 carboxylation in CAM-phase IV contributes to CO_2 uptake. We studied two obligate CAM species: *Phalaenopsis* "Sacramento" and *Kalanchoe blossfeldiana* "Saja." *Phalaenopsis*, in particular, is considered economically important in the horticultural sector (Davis et al., 2019). The *Kalanchoe* genus, in general, is relatively well studied due to its potential as CAM model plant and its wide range of CAM phenotypes, ontogenetically shifting from C_3 to CAM, makes it an interesting object of study (Winter, 2019; Yang et al., 2015). Species used in this study are both cultivated for their ornamental value. *Phalaenopsis* is an epiphytic orchid that can be mainly found in forests in tropical Asia (Tsai, 2011), whereas *K. blossfeldiana* originates in Madagascar, where it grows at higher altitudes and in humid forests at moderate temperatures (Smith et al., 2019). In *K. blossfeldiana*, short days induce flowering and accelerate the switch to CAM (Winter & Holtum, 2014). In the current study, these plants were grown in climate chambers. We showed that CO_2 taken up in phase IV in *K. blossfeldiana* "Saja" was mainly fixed via Rubisco, while PEPC seemed to be the main carboxylase in *Phalaenopsis* "Sacremento." However, in *Phalaenopsis*, Rubisco remained active as well, which may lead to futile cycling because of double carboxylation but could help to avoid photoinhibition.

2 | MATERIAL AND METHODS

2.1 | Plant material and growth conditions

Vegetative *Phalaenopsis* hybrid cv. "Sacramento" (further referred to as *Phalaenopsis*) and *Kalanchoe blossfeldiana* cv. "Saja" (further referred to as *K. blossfeldiana*) were grown in two separate climate chambers. Plants were grown in the greenhouse before transfer to the climate chambers. Vegetative *Phalaenopsis* plants were potted in 12 cm pots and grown in a Venlo type greenhouse (Bleiswijk, The Netherlands) for 21 weeks, after propagation in the lab. Cuttings of *K. blossfeldiana* plants were potted in 10.5 cm pots and grown for 8 weeks, of which 5 weeks in short-day conditions, in a Venlo type greenhouse ('s-Gravenzande, The Netherlands). Plants were allowed to acclimate to climate chamber conditions for 1 week before starting measurements. In the time range where the experimental work was conducted, *K. blossfeldiana* plants were expected to show maximum CAM functioning and malate carboxylation (40–60 days, Queiroz & Morel, 1974). *Phalaenopsis* was grown at a temperature of 27°C and PPFD of 140 μmol photons m^{-2} s^{-1} between 05:00 and 21:00. *Kalanchoe blossfeldiana* was grown at 20°C with a PPFD of 140 μmol photons m^{-2} s^{-1} between 08:00 and 18:00. Growth conditions for each species were comparable to those applied by commercial growers. The shorter day length used for growth of *K. blossfeldiana* induces CAM in young leaves, as well as flowering (Queiroz & Morel, 1974). Plants were grown under red/white LED modules with supplementary

far red (23 μmol photons m^{-2} s^{-1}) (Philips GreenPower LED production module deep red/white and GreenPower LED research module far red) at a photostationary state of 0.83 (Sager et al., 1988) (Appendix S1). Spacing was done in such a way that no shading due to nearby plants occurred. For both species, CO_2 concentration was kept at 600 ppm, as is common practice in commercial greenhouses, and vapor deficit of the air at 7.2 g m^{-3}. Plants were watered every 5 days with a nutrient solution that had an EC 1.2 mS cm^{-1} and pH of 5.7. For the composition of the nutrient solution, see Appendix S2. For all measurements, either the youngest mature leaf (*Phalaenopsis)* or the third leaf pair from the apex, present at the start of the experiment (*K. blossfeldiana*), was used.

2.2 | Gas exchange and chlorophyll fluorescence measurements

Gas exchange measurements were done using LI-6400XT (Li-Cor Inc., Lincoln) using the LCF chamber with a flow rate of 200 μmol m^{-2} s^{-1}, light intensity of 140 μmol photons m^{-2} s^{-1} (10% blue), block controlled temperature of either 27°C (*Phalaenopsis*) or 20°C (*K. blossfeldiana*). CO_2 was controlled at 600 ppm. To ensure continuous and steady CO_2 supply, an external gas cylinder and a setup to humidify the air to ambient conditions were used. For chlorophyll fluorescence measurements, a rectangular flash was used. Intensities of the 0.8 s saturating flash (>6200 μmol photons m^{-2} s^{-1} for *Phalaenopsis* and >6300 μmol photons m^{-2} s^{-1} for *K. blossfeldiana*) and measuring light were tested beforehand to avoid photo-inhibition and reduction of Q_A, respectively. Measurements were made in situ in the climate chamber.

2.2.1 | Diel profiles

Data were collected starting in phase III and continued throughout the subsequent night and morning under conditions similar to those in the climate chamber to record the "steady-state" data of plants (n = 8). Data were logged every minute, while matching of IRGAs was done every 5 min. For readability of the figures, plotted results contain one data point every 10 min. Each data point represents a single measured value. During the day, chlorophyll fluorescence measurements were made every 15 minutes, in the night every hour. These data were also used to determine the beginning of phase IV. When the net CO_2 uptake rate was higher than 0.1 μmol CO_2 m^{-2} s^{-1} for at least three consecutive minutes and remained positive, this was marked as the start of phase IV.

2.2.2 | Procedures to distinguish C_3- and C_4-driven CO_2-uptake during phase IV

Short blackout period

Light in the leaf chamber was switched off for 5 min three (*K. blossfeldiana*: 210, 120 and 30 min before lights off) or four (*Phalaenopsis*: 300, 210, 120 and 30 min before lights off) times during the progression of phase IV. Data were logged every minute, but from 10 min before until 10 min after lights off, the frequency of logging was increased to every 5 s. When logging data under changing conditions, the lag of the system to changes in water vapor content has to be considered. This is especially true for changes in stomatal conductance caused by disabling C_3 uptake due to switching lights off. Therefore, from the high logging frequency only one data point per 100 s was used in data analysis and figures. To ensure that switching off the lights was not the reason for PEPC induction, a separate set of measurements was conducted, where lights were only switched off at the last time point (Appendix S3).

2% O_2 measurements

In addition to short blackout-period measurements, the part of the leaf clamped in the chamber was subjected to 2% O_2. This was done to (1) determine whether photorespiration occurred and (2) determine the contribution of Rubisco/PEPC carboxylation throughout phase IV. Settings in the leaf chamber were as described previously. The leaf chamber was sealed 15 min prior to the start of the first measurement. During every measurement, IRGAs were matched and gas exchange and fluorescence data were logged. After an initial steady-state measurement, CO_2 was mixed with 2% O_2, using a separate gas mixing system with mass flow meters. Plants were exposed to this air mixture for 5 min, after which data were logged again. This was done twice for *K. blossfeldiana* (150 and 45 min before lights off) and three times for *Phalaenopsis* (255, 150 and 45 min before lights off) (n = 8). New plants were used at every time step. Photosynthetic operating efficiency (ΦPSII) was calculated according to Baker (2008). To calculate NPQ, a dark-adapted leaf is required, in which NPQ is fully relaxed. However, Tietz et al. (2017) demonstrated that NPQ can also be derived without having a fully dark-adapted leaf. This new fluorescence parameter is characterized as $NPQ_{(T)}$ (Equation 1) and was used accordingly in the current study.

$$NPQ_{(T)} = \frac{4.88}{\left(\frac{F'_m}{F_0}\right) - 1} - 1 \quad (1)$$

2.3 | Sampling and analysis of metabolites

Leaf discs were taken for biochemical analysis at several time points throughout phase IV, and immediately after lights off. For *K. blossfeldiana*, sampling in phase IV was done every hour, i.e., 3, 2, and 1 h before lights off. *Phalaenopsis* was sampled every 2 h, i.e., 6, 4, and 2 h before lights off. New plants were used at every time point. At each time point, we measured three different plants (three biological replicates; n = 3). Per plant, three leaf discs were taken (three sampling replicates per biological replicate) and immediately placed in liquid nitrogen before placing them in the −80°C freezer. Leaf discs were freeze-dried and ground to powder with two metal pellets (3 mm) in an Eppendorf for a minute using a ball mill. Samples were stored in a desiccator until further processing.

2.3.1 | Sample preparation for anion and sugar analysis

The freeze-dried powder (15 mg) was dissolved in 5 ml 75% ethanol. Samples were heated in a shaking water bath at 80°C for 20 min and centrifugated for 5 min (8500 g, 4°C, Universal 320R, Hettich). One milliliter of supernatant was dried in the Speed Vac at 55°C for 2 h or until dry. The evaporated ethanol was replaced by 1 ml of MilliQ water and mixed for 10 min in an ultrasonic bath at 50 Hz, followed by vortex and centrifuge for 10 min. This protocol was used for both analyzes of anions and sugars. Samples were then **diluted,** 5 times for anions and 10 times for soluble sugars.

2.3.2 | Sample preparation for starch analysis

Of the previously prepared samples for anions and sugars, the remaining supernatant was discarded and 3 ml of 80% ethanol was added. Samples were centrifugated 15 min (8500 *g*, 4°C), then washed twice with 80% ethanol. After discarding the supernatant again, pellets were placed in the Speed Vac at 55°C for 25 min. After drying, 2 ml of thermostable α-amylase solution (1 mg Rohalase ml^{-1}) was added to the pellet, and the sample was placed in a shaking water bath at 90°C for 30 min. One milliliter of amyloglucosidase (0.5 mg ml^{-1} citrate buffer, 50 mM at a pH of 4.6) was added, again placed in a shaking water bath at 60°C for 15 min. One milliliter was put into an Eppendorf tube that was centrifuged for 15 min (8500 *g*, 4°C) and diluted 20 times before analysis.

2.3.3 | Sample analysis

Soluble sugars and starch were quantified using High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD; Dionex ICS5000, Thermo Fisher Inc.), equipped with a CarboPac1 column (250 × 2 mm) eluted with 100 mM NaOH. Anions were quantified using a Dionex HPAEC system (Dionex Corporation) equipped with a GS50 pump, an ED50 detector and with an IonPac AS11-HC (250 x 2mm) column and eluted with a gradient starting at 16 mM NaOH. The total accumulation of malate, citrate, sucrose, fructose, glucose, and starch was calculated in mmol m^{-2} using leaf mass area of dried leaf discs.

2.4 | Statistical analysis

Measurements at low O_2 were analyzed using a paired *t*-test for samples within one species at one timepoint. Simple linear regression was conducted on the results of metabolite analysis at different time points in phase IV. For all analyzes, Genstat 19th edition (VSN International Ltd) was used. Treatment effects within species were considered significant at $P < 0.05$. All data are expressed as mean ± standard error of the mean.

3 | RESULTS

3.1 | Diel profiles of CO_2 uptake

When lights were switched off (start of phase I), there was a direct, sharp drop in net CO_2 uptake in *K. blossfeldiana* "Saja," even resulting in the release of CO_2 for the first few hours of phase I. This indicated a strong Rubisco-mediated uptake during phase IV, and negligible PEPC-mediated CO_2-uptake (Figure 1A). After a few hours, net CO_2 uptake became positive, but CO_2 uptake rates stayed low during the night period. In *Phalaenopsis* "Sacremento," transitioning from light to dark did not result in a decrease in the CO_2 uptake rate (Figure 1B). Instead, an increase occurred during the first hour after the lights switched off. This indicates a large contribution of PEPC-mediated CO_2 uptake in phase IV, which is

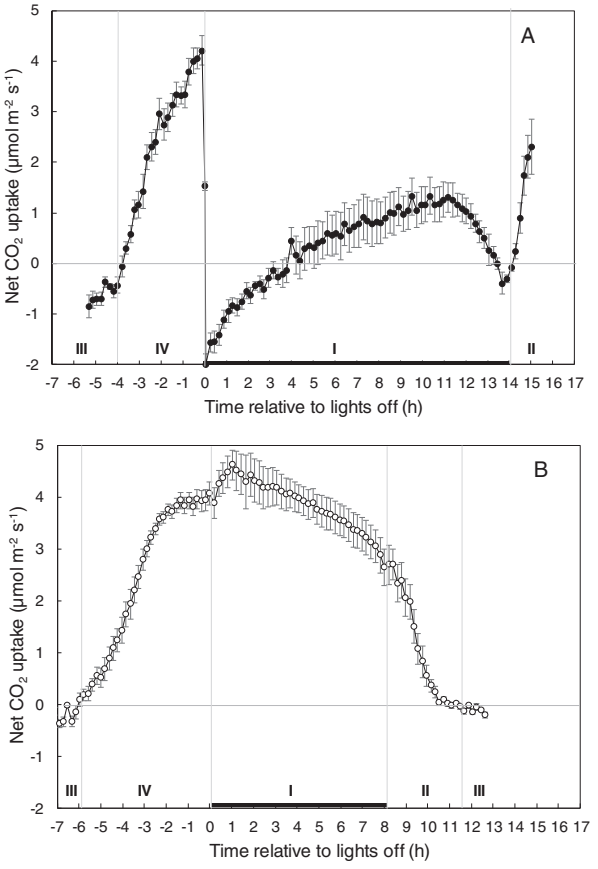

FIGURE 1 Diel profiles of CO_2 exchange rates of *Kalanchoe blossfeldiana* cv. Saja (A) and *Phalaenopsis* cv. Sacramento (B) leaves. The third leaf pair from the apex of *K. blossfeldiana* and the youngest mature leaf of *Phalaenopsis* were used. Solid bar on x-axis indicates night period. CAM phases, as described by Osmond (1978), are indicated by roman numerals. Data represent the average of eight replicates with SEM. Plants were grown in climate chambers at 660 ppm CO_2 and a PPFD of 140 µmol photons m^{-2} s^{-1} with additional far-red of 23 µmol photons m^{-2} s^{-1}. *Kalanchoe blossfeldiana* was grown at 20°C and a day length of 10 h, and *Phalaenopsis* was grown at 27°C and a day length of 16 h

opposite to *K. blossfeldiana*. Fluorescence measurements showed that the photosynthetic operating efficiency (ΦPSII) (Baker, 2008) decreased progressively in *Phalaenopsis* throughout phase IV, which implies a decreasing Rubisco activity over time. However, *K. blossfeldiana* maintained a constant photosynthetic efficiency until the end of the day (Figure 2).

3.2 | Multiple short blackout periods to distinguish C_3- and C_4-driven CO_2-uptake during phase IV

Momentarily switching off the light throughout phase IV always resulted in a reversal of CO_2-exchange direction from uptake towards release in *K. blossfeldiana*, which suggests that all CO_2 uptake in phase IV is via C_3 photosynthesis (Figure 3). The immediate drop in CO_2 exchange was followed by a delayed closing response of the stomata (Figure 3B,C). Stomatal closure was not as visible the first time, most likely because stomatal conductance was very low (Figure 3A). After lights on, CO_2 uptake was not immediately back at the same rate as before (when lights were off) due to a delayed response of the stomata. Stomatal conductance started to decrease a few minutes after lights were switched off but took more than 20 min to fully recover. In *Phalaenopsis*, CO_2 uptake dropped when lights switched off at the beginning of phase IV (Figure 4A,B) but not later on (Figure 4C,D). During the second drop, there was a decrease in CO_2 exchange, but the net uptake remained positive, suggesting PEPC-carboxylation was already induced. At the last two time points, there was no decrease in CO_2 exchange anymore, indicating full PEPC-carboxylation. A separate set of measurements with switching the lights off only at the last time point showed that lights off early in phase IV was not the reason CAM was induced at later time points (Appendix S3).

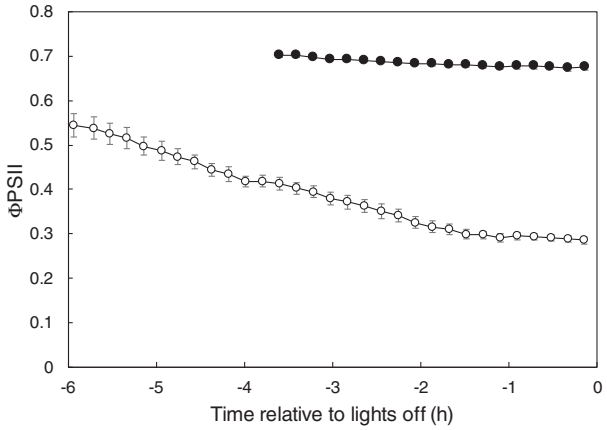

FIGURE 2 **Photosynthetic** operating efficiency (ΦPSII) in *K. blossfeldiana* (closed circles) and *Phalaenopsis* (open circles) leaves in phase IV. Data represent the average of eight replicates with SEM. Plant growth conditions as described in Figure 1

3.3 | 2% O_2 to detect photorespiration during phase IV

In both species, switching to 2% oxygen resulted in an increase in CO_2 uptake and in a drop in ETR, meaning photorespiration occurs at all times in both species, except for *Phalaenopsis* at 225 min before lights off (Table 1), which is just after the start of phase IV. Here, ETR did decrease, although net CO_2 uptake was not different. The CO_2 uptake at this point was probably too low to measure differences. However, the change in ETR suggests Rubisco oxygenase activity behind closed stomata did occur. Switching to 2% oxygen resulted in an increased $NPQ_{(T)}$ in both species at all-time points, but the relative increase in $NPQ_{(T)}$ was larger in *K. blossfeldiana* than in *Phalaenopsis*.

3.4 | Metabolite analysis

In *K. blossfeldiana*, there was no accumulation of malate throughout phase IV (Figure 5A), suggesting that only C_3 photosynthesis occurred. There was some breakdown of citrate at the beginning of phase IV (P = 0.015), although levels were generally very low and not considered to contribute significantly to the CAM pathway. No significant changes during Phase IV were found for soluble sugars or starch in *K. blossfeldiana* (Figure 5C–F). *Phalaenopsis* showed some malate buildup, but differences between the beginning and end of phase IV were not significant due to large variation between samples (P = 0.159). Data from a similar experiment on vegetative *Phalaenopsis* plants did show a significant buildup of malate in phase IV (P = 0.009, see Appendix S4). In this complementary dataset, sampling was done on a higher number of biological replicates with a sampling from the same leaf throughout the time of the experiment (one leaf, one replicate). This greatly reduced variance compared to the current dataset, and therefore showed a significant difference, which presumably could also be the case for the current dataset, if the same sampling methodology was used. There was no change in citrate levels in phase IV nor in soluble sugars (Figure 5B-E). However, there was an accumulation of starch (P = 0.041) in *Phalaenopsis* (Figure 5F).

4 | DISCUSSION

4.1 | PEPC and not Rubisco is the main carboxylase in Phalaenopsis

In general, Rubisco is assumed to be the main carboxylase in CAM plants in phase IV (Osmond & Holtum, 1981). In C_3 plants, switching off the lights would result in a sharp drop in CO_2 uptake because light is needed to keep the Calvin-Benson cycle running and Rubisco carbamylated (Von Caemmerer & Edmondson, 1986). The CO_2 gas exchange profile measured in *K. blossfeldiana* confirmed that CO_2 uptake ceased when lights were switched off (Figure 1A). This is in line with previous studies on *K. daigremontiana* (e.g. Griffiths et al., 2002; Wyka & Lüttge, 2003) and *K. fedtschenkoi* (Borland

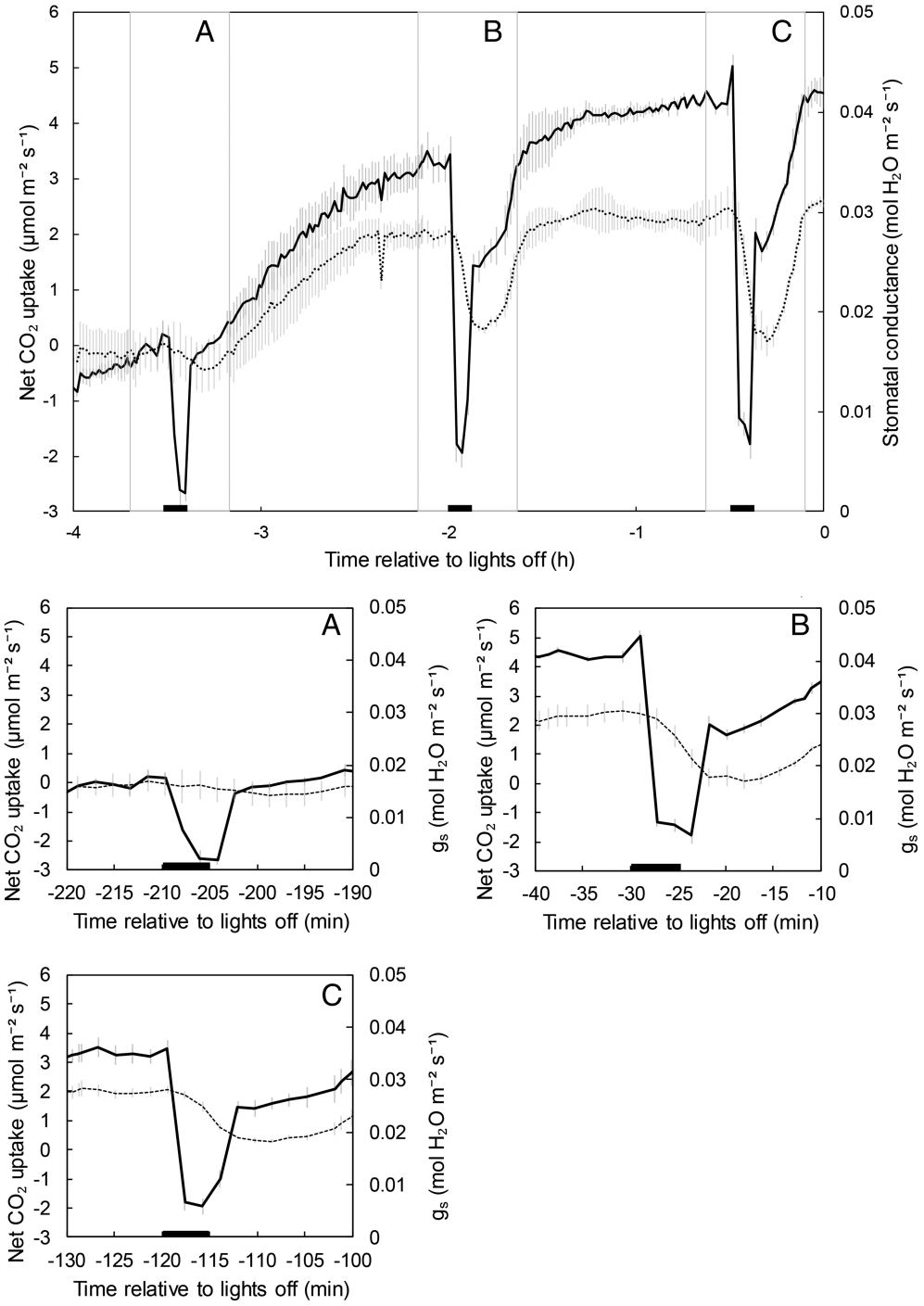

FIGURE 3 Response of CO_2 uptake (solid line) and stomatal conductance (g_s, dashed line) of *K. blossfeldiana* leaves (third leaf pair from the apex) to a 5-min dark period in phase IV. 3.5 h (A), 2 h (B), or 0.5 h (C) before end of day, the light in the leaf chamber was switched off for 5 min before returning to 140 µmol photons m^{-2} s^{-1}, as indicated by solid black bar. Data represent the average of three replicates with SEM. Plant growth conditions as described in Figure 1

et al., 2009) but also with studies on other CAM species such as *Mesembryanthemum crystallinum* (Dodd et al., 2003), *Clusia minor* (Grams & Thiel, 2002), and *Guzmania lingulata* (Maxwell et al., 1999). It is therefore not surprising that a similar gas exchange profile is shown in review papers on CAM plants (Borland et al., 2011; Lüttge, 2001; Winter, 2019). Notably, a robust nocturnal CAM profile was maintained for both species, without applying a day-night difference in temperature. Optimum nocturnal temperature for CO_2 fixation in CAM plants typically is between 10–20°C (Yamori et al., 2014), and in general it is believed that either low night temperatures or a temperature differential is required for CAM to function (Buchanan-Bollig & Kluge, 1981; Nimmo, 2000). Additionally, we temporarily switched off the lights in the leaf chamber for 5 min at several moments throughout phase IV and continued to measure gas exchange. These blackout periods always resulted in substantial CO_2 release in *K. blossfeldiana*, indicating that Rubisco was the main carboxylase (Figure 3). Diurnal gas exchange profiles in *Phalaenopsis* showed a continued rate of CO_2 uptake without a drop when lights were switched off at the end of the day (Figure 1B). This is in line with gas exchange profiles measured by Lootens and Heursel (1998) in

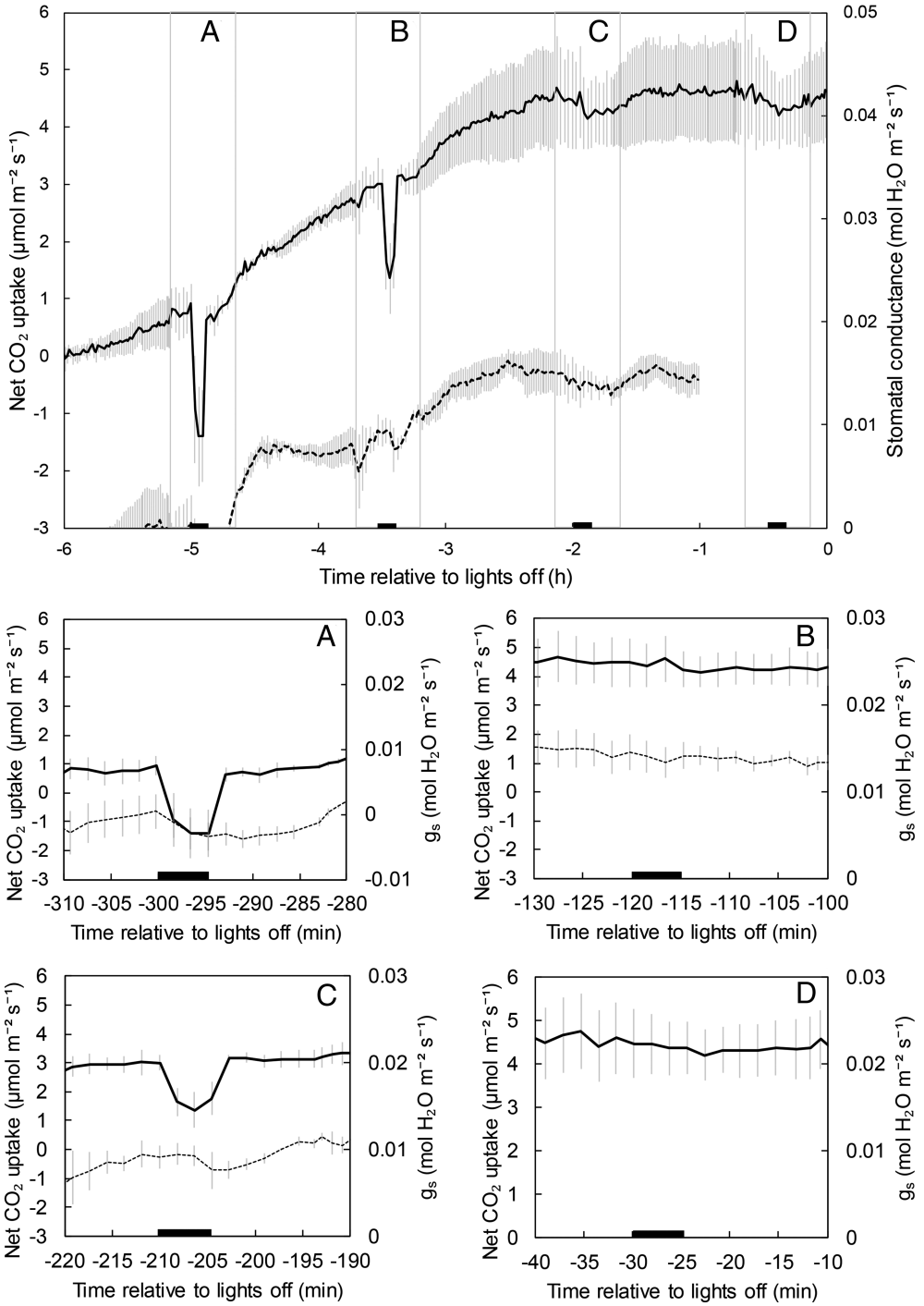

FIGURE 4 Response of CO_2 uptake (solid line) and stomatal conductance (g_s, dashed line) of *Phalaenopsis* leaves to a 5-min dark period in phase IV. 5 h (A), 3.5 h (B), 2 h (C), or 0.5 h (D) before end of day, the light in the leaf chamber was switched off for 5 min before returning to 140 µmol photons m^{-2} s^{-1}, as indicated by solid black bar. Data represent the average of three replicates with SEM. g_s data from the last hour are missing due to irregularities in climate chamber humidity control. Plant growth conditions as described in Figure 1

Phalaenopsis hybrids "70" and "L," but these were never placed in the context of carboxylation type in phase IV. In *Phalaenopsis*, blackout periods during phase IV showed a decrease in CO_2 uptake early on (Figure 4A). However, this no longer resulted in CO_2 release already during the second blackout. Later on, in phase IV (Figure 4C,D), the continued rates of CO_2 uptake suggested that PEPC was the primary carboxylase in *Phalaenopsis*, at a time when CO_2 uptake rates were highest.

Kalanchoe blossfeldiana retained a constant ΦPSII throughout phase IV until the end of the day, indicating that Rubisco activity did not decrease (Griffiths et al., 2008), which is in line with the CO_2 release measurements due to a short blackout. The chlorophyll fluorescence profiles for *Phalaenopsis* showed a gradual decrease of the photosystem II operating efficiency (ΦPSII) towards the end of the day, while the CO_2 uptake simultaneously increased (Figure 2). This indicated changes in the regulation and activity levels of both carboxylases, as a decreasing ΦPSII at steady irradiance can be directly linked to reduced Rubisco activity and increasing PEPC activity (Griffiths et al., 2008). These fluorescence data indicate that in *Phalaenopsis*, the upregulation of PEPC occurs early in phase IV. This may mean that

TABLE 1 Net CO_2 uptake rate (μmol CO_2 m^{-2} s^{-1}), electron transport rate (ETR), and non-photochemical quenching ($NPQ_{(T)}$) in young mature Phalaenopsis and Kalanchoe leaves at several time points (minutes relative to lights off) during phase IV in air with 21% O_2 or 2% O_2

	Net CO_2 uptake			ETR			$NPQ_{(T)}$		
Time before lights off	21% O_2	2% O_2	Sig.	21% O_2	2% O_2	Sig.	21% O_2	2% O_2	Sig.
	Phalaenopsis								
−225	0.16 ± 0.25	−0.03 ± 0.41	NS	27.1 ± 1.1	23.6 ± 1.3	**	2.31 ± 0.18	2.96 ± 0.15	***
−150	2.23 ± 0.41	3.34 ± 0.51	***	21.8 ± 1.2	18.8 ± 1.2	***	3.15 ± 0.17	3.58 ± 0.18	***
−45	2.32 ± 0.38	3.03 ± 0.26	*	19.2 ± 1.4	15.5 ± 0.77	***	3.52 ± 0.20	4.14 ± 0.22	***
	Kalanchoe								
−150	0.42 ± 0.16	0.84 ± 0.17	**	38.1 ± 1.1	33.9 ± 1.5	***	0.82 ± 0.12	1.32 ± 0.26	*
−45	1.88 ± 0.22	2.90 ± 0.40	**	34.7 ± 0.9	29.5 ± 1.1	***	1.38 ± 0.12	2.15 ± 0.15	***

Note: Data are means ± SE, measurements within one timepoint and within one species (n = 8). *,**, and *** are significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
Abbreviations: NS, not significant; Sig, significance.

PEPC was the main carboxylase during phase IV, and not only at the end of the day, as was previously suggested (e.g. Borland & Taybi, 2004; Osmond & Holtum, 1981). These fluorescence data over phase IV are in full agreement with the effect of the blackout periods in phase IV on CO_2 exchange (Figures 3 and 4). Differences in the response of CO_2 uptake, as well as in stomatal behavior due to blackouts in phase IV (Figures 3 and 4), suggest that two different pathways were indeed active in *K. blossfeldiana* and *Phalaenopsis*. Metabolite control (Dever et al., 2015), degree of succulence (Von Caemmerer & Griffiths, 2009) and in particular levels of C_i may play a role in determining the stomatal conductance in CAM plants. The drawdown of C_i is linked to exhaustion of the supply of malate. This process is associated with stomatal opening, which marks the start of phase IV (Males & Griffiths, 2017). Bearing this in mind, the closure response of stomata after switching the lights off in *K. blossfeldiana* might be due to changes in C_i, which would not occur in *Phalaenopsis* when PEPC, carboxylating CO_2 independent of light, is active. For *K. daigremontiana*, it was found that Rubisco activity was highest early in phase IV, most closely resembling C_3 photosynthesis (Maxwell et al., 1999). By closing stomata in response to switching off the light, the response of *K. blossfeldiana* appeared indeed similar to what is seen in C_3 plants (e.g. Lawson & Blatt, 2014). If C_i and C_c during phase IV in *K. blossfeldiana* would be very low and Rubisco highly active, this can lead to a high level of photorespiration (Borland et al., 2000; Maxwell et al., 1999). Carboxylation of CO_2 (in the form of PEP) via PEPC is more effective in fixing CO_2 than Rubisco due to its higher affinity for CO_2 (Wyka & Lüttge, 2003), and because it does not induce photorespiration. This makes CO_2 uptake via PEPC at low C_i more efficient than via Rubisco, even though this would come with a slightly higher energetic cost per CO_2 fixed (Shameer et al., 2018). CO_2 plays a role in the activation of Rubisco, higher ambient CO_2 can increase carbamylation of Rubisco in C_3 plants (Von Caemmerer & Edmondson, 1986). In CAM plants, this could result in an increased end of day fixation via Rubisco. It is likely that CO_2 uptake via Rubisco would be more favorable compared to PEPC at 600 ppm, due to its lower affinity Rubisco mediated CO_2 uptake would benefit more from increased [CO_2]. Furthermore, it could increase carbamylation status, which would further enhance the CO_2 uptake by Rubisco, but PEPC might still be the main carboxylase in *Phalaenopsis*.

4.2 | Effect of light quality and daylength on carboxylation activity in phase IV

Light quality can affect the functioning of CAM photosynthesis, although little research has been conducted in this area. Exposing *Phalaenopsis* plants to either narrow-band red or white light did not affect PEPC activity during phase IV (Zheng et al., 2019). In another recent study on *Phalaenopsis* 'Sacramento', the same cultivar as in the current study, the response for CO_2 uptake during phase IV was similar to ours, though we are using a more dominant red light (Hogewoning et al., 2020, in press). Obviously, growing plants under a narrow-band light affects photosynthesis and plant growth, regardless of species or photosynthetic type (e.g. Wang et al., 2009).

It is unclear to what degree the contribution of PEPC fixation in phase IV is influenced by an extension of natural daylength, as results from previous studies have proven inconclusive (Ceusters et al., 2010; Sekizuka et al., 1995). For *Phalaenopsis amabilis*, a 16-h day resulted in continuous CO_2 uptake from phase IV to phase I, suggesting fixation via PEPC at the end of the day. This was not the case for plants with a 12-h day (Guo et al., 2012). Diel PEPC activity is regulated at transcriptional and posttranslational levels via the circadian clock through phosphorylation (Hartwell, 2005; Nimmo, 2000; Taybi, 2004). This reduces the sensitivity of PEPC to inhibition by malate, which enhances (nocturnal) CO_2 fixation. Boxall et al. (2017) showed that CO_2 fixation and malate accumulation occurred also without PEPC phosphorylation, albeit to a lesser extent. PEPC activity is highest during the night, but phosphorylation can already occur in phase IV, especially in a relatively longer light period (Borland & Taybi, 2004).

Lootens and Heursel (1998) showed a continuous CO_2 uptake from phase IV to phase I with a 12-h day in vegetative *Phalaenopsis*

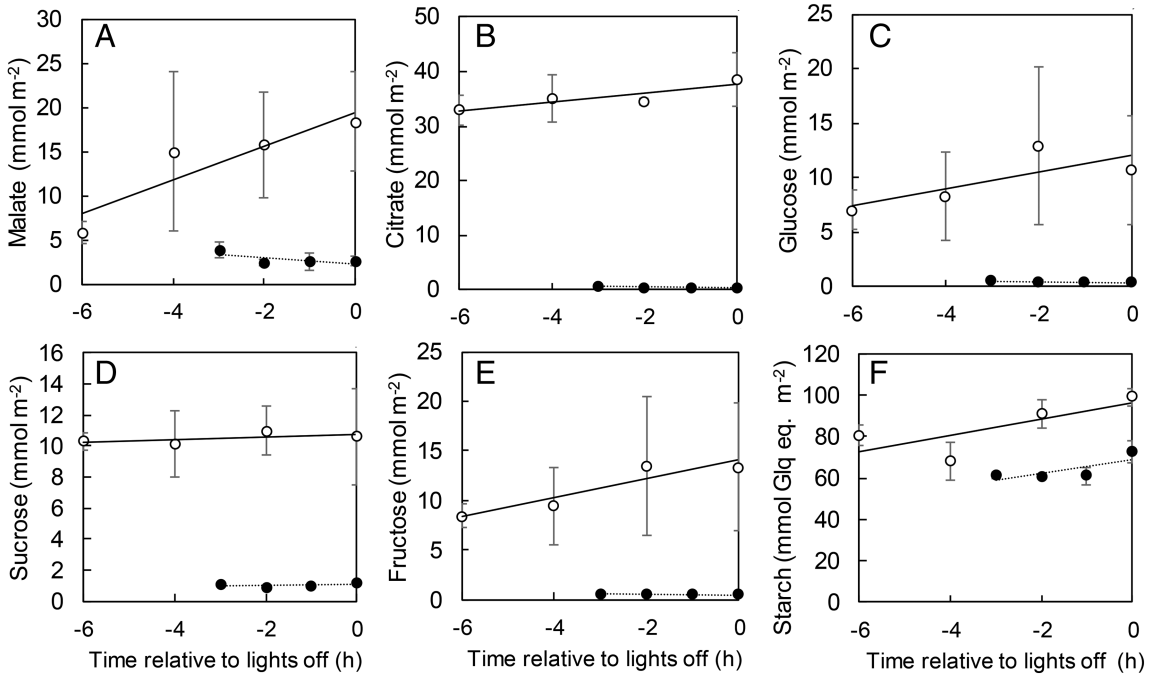

FIGURE 5 Levels of malate (A), citrate (B), glucose (C), sucrose (D), fructose (E), and starch (F) during phase IV in leaves of *K. blossfeldiana* (closed circles) and *Phalaenopsis* (open circles). Data represent the average of three independent replicates with SEM bars. Plant growth conditions as described in Figure 1. See also Appendix S4 for results on malate and starch from a complementary experiment in *Phalaenopsis*. In this supporting dataset, the error bars are much smaller, and the malate concentration increased significantly in leaves of young vegetative *Phalaenopsis* plants during phase IV

hybrids "70" and "L," indicative of mainly PEPC-mediated uptake at the end of day in shorter days. A more recent study with a 12-h day in *Phalaenopsis* hybrid "Exquisite Edessa" showed a similar continuation of CO_2 uptake in darkness, combined with a decreasing ΦPSII towards the end of the day (Zheng et al., 2019). This showed that PEPC can play a large role in CO_2 fixation during phase IV in *Phalaneopsis*.

4.3 | Futile cycling might serve as a mechanism to avoid photoinhibition

Under natural daylight conditions, Rubisco activity decreases toward the end of the day at lower light intensities, as carbamylation of active sites is light-dependent (Von Caemmerer & Edmondson, 1986). However, plants grown in a greenhouse with additional supplemental lighting, or in a climate chamber with constant light conditions until the end of the day, require a form of energy dissipation to prevent photoinhibition when Rubisco activity and C_3 carboxylation are downregulated. If Rubisco activity stays high, the Calvin-Benson cycle can function as an (indirect) electron sink to dissipate light energy (Griffiths et al., 2002). Carbon isotope discrimination data show that PEPC and Rubisco can be active at the same time (Ritz et al., 1986; Roberts et al., 1997), but this has so far been dismissed as playing only a small role in CO_2 uptake, as it is sub-optimal and increases chances of futile cycling (Griffiths et al., 1990; Maxwell et al., 1999; Winter et al., 2015). Futile cycling is the concurrent production and degradation of malate, which can occur if both carboxylation enzymes are active at the same time (Dodd et al., 2002). Instead of malate being stored in the vacuole after carboxylation via PEPC, it is immediately decarboxylated again into CO_2 and PEP via pyruvate (Osmond et al., 1996). This CO_2 might then be carboxylated again by PEPC, or it diffuses into the chloroplast where it is fixed by Rubisco, resulting in net carbon gain. When net CO_2 uptake occurred, the response of *Phalaenopsis* to 2% O_2 was similar to *K. blossfeldiana*, in the sense that CO_2 uptake increased, while ETR decreased (Table 1). These results indicate that photorespiration was occurring in both species. This makes sense in *K. blossfeldiana*, where Rubisco was the main carboxylase. In *Phalaenopsis*, there was also an increase in CO_2 uptake at 2% O_2, although this increase was less than in *K. blossfeldiana*. This indicated that Rubisco was also still active, alongside PEPC. The relative increase in $NPQ_{(T)}$ when switching to 2% O_2 was much larger in *K. blossfeldiana* than in *Phalaenopsis*. Photon costs per mol CO_2 fixed for CAM are higher than for C_3 photosynthesis (Winter & Smith, 1996). With PEPC active, this explains why photochemistry in *Phalaenopsis* can dissipate more energy than in *K. blossfeldiana*, in a situation where CO_2 might be limiting. These results are in line with the buildup of starch in *Phalaenopsis* at the end of phase IV (Figure 5). In *Phalaenopsis*, the amount of net CO_2 uptake in the last 2 h of phase IV, when PEPC was the main carboxylase based on switching

off the lights (Figure 4), is well over the amount of malate accumulated over this period (Figure 5). This indicated that both carboxylases were probably active, and that futile cycling may be indeed occurring (Borland & Griffiths, 1997). While futile cycling is considered inefficient from an energy use perspective, it might help to maintain electron sink strength in phase IV, to dissipate absorbed light energy and thus to avoid photoinhibition while still contributing to net carbon gain.

5 | CONCLUSION

With these results, we showed in several ways that PEPC can be the main carboxylase in *Phalaenopsis* "Sacramento" in phase IV while Rubisco remained active throughout the whole phase as well. This contrasted with the findings in *K. blossfeldiana* "Saja," and what is generally assumed for CAM plants. This conclusion is based on: (1) the continuous CO_2 uptake pattern at the end of the day in *Phalaenopsis* when the light was turned off, showing that CO_2 uptake was light-independent, whereas *K. blossfeldiana* showed a drop in CO_2 uptake rates. Temporarily switching off the lights while continuing to measure gas exchange showed that a decrease in CO_2 uptake occurred only at the beginning of phase IV in *Phalaenopsis* but not later on, whereas *K. blossfeldiana* showed a decrease in CO_2 uptake when lights were switched off at all-time points in phase IV. The distinct response was confirmed by differences in patterns of stomatal opening from the same dataset. This was also confirmed by (2) a decrease in ΦPSII in *Phalaenopsis* throughout phase IV, indicative of a decreasing Rubisco activity while a steady ΦPSII was measured in *K. blossfeldiana*. (3) Measurements at 2% O_2 showed that photorespiration occurred throughout phase IV in both *Phalaenopsis* and *K. blossfeldiana*. Lastly, (4) accumulation of both malate and starch occurred in phase IV in *Phalaenopsis* but not in *K. blossfeldiana*. Combining the results of gas exchange, fluorescence, and biochemical analysis, we suggest that PEPC was the main carboxylase in *Phalaenopsis* in phase IV, even though Rubisco also remained active. This seems to lead to futile cycling because of double carboxylation but can help to avoid photoinhibition. Additional research, including, e.g., online carbon isotope discrimination, would help to further strengthen these statements.

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AUTHOR CONTRIBUTIONS

Evelien van Tongerlo conducted the experiments, performed data analysis, and wrote the main part of the manuscript. Govert Trouwborst, Sander W. Hogewoning, and Wim van Ieperen contributed in the experimental planning, and together with Janneke A. Dieleman and Leo F.M. Marcelis all contributed in the writing process. All authors discussed the results and commented on the manuscript throughout the process.

DATA AVAILABILITY STATEMENT

The data that further supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

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