RESEARCH ARTICLE

Kalanchoë PPC1 is Essential for Crassulacean Acid Metabolism and the Regulation of Core Circadian Clock and Guard Cell Signaling Genes

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Short Title: Silencing PPC1 in an obligate CAM plant

One-sentence Summary: Silencing phosphoenolpyruvate carboxylase in a Crassulacean acid metabolism species prevented nocturnal CO2 fixation and malate accumulation and perturbed the circadian clock and guard cell signalling.

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ABSTRACT

Unlike C3 plants, Crassulacean acid metabolism (CAM) plants fix CO2 in the dark using phosphoenolpyruvate (PEP) carboxylase (PPC; EC 4.1.1.31). PPC combines PEP with CO2 (as HCO3-), forming oxaloacetate. The oxaloacetate is converted to malate, leading to malic acid accumulation in the vacuole, which peaks at dawn. During the light period, malate decarboxylation concentrates CO2 around RuBisCO for secondary fixation. CAM mutants lacking PPC have not been described. Here, we employed RNAi to silence the CAM isogene PPC1 in Kalanchoë laxiflora. Line rPPC1-B lacked PPC1 transcripts, PPC activity, dark period CO2 fixation, and nocturnal malate accumulation. Light period stomatal closure was also perturbed, and the plants displayed reduced but detectable dark period stomatal conductance and arrhythmia of the CAM CO2 fixation circadian rhythm under constant light and temperature free-running conditions. By contrast, the rhythm of delayed fluorescence was enhanced in plants lacking PPC1. Furthermore, a subset of gene transcripts within the central circadian oscillator were upregulated and oscillated robustly in this line. The regulation of guard cell genes involved in controlling stomatal movements was also perturbed in rPPC1-B. These findings provide direct evidence that the regulatory patterns of key guard cell signaling genes are linked with the characteristic inverse pattern of stomatal opening and closing during CAM.

INTRODUCTION

Crassulacean acid metabolism (CAM) is a pathway of photosynthetic CO2 fixation found in species adapted to low rainfall and/or periodic drought, such as the
Madagascan-endemic succulent, *Kalanchoë laxiflora* Baker (Family: Crassulaceae; Order: Saxifragales) (Hartwell et al., 2016). CAM species open their stomata for primary atmospheric CO$_2$ fixation in the dark, when their environment is cooler and more humid, and close their stomata in the light, when the atmosphere is at its hottest and driest (Osmond, 1978). The increased water use efficiency (WUE) and CO$_2$ fixation efficiency of CAM species have led to the proposal that the use of productive CAM crop species, including certain *Agave* and *Opuntia* species, represents a viable approach to generating biomass for biofuels and renewable platform chemicals for industry through their cultivation on seasonally-dry lands that are not well-suited to food crop production (Borland et al., 2009; Cushman et al., 2015). Furthermore, efforts are underway to engineer CAM into key C$_3$ crops (Borland et al., 2014; DePaoli et al., 2014; Borland et al., 2015; Lim et al., 2019).

Recently, genome, transcriptome, proteome, and metabolome datasets for a phylogenetically diverse range of CAM plants of independent origin have started to open up a large catalogue of putative CAM genes. CAM species represented by published 'omics datasets span orchids (*Phalaenopsis equestris* and *Erycina pusilla*, Orchidaceae, monocots), pineapple (*Ananas comosus*, Bromeliaceae, monocots), *Agave* (*A. americana*, *A. desertii*, *A. tequilana*, Agavaceae, monocots), *Yucca* (*Y. aloifolia*, Asparagaceae, monocots), *Kalanchoë* (*K. fedtschenkoii* and *K. laxiflora*, Crassulaceae, eudicots), *Mesembryanthemum* (*M. crystallinum*, Aizoaceae, eudicots), and waterleaf (*T. triangulare*, Portulacaceae, eudicots) (Cushman et al., 2008; Gross et al., 2013; Cai et al., 2015; Ming et al., 2015; Abraham et al., 2016; Brilhaus et al., 2016; Hartwell et al., 2016; Yang et al., 2017; Heyduk et al., 2018; Heyduk et al., 2019). The optimal exploitation of CAM will be facilitated through decoding the molecular-genetic blueprint for CAM from these genomes and transcriptomes. In turn, to be fully realised, this opportunity requires functional genomics approaches using transgenic and/or mutant lines of model CAM species to test and define in detail candidate CAM gene functions (Dever et al., 2015; Hartwell et al., 2016; Boxall et al., 2017).

During CAM, primary nocturnal CO$_2$ assimilation is catalyzed by phosphoenolpyruvate (PEP) carboxylase (PPC), generating oxaloacetate, which is rapidly converted to malate and stored in the vacuole as malic acid (Borland et al., 2009). At dawn, malic acid is transported out of the vacuole and malate is decarboxylated in the mitochondria, chloroplasts and/or cytosol, and the released
CO₂ is re-fixed by RuBisCO behind closed stomata (Borland et al., 2009). Strict temporal control prevents a futile cycle between the enzymes and metabolite transporters driving malate production in the dark and those driving malate decarboxylation in the light (Hartwell, 2006). PPC regulation is central to this temporal control. PPC is activated allosterically by glucose 6-phosphate (G6P) and inhibited by malate, aspartate and glutamate (O’Leary et al., 2011). The circadian clock optimizes the timing of the CAM carboxylation and decarboxylation pathways to prevent futile cycling (Wilkins, 1992; Hartwell, 2005).

Temporal optimization of PPC involves protein phosphorylation in the dark period, a process catalysed by the circadian clock-controlled protein kinase, phosphoënoëpyruvate carboxylase kinase (PPCK) (Carter et al., 1991; Hartwell et al., 1996; Hartwell et al., 1999; Taybi et al., 2000; Boxall et al., 2005). Phosphorylated PPC is less sensitive to feedback inhibition by malate, which in turn ensures sustained CO₂ fixation as malic acid accumulates throughout the dark period (Nimmo et al., 1984; Carter et al., 1991; Boxall et al., 2017). In the light, PPC becomes more sensitive to inhibition by malate due to dephosphorylation by a protein phosphatase type 2A (PP2A), which is not known to be subject to circadian control (Carter et al., 1990).

In CAM species such as Kalanchoë, the PEP substrate required for nocturnal atmospheric CO₂ fixation by PPC is generated through starch breakdown and glycolysis (Borland et al., 2016). In the C₃ model species Arabidopsis thaliana, nocturnal degradation of leaf starch begins with the phosphorylation of glucan chains by GLUCAN WATER DIKINASE (GWD) and PHOSPHOGLUCAN WATER DIKINASE (PWD) (Ritte et al., 2006). The phosphorylated glucan chains are then further degraded by ALPHA-AMYLASES (AMYs) and BETA-AMYLASES (BAMs) to maltose and glucose. Nocturnal starch hydrolysis by BAMs is the predominant pathway in C₃ leaves, with chloroplastic BAM3 being the major BAM isozyme driving nocturnal starch degradation in photosynthetic leaf mesophyll cells of Arabidopsis (Fulton et al., 2008). BAM1, BAM2 and BAM5-9 are not required for nocturnal starch degradation in Arabidopsis leaf mesophyll cells (Santelia and Lunn, 2017). Maltose and glucose are exported from chloroplasts by MALTOSE EXCESS1 (MEX1) and PLASTIDIC GLUCOSE TRANSPORTER (pGlcT), respectively, with MEX1 being the predominant C₃ route for carbon export (Smith et al., 2005). In the facultative CAM
species *M. crystallinum*, chloroplasts possess transporters for triose phosphate (TPT), G6P (GPT), glucose (pGlcT) and maltose (MEX1) (Neuhaus and Schulte, 1996; Kore-eda et al., 2005; Kore-eda et al., 2013). Chloroplasts from C_3 leaves of *M. crystallinum* exported maltose during starch degradation, whereas chloroplasts isolated from CAM-induced leaves predominantly exported G6P, supporting the proposal that starch is broken down via plastidic starch phosphorylase (PHS1) during nocturnal CO_2 fixation (Neuhaus and Schulte, 1996).

A further defining characteristic of CAM relates to nocturnal stomatal opening and CO_2 uptake, and light period stomatal closure during malate decarboxylation and peak internal CO_2 supply (Males and Griffiths, 2017). Stomatal control in CAM species is the inverse of the stomatal regulation observed in C_3 species (Borland et al., 2014). The opening and closing of stomata are driven by the turgor of the guard cell (GC) pair that surrounds the stomatal pore. High turgor drives stomatal opening, and a reduction in turgor leads to stomatal closure. The increase in turgor during opening is driven by the accumulation of K^+, Cl^- and malate^{2-} ions plus sugars in the GCs (Jezek and Blatt, 2017). The closure of stomata is driven by a reversal of GC ion channels and metabolism, with K^+ and Cl^- being transported out, and metabolites being turned over within the GCs. Stomatal aperture responds to changing light, CO_2, abscisic acid (ABA), solutes and water availability (Kim et al., 2010; Daloso et al., 2016; Horrer et al., 2016; Zhang et al., 2018; Yoshida et al., 2019).

In addition to its role in CAM and C_4 plants, PPC performs an anapleurotic function by replenishing tricarboxylic acid cycle intermediates utilized for amino acid biosynthesis (Chollet et al., 1996). PPC also functions in the formation of malate as a counter anion for light period opening in C_3 GCs and supports nitrogen fixation into amino acids in legume root nodules (Chollet et al., 1996). The major leaf PPCs in Arabidopsis, which are encoded by *PPC1* (AT1G53310) and *PPC2* (AT2G42600), are crucial for leaf carbon and nitrogen metabolism (Shi et al., 2015). Compared to the wild type, the double *ppc1 ppc2* null mutant accumulates less starch and sucrose and has reduced malate, citrate, and ammonium assimilation; these metabolic changes lead to a severe, growth-arrested phenotype (Shi et al., 2015).

Although PPC catalyses primary CO_2 fixation in CAM and C_4 plants, the only reported PPC mutants are for the C_4 eudicot grain amaranth (*Amaranthus edulis*) and the C_4 monocot green foxtail (*Setaria viridis*) (Dever et al., 1995; Alonso-Cantabrana et al., 2018). C_4 PPC catalyzes light period primary CO_2 fixation in
mesophyll cells, generating malate or aspartate, which is shuttled to bundle sheath cells for decarboxylation (von Caemmerer and Furbank, 2003). This leads to the concentration of CO$_2$ around RuBisCO, which is only present in bundle sheath cells. Photorespiration is thus minimised. The loss of the C$_4$ PPC isogene in A. edulis caused a severe and lethal growth phenotype in normal air, with the homozygous mutant plants only managing to reach flowering and set seed when grown at highly elevated CO$_2$ (Dever et al., 1995). In S. viridis, the C$_4$ PPC expression was reduced to very low levels using RNAi in transgenic lines (Alonso-Cantabrana et al., 2018). These lines grew very slowly even at 2% CO$_2$ (normal air is 0.04%) and developed increased numbers of plasmodesmatal pit fields at the mesophyll-bundle sheath interface (Alonso-Cantabrana et al., 2018). By contrast, no CAM mutants lacking PPC have been described, but transgenic lines of Kalanchoë lacking the light-period, decarboxylation pathway enzymes mitochondrial NAD-malic enzyme (NAD-ME) and pyruvate orthophosphate dikinase (PPDK) displayed a near complete loss of dark CO$_2$ fixation and failed to turnover significant malate during the light period (Dever et al., 2015). In Kalanchoë, NAD-ME catalyses the conversion of malate to pyruvate and CO$_2$ during the light period, and PPDK converts the pyruvate to PEP, allowing subsequent recycling of the by-product of malate decarboxylation through gluconeogenesis to stored starch (Dever et al., 2015). Another CAM mutant lacking the starch synthesis pathway enzyme plastidic phosphoglucomutase has been reported in the inducible CAM species M. crystallinum (Cushman et al., 2008).

In addition, the CAM-associated PPCK1 gene in Kalanchoë was silenced using RNAi, which not only led to a reduction in dark period CO$_2$ fixation, but also perturbed the operation of the central circadian clock (Boxall et al., 2017). However, even the strongest PPCK1 RNAi line was still able to achieve ~33% of the dark period CO$_2$ fixation observed in the wild type (Boxall et al., 2017). These findings led us here to develop transgenic K. laxiflora lines in which the CAM-associated isogene of PPC itself (isogene PPC1) was downregulated using RNAi. The most strongly silenced line, rPPC1-B, lacked PPC1 transcripts and activity, and this resulted in the complete loss of dark CO$_2$ fixation associated with CAM, and arrhythmia of the CAM CO$_2$ fixation rhythm under constant light and temperature (LL) free-running conditions. Growth of rPPC1-B plants was reduced relative to wild type under both well-watered and drought-stressed conditions. The plants reverted to fixing CO$_2$ in the light, especially in their youngest leaf pairs. Although the circadian rhythm of CO$_2$
fixation dampened rapidly towards arrhythmia in the \textit{rPPC1-B} line, the distinct circadian clock output of delayed fluorescence, and the oscillations of the transcript abundances of a subset of core circadian clock genes, were enhanced. Furthermore, the temporal phasing of a wide range of GC specific signaling genes involved in opening and closing was perturbed relative to the wild type in \textit{rPPC1-B}. These findings shed light on important regulators underpinning the inverse stomatal control associated with CAM.

\textbf{RESULTS}

\textbf{Initial Screening and Characterisation of \textit{PPC1} RNAi Lines of \textit{K. laxiflora}}

As \textit{K. laxiflora} is relatively slow-growing, with flowering and seed set taking approximately 9-months seed-to-seed (Hartwell et al., 2016), data are presented for independent primary transformants that were propagated clonally via leaf margin plantlets and/or stem cuttings. We initially screened primary transformants using high-throughput leaf disc tests for starch and acidity at dawn and dusk (Cushman et al., 2008; Dever et al., 2015). We screened independent transgenic lines that acidified less during the dark period than the wild type for the steady-state abundance of \textit{PPC1} transcripts using RT-qPCR (Figure 1). Line \textit{rPPC1-B} displayed a complete loss of \textit{PPC1} transcripts, whereas \textit{rPPC1-A} had an intermediate level of \textit{PPC1} transcripts (Figure 1A). The other plant-type \textit{PPC} genes (\textit{PPC2}, \textit{PPC3} and \textit{PPC4}) were up-regulated relative to the wild type, with their peak phased to dawn (Figure 1B to 1D). \textit{PPC2} was up-regulated in line \textit{rPPC1-B} at 6 and 10 h into the 12 h dark period (Figure 1B).

In line \textit{rPPC1-B}, \textit{PPCK1}, encoding the protein kinase that phosphorylates the CAM-associated protein \textit{PPC1}, was down-regulated during its dark period phased peak and appeared to be slightly up-regulated at 2 h after dawn (Figure 1E). The other two detectable \textit{PPCK} genes, \textit{PPCK2} and \textit{PPCK3}, were up-regulated in \textit{rPPC1-B}, with \textit{PPCK2} induced 5-fold when it reached its 24 h peak in the middle of the dark period, 4 h after the wild-type peak (Figure 1F). \textit{PPCK3} peaked in the middle of the light period, when it reached a level almost 8-fold greater than the wild type (Figure 1G).
Loss of PPC1 Transcripts lead to Loss of PPC Protein and Activity

Immunoblotting using an antibody raised against purified CAM-specific PPC protein from K. fedtschenkoi leaves (Nimmo et al., 1986) (Figure 2) revealed reduced levels of PPC protein in rPPC1-A and no detectable PPC in rPPC1-B (Figure 2A).

We also measured the level of phospho-PPC using immunoblotting (Figure 2B). Phospho-PPC levels were lower in rPPC1-A than the wild type, whilst rPPC1-B lacked detectable phospho-PPC (Figure 2B), which was consistent with the level of PPC (Figure 2A). Although PPC2 was up-regulated in rPPC1-B (Figure 1B), it was not detected at the protein level by the PPC antibody (Figure 2A). Furthermore, despite PPCK2 and PPCK3 being up-regulated by 5- to 8-fold in rPPC1-B (Figure 1F and 1G), any protein produced from these transcripts did not phosphorylate any PPC2 protein that may have resulted from the induced PPC2 transcripts (Figure 2B), at least not within the limits of detection with this immunoblotting technique. We used rapidly desalted leaf extracts to measure the apparent K_i of PPC for L-malate. The K_i was higher in the dark than the light for wild type and rPPC1-A, but no change in the K_i was detected for rPPC1-B (Supplemental Figure 1). Furthermore, PPC activity was not detected in rapidly desalted extracts from rPPC1-B leaves, whereas rPPC1-A displayed reduced but detectable PPC activity, at 43% of the wild-type level (Figure 2C and Supplemental Table 1).

Pyruvate orthophosphate dikinase (PPDK), which functions in concert with NAD-ME during the light-period as part of the CAM malate decarboxylation pathway (Dever et al., 2015), was measured on immunoblots using specific antibodies against PPDK and phospho-PPDK (Chastain et al., 2000; Chastain et al., 2002). The blots showed a similar amount of PPDK protein over the diel cycle in wild type, rPPC1-A and rPPC1-B (Figure 2D), confirming the even protein loading of the blots, which was further supported by the images of Coomassie stained gels (Supplemental Figure 1). In Kalanchoë, PPDK is inactivated in the dark by phosphorylation by PPDK-regulatory protein (PPDK-RP), which also activates PPDK in the light through dephosphorylation (Dever et al., 2015). In the wild type, immunoblotting of phospho-PPDK revealed that PPDK was dephosphorylated, and therefore likely to be fully active, between 02:00 and 06:00 in the light when it is required for the conversion of pyruvate, from malate decarboxylation, to PEP, thereby facilitating the recycling of carbon through gluconeogenesis to starch (Figure 2E). Line rPPC1-A showed the
same pattern of PPDK phosphorylation/de-phosphorylation as the wild type (Figure 2E). However, PPDK was phosphorylated throughout the 24 h cycle in rPPC1-B (Figure 2E), and was therefore likely to be inactive. Consistent with this prediction, loss of the light period dephosphorylation of PPDK in rPPC1-B correlated with a significant decrease in PPDK activity in the light, whereas the wild type and line rPPC1-A showed strongly light-induced levels of PPDK activity that correlated with the detected level of PPDK dephosphorylation in the light (Figure 2F and Supplemental Table 2).

**Malate, Starch, and Soluble Sugar Levels**

During CAM in *Kalanchoë*, primary nocturnal fixation of atmospheric CO$_2$ (as HCO$_3^-$) results in vacuolar malic acid accumulation throughout the dark period. Starch accumulates during the light period and is broken down during the dark period to provide PEP as the substrate for carboxylation by PPC. Starch is also broken down in a rapid burst at dawn to form soluble sugars (Wild et al., 2010; Boxall et al., 2017). As the lack of CAM-associated PPC1 was predicted to prevent primary nocturnal carboxylation, we measured metabolites including malate, starch and soluble sugars every 4 h over the 24 h cycle (Figure 3).

Wild-type plants accumulated 130 µmols gFW$^{-1}$ malate by dawn, whereas rPPC1-A and rPPC1-B accumulated 75 µmol gFW$^{-1}$ and 19.5 µmols gFW$^{-1}$, respectively (Figure 3A). The Δ-malate values for wild type, rPPC1-A and rPPC1-B were 124.0, 64.3 and 16.2 µmol gFW$^{-1}$, respectively (Figure 3A). During the diel cycle, rPPC1-A and rPPC1-B synthesized 100% and 41% of the amount of starch accumulated by the wild type, respectively (Figure 3B). The Δ-starch values for wild type, rPPC1-A and rPPC1-B were 8.5, 8.5 and 3.5 mg starch gFW$^{-1}$ (Figure 3B). Lines rPPC1-A and rPPC1-B accumulated 51% and 15% of the amount of sucrose accumulated by the wild type (Figure 3C), and 83% and 69% of the level of glucose, respectively (Figure 3D). Glucose accumulated 4 h after the sucrose peak in the wild type, whereas glucose levels peaked at the same time as sucrose in lines rPPC1-A and rPPC1-B (Figure 3C and 3D). Finally, lines rPPC1-A and rPPC1-B accumulated, respectively, 113% and 61% of the amount of fructose compared with the wild type (Figure 3E). In rPPC1-B, the daily maxima for fructose and glucose coincided with
that of sucrose at 2 h after dawn (Figure 3C to 3E). Whilst no direct evidence was obtained to explain these shifts in the timing of the sucrose, glucose and fructose daily maxima, the most straightforward explanation is that the observed changes in the timing of photosynthetic CO$_2$ fixation and starch accumulation and turnover in the \textit{rPPC1-B} transgenic line resulted in the observed changes in the timing of the soluble sugar peaks.

**Growth Analysis in Well-Watered versus Drought-Stressed Conditions**

CAM is widely regarded as an adaptation to drought, and so it was important to compare the growth performance of wild type (full CAM) with that of \textit{rPPC1-A} (small reduction in CAM) and \textit{rPPC1-B} plants (no CAM) under both well-watered (WW) and drought-stressed (D-S) conditions (Figure 3F, Supplemental Figure 2 and Supplemental Data Set 1). \textit{rPPC1-B} plants were significantly smaller than wild type in both WW and D-S conditions (Figure 3F and Supplemental Figure 2). In WW conditions, the shoot dry weight of \textit{rPPC1-B} was 21% less than that of the wild type (Figure 3F), but the shoot dry weight of \textit{rPPC1-A} was not significantly different from that of the wild type. Under D-S, the shoot dry weight of line \textit{rPPC1-B} was 12% lower than that of the wild type (Figure 3F). A visual inspection of representative 4-month-old plants demonstrated that \textit{rPPC1-B} was smaller than wild type and \textit{rPPC1-A} (Figure 3G), which is consistent with the shoot dry weight data (Figure 3F). Shoot fresh weight was also significantly reduced in WW and D-S \textit{rPPC1-B}, whereas the only significant difference in below-ground root tissues was for \textit{rPPC1-B} under drought-stress, which displayed an increase in root fresh weight that was only just significant (p = 0.0483; Supplemental Figure 2). Furthermore, \textit{rPPC1-B} displayed a reduced degree of leaf in-rolling in response to drought relative to the wild type (Supplemental Figure 3), even though it lost just as much water during drought as the wild type (Supplemental Figure 2).

**Gas Exchange Characteristics under Light/Dark Cycles**

We measured gas exchange in mature CAM leaves (leaf pair 6, LP6) of each line over a 12-h-light, 25°C, 60% humidity/ 12-h-dark, 15°C, 70% humidity cycle using an infra-red gas analyser (IRGA; LI-COR LI-6400XT) (Figure 4; 4A to 4C). Over the 24
275 diel cycle, wild type fixed 297 µmol CO₂ m⁻², rPPC1-A fixed 320 µmol CO₂ m⁻², and 276 rPPC1-B fixed 230 µmol CO₂ m⁻² (Figure 4A). Net CO₂ fixation was 23% less than 277 the wild type in line rPPC1-B, whereas it was 15% greater in line rPPC1-A (Figure 278 4A).

279 The four phases of CAM (Osmond, 1978) are indicated on the CO₂ exchange 280 graph in Figure 4A. Phase I corresponds to the period of nocturnal atmospheric CO₂ 281 fixation to vacuolar malic acid in the wild type, and phases II, III and IV define the 282 peaks and trough of CO₂ fixation and stomatal conductance across the 12-h-light 283 period (Figure 4A and 4B). Specifically, phase II refers to the sharp peak of CO₂ 284 fixation in the 1 to 2 hours after dawn, phase III defines the period of refixation CO₂ 285 from malate decarboxylation by RuBisCO behind closed stomata that spans the 286 middle of the light period, and phase IV occurs in younger leaves of well-watered 287 plants and corresponds to the period in the late afternoon when stomata re-open and 288 atmospheric CO₂ is fixed directly by RuBisCO (Osmond, 1978). Note that the data in 289 blue for rPPC1-A provide the best example of this four phases framework for CAM 290 CO₂ fixation and stomatal physiology (Figure 4A). By contrast, LP6 of the wild type 291 perform full CAM defined by phase I in the dark and phase III in the light (Figure 4A). 292 By calculating the total area under or over the CO₂ exchange curves in Figure 4A, 293 and calculating these areas separately for the 12-h-light and the 12-h-dark periods, it 294 was possible to quantify the amounts of CO₂ fixed by each line during either the light 295 period or the dark period. In the light period (phases II through IV), wild type leaves 296 fixed negligible amounts of atmospheric CO₂, whereas rPPC1-B fixed a total of 265 297 µmol m⁻², and rPPC1-A fixed 89 µmol m⁻² (Figure 4A). In the dark period (phase I), 298 wild type fixed 297 µmol CO₂ m⁻² and rPPC1-A fixed 226 µmol CO₂ m⁻², but rPPC1-B 299 respired 35 µmol CO₂ m⁻² (Figure 4A). The loss of nocturnal CO₂ fixation in rPPC1-B 300 (Figure 4A) corresponded with the lack of the CAM-associated PPC1 (Figure 1 and 301 2). LP6 of rPPC1-A fixed 24% less nocturnal CO₂ than wild type, but, in contrast to 302 wild type, continued some light period CO₂ capture (Figure 4A).

303 Apart from opening briefly for phase II, just after lights-on, the attached LP6 of 304 the wild type closed its stomata in the light and opened them throughout the dark, 305 when stomatal conductance (gₛ) tracked CO₂ uptake (Figure 4B). In rPPC1-B, 306 stomata stayed open in the light, closed briefly at dusk, and opened slightly 307 throughout the dark, with a small peak prior to dawn (Figure 4B). The dark 308 conductance of rPPC1-B corresponded with the release of respiratory CO₂ (Figure
The calculated internal partial pressure of CO\textsubscript{2} inside the leaf (C\textsubscript{i}) was highest for the wild type in the light period, when stomata were closed, which is consistent with the expected high level of CO\textsubscript{2} inside the leaf during malate decarboxylation (Figure 4C). By contrast, in rPPC1-B, C\textsubscript{i} peaked during the dark period, when stomata were slightly open, but the leaves failed to fix atmospheric CO\textsubscript{2}, and respiratory CO\textsubscript{2} escaped (Figure 4C).

**Impact of Drought on CO\textsubscript{2} uptake in Plants lacking PPC1**

To test the importance of PPC1 for carbon assimilation during drought, we measured CO\textsubscript{2} uptake continuously in whole, young plants (9-leaf-pairs; 2-months-old) over 22-days of drought, followed by re-watering (Figure 4D). The soil was watered to its holding capacity prior to placing the plants in the gas exchange cuvettes at the start of the experiment, and then no further water was added until re-watering occurred on day 22. CAM develops with leaf age in K. laxiflora; the leaves gradually reduce light period CO\textsubscript{2} fixation and increase nocturnal CO\textsubscript{2} fixation as they mature (Supplemental Figure 4). A 2-month-old wild-type plant with 9-leaf-pairs thus includes young leaves fixing CO\textsubscript{2} mainly in the light via the C\textsubscript{3} pathway, and older leaves fixing the majority of their atmospheric CO\textsubscript{2} in the dark via PPC and CAM.

When WW on day 1, wild type, rPPC1-A and rPPC1-B fixed, respectively, 7%, 6% and -26% of their CO\textsubscript{2} during the dark period and 93%, 94% and 126% during the light period (Table 1). This indicated that, in well-watered conditions, the young leaves of these young plants performed the majority of the 24 h CO\textsubscript{2} uptake (Table 1). On day 1, wild type, rPPC1-A and rPPC1–B fixed a total of 1093, 1008 and 1078 µmol of atmospheric CO\textsubscript{2} m\textsuperscript{-2} over 24 h, respectively (Table 1). After 7-days without watering, 24 h CO\textsubscript{2} fixation was 1345, 1367 and 1506 µmol m\textsuperscript{-2}, respectively. It should be noted that total leaf area was measured at the end of the experiment, so leaf growth and expansion during the experiment could not be accounted for.

After 7-days without water, there was a substantial increase in nocturnal CO\textsubscript{2} uptake in wild type and rPPC1-A relative to day 1 (52% and 40% of CO\textsubscript{2} fixation occurred in the dark, respectively; Table 1). rPPC1-B respired less (-14%) after 7-days of drought relative to the -26% dark respired CO\textsubscript{2} level on day 1 (Table 1). After 13-days of drought, wild type, rPPC1-A and rPPC1-B fixed, respectively, 795, 802 and 145 µmol CO\textsubscript{2} m\textsuperscript{-2} over the 24 h cycle (Table 1). Thus, plants performing CAM (wild type and rPPC1-A) were able to fix over 5-fold more CO\textsubscript{2} after 13-days of
drought compared to \textit{rPPC1-B}. Furthermore, after 22-days of drought, wild type, \textit{rPPC1-A} and \textit{rPPC1-B} fixed 76, 130 and -30 µmol m\(^{-2}\) during the 24 h light/dark cycle, respectively.

On day 22 without water, the drought-stressed plants were re-watered (see photos in Supplemental Figure 3). After re-watering, CO\(_2\) fixation increased rapidly for all plants (Figure 4D). In addition, wild type and \textit{rPPC1-A} displayed pronounced phase III of CAM after re-watering (Figure 4D). Following soil rehydration, the wild type fixed 931 µmol m\(^{-2}\) CO\(_2\) in the dark, compared to 768 µmol m\(^{-2}\) for \textit{rPPC1-A}, whereas prior to drought, \textit{rPPC1-A} fixed more atmospheric CO\(_2\) (Table 1). \textit{rPPC1-B} fixed CO\(_2\) throughout the light period following re-watering, and it also resumed respiratory CO\(_2\) loss throughout the dark period (Figure 4D).

As there was an increase in C\(_3\) photosynthesis in \textit{rPPC1-B}, we measured chlorophyll \textit{a} and \textit{b} levels in LP2 through LP7 (Figure 4E and 4F and Supplemental Table 3). Line \textit{rPPC1-A} contained significantly more chlorophyll \textit{a} and \textit{b} than wild type in LP2 (Figure 4E and 4F). \textit{rPPC1-B} contained significantly more chlorophyll \textit{a} than the wild type in LP2, but significantly less chlorophyll \textit{a} in LP3 through LP7, and significantly less chlorophyll \textit{b} in LP3 to LP6 (Figure 4E and 4F). This decreased level of chlorophyll \textit{a} and \textit{b} in LP3 to LP7 or LP6 in \textit{rPPC1-B} was a surprising finding. In general, CAM species contain reduced levels of gene transcripts and encoded proteins associated with C\(_3\) photosynthesis, such as RuBisCO and light harvesting complex components, when the level of CAM is induced by stress. Thus, reducing the level of CAM in leaf pair 3 through 7 due to the silencing of \textit{PPC1} was predicted to result in the induction of core C\(_3\) photosynthesis genes and chlorophyll content in parallel with the measured increase in light period atmospheric CO\(_2\) fixation via the C\(_3\) pathway. Further elucidation of the reasons for this unexpected chlorophyll response may come from transcriptome-wide analysis of the differentially abundant transcripts in \textit{rPPC1-B} compared to the wild type.

**Characterisation of CAM Gene Transcript Abundance in \textit{rPPC1 Lines}**

Having established that \textit{rPPC1-B} lacked nocturnal CO\(_2\) fixation (Figure 4A), it was important to investigate the temporal regulation of other CAM-associated genes in the \textit{rPPC1} RNAi lines. We investigated the transcript abundance of CAM genes in CAM leaves (LP6) using samples collected every 4 h over a 12-h-light/12-h-dark
cycle (Figure 5). PPDK transcript levels were unchanged relative to the wild type in both rPPC1 lines (Figure 5A), but its regulator gene PPDK-RP was up-regulated in line rPPC1-B (Figure 5B), which is consistent with the continuous phosphorylation and inactivation of PPDK (Figure 2E and 2F). β-NAD-ME transcript levels appeared to be only slightly different from the wild type in the rPPC1 lines but appeared to be lower in rPPC1-B at 22:00 (Figure 5C).

In light of the marked reduction in starch to half the wild-type level in rPPC1-B (Figure 3B), we also measured transcripts associated with starch turnover. In rPPC1-B, dusk-phased starch breakdown-associated genes (GWD, AMY3a and 3b, PHS1; Figure 5D to 5F and 5J) and sugar transporter genes (MEX1, pGlcT; Figure 5L and 5M) were up-regulated, whereas dawn-phased starch breakdown genes (BAM1, BAM3, BAM9; Figure 5G to 5I) and sugar transporter genes (GPT2; Figure 5K) were down-regulated compared to the wild type.

Finally, a CHLOROPHYLL A/B BINDING PROTEIN (CAB1) gene was up-regulated in rPPC1-B at dawn (Figure 5N), whereas a gene encoding a potential sucrose sensor connecting growth and development to metabolic status, TREHALOSE 6-PHOSPHATE SYNTHASE7 (TPS7) (Schluepmann et al., 2003), was down-regulated relative to the wild type at 6 and 10 h into the 12-h-dark period (Figure 5O).

Characterisation of Diel Regulation of Circadian Clock Genes in rPPC1 Lines

Recent studies using PPCK1 RNAi lines in Kalanchoë reported that a reduced temporal peak of sucrose content phased to 2 h after dawn correlated with perturbation of the central circadian clock (Boxall et al., 2017). Of the two CIRCADIAN CLOCK ASSOCIATED1 (CCA1) genes in K. laxiflora, each of which is represented by two homeologous copies in the tetraploid genome, only the two homeologs of CCA1-2 were down-regulated in rPPC1-B (Figure 5P and 5Q), whereas both homeologs of both TIMING OF CHLOROPHYLL A/B BINDING PROTEIN1 (TOC1) genes were up-regulated in both rPPC1-A and rPPC1-B (Figure 5R and 5S). Two other pseudo-response regulators (PRRs) related to TOC1, namely PRR7 and PRR3/7, were up-regulated in both rPPC1-A and rPPC1-B (Figure 5T and
5U), and PRR9 was induced almost 5-fold in the middle of the light period, specifically in line rPPC1-B (Figure 5V).

Other clock associated genes, including JUMONJI DOMAIN CONTAINING30/5 (JMJD30/ JMJD5), EARLY FLOWERING3 (ELF3) and CYCLING DOF-FACTOR2 (CDF2) were also up-regulated in line rPPC1-B (Figure 5W to 5Y) vs. the wild type, whereas the single MYB-repeat transcription factor genes REVEILLE1-like (RVE1-like) and EARLY-PHYTOCHROME-RESPONSIVE1 (EPR1), and LIGHT NIGHT-INDUCIBLE AND CLOCK-REGULATED3-like (LNK3-like) were down-regulated (Figure 5Z to 5BB). Finally, CCA1-1, FLAVIN-BINDING KELCH-REPEAT F-BOX PROTEIN1 (FKF1) and GIgANTEA (GI) transcript levels were consistent among all three lines (Figure 5P, 5CC and 5DD).

Gas Exchange Characteristics of rPPC1 Lines under Circadian Free Running Conditions

Under constant light and temperature (LL) free-running conditions, detached K. laxiflora wild type CAM leaves (LP6) with their petioles in water displayed a robust circadian rhythm of CO₂ uptake with a period of approximately 20 h (Figure 6; 6A). This rhythm was entirely consistent with CAM rhythms reported previously for detached LP6 of closely related Kalanchoë species with obligate CAM (Lüttge and Ball, 1978; Anderson and Wilkins, 1989). The rhythm dampened rapidly in line rPPC1-B, whereas rPPC1-A maintained a rhythm that was very similar to that of the wild type (Figure 6A).

When LL CO₂ uptake was measured for well-watered whole plants (2 months old; 9-leaf pairs), rPPC1-B fixed more CO₂ than the wild type and line rPPC1-A, with levels of 11,269, 7414, 8383 µmol CO₂ m⁻², respectively (Figure 6B). Wild type maintained robust oscillations of CO₂ exchange under LL conditions, whereas rPPC1-A dampened to arrhythmia after 3-days, and rPPC1-B was arrhythmic (Figure 6B). It should be noted that the LL CO₂ exchange data for intact young plants with their roots in soil (Figure 6B) differs from the data for detached LP6 (Figure 6A) because the whole plants measured in Figure 6B included leaf pairs from very young LP1, which perform C₃, through to LP6 to LP9 that perform full CAM (phase I in the dark and III in the light). The detached LP6 measured in Figure 6A were full CAM leaves only, at least for the wild type (Supplemental Figure 4). Thus, the more rapid
and substantial dampening of the free-running CO$_2$ fixation rhythm towards arrhythmia in lines $rPPC1$-A and $rPPC1$-B relative to the wild type (Figure 6B), suggests that the younger leaves (LP1 to LP5) made a dominant contribution to the robust circadian rhythm in wild-type whole plants.

**Delayed Fluorescence Rhythms are Enhanced in $rPPC1$-B Despite the Loss of CAM-Associated CO$_2$ Uptake Rhythms**

Delayed fluorescence (DF) displays a robust circadian rhythm in *Kalanchoë* and provides a measure of a chloroplast-derived clock-output that can be used for statistical analysis of circadian period, robustness and accuracy (Gould et al., 2009; Boxall et al., 2017). We therefore measured and analysed DF under LL (Figure 6C) to calculate rhythm statistics using Biodar (Moore et al., 2014; Zielinski et al., 2014). Wild type DF oscillations were very similar to those reported previously for *K. fedtschenko* (Figure 6C; Gould et al., 2009; Boxall et al., 2017). $rPPC1$-A and $rPPC1$-B had more robust oscillations than the wild type (Figure 6C). The rhythm amplitude increased slightly with time in $rPPC1$-A and $rPPC1$-B but remained relatively constant in the wild type. The relative amplitude error (RAE) plot showed a wider spread of period for the wild type than for the $rPPC1$ lines (Figure 6D). Mean periods were between 21.5 h and 22.1 h when calculated using spectral resampling or fast Fourier transform (nonlinear least squares) methods, respectively (Figure 6E). Average periods were similar between wild type and the $rPPC1$ lines. A lower mean RAE was calculated for $rPPC1$-A and $rPPC1$-B compared to the wild type (Figure 6F), supporting statistically the visibly robust and high-amplitude DF rhythm in the $rPPC1$ lines (Figure 6C).

**Rhythm Characteristics of Core Circadian Clock and Clock-Controlled Genes**

Having established that circadian control of CO$_2$ fixation was dampened under LL in plants lacking *PPC1*, whereas the circadian control of DF was enhanced under LL (Figure 6A to 6F), it was important to investigate the regulation of both core clock genes and circadian clock-controlled genes in the $rPPC1$ lines under LL (Figure 7). Wild type displayed a rhythm in the transcript abundance of *PPC1* that was absent in $rPPC1$-B (Figure 7A). *PPC2* was rhythmic in wild type and $rPPC1$-B, but its
abundance was lower in rPPC1-B (Figure 7B). The PPCK1 rhythm was of greater amplitude in rPPC1-B, and the daily transcript peaks occurred 4 to 8 h later than the wild type after the first 24 h of LL (Figure 7C). PPCK2 and PPCK3 were induced in line rPPC1-B and oscillated with higher amplitude (Figure 7D and 7E). PPCK2 peaked after the wild type on the second and third 24 h cycles under LL (Figure 7D and 7E), which is consistent with the induction detected under light/dark cycles (Figure 1F and 1G). GPT2, which is involved in the transport of G6P across the chloroplast membrane, was down-regulated and had lower amplitude in rPPC1-B than the wild type (Figure 7F), whereas CAB1 was induced and oscillated robustly (Figure 7G).

In rPPC1-B, the core clock gene CCA1-1 was down-regulated, phase delayed, and had lower amplitude than the wild type (Figure 7H), whereas CCA1-2, TOC1-1 and TOC1-2 were all up-regulated and phase delayed relative to the wild type for the latter two peaks of LL (Figure 7I, 7J and 7K). PRR7 was up-regulated and more robustly rhythmic in rPPC1-B (Figure 7L), whereas the rhythms of PRR3/7 and PRR9 dampened in rPPC1-B (Figure 7M and 7N). Finally, JMJD30/JMJD5, ELF3, CDF2, RVE1-like, EPR1, FKF1 and GI were up-regulated in rPPC1-B, displaying higher amplitude, phase delays and/or period lengthening during the LL time course (Figure 7P to 7V), whereas LNK3-like was down-regulated but remained rhythmic, with a lengthening period compared to the wild type (Figure 7O).

**Diel Regulation of GC-Signalling and Ion Channel Genes in GC-Enriched Epidermal Peels of rPPC1-B**

As CO₂ fixation and associated stomatal opening were shifted to the light period in rPPC1-B, it was important to investigate the temporal regulation of known guard cell signalling genes in wild type and the rPPC1-B line (Figure 8). Since epidermal peels of Kalanchoë leaves are enriched for intact guard cells, we isolated RNA from epidermal peels from the upper and lower leaf surfaces of LP6 every 4 h over the 12-h-light/12-h-dark cycle and used them for RT-qPCR.

**PHOTOTROPIN1 (PHOT1)** encodes a protein kinase that acts as a blue light (BL) photoreceptor in the signal-transduction pathway leading to BL-induced stomatal movements (Kinoshita et al., 2001). In rPPC1-B, PHOT1 transcripts were up-regulated relative to the wild type and peaked 8 h into the 12-h-light period.
CRYPTOCHROME2 (CRY2) encodes a photoreceptor that regulates BL responses, including the entrainment of endogenous circadian rhythms (Somers et al., 1998), and stomatal conductance via an indirect effect on ABA levels (Boccalandro et al., 2012). In rPPC1-B epidermal peels, CRY2 transcript levels were up-regulated and peaked at dusk, whereas CRY2 peaked 8 h into the dark in the wild type (Figure 8B).

In Arabidopsis, GC-localised β-CARBONIC ANHYDRASE1 (CA1) and CA4 are involved in CO₂ sensing in GCs, with the ca1 ca4 mutant displaying impaired stomatal control in response to CO₂ (Hu et al., 2010). In rPPC1-B, a β-carbonic anhydrase gene was much more strongly induced at dawn relative to the wild type (Figure 8C). PATROL1 controls the tethering of the proton ATPase AHA1 to the plasma membrane and is essential for stomatal opening in response to low CO₂ and light levels (Hashimoto-Sugimoto et al., 2013). The cycle of PATROL1 in epidermal peels was slightly different between rPPC1-B and wild type, with rPPC1-B peaking 4 h into the dark, but the wild type peak at 4 h into the light period (Figure 8D).

CONVERGENCE OF BLUE LIGHT AND CO₂ 1/2 (CBC1/2) stimulate stomatal opening by inhibiting S-type anion channels in response to both BL and low CO₂ and connect BL signals perceived by PHOT1 with the protein kinase HIGH LEAF TEMPERATURE1 (HT1) (Hiyama et al., 2017). Both proteins interact with and are phosphorylated by HT1. In rPPC1-B, CBC1/2 was up-regulated compared to the wild type both in the second half of the dark period and at dawn (Figure 8E). HT1 acts as a negative regulator of high CO₂ induced stomatal closure (Hashimoto et al., 2006). HT1 transcript levels were reduced throughout the light/dark cycle relative to the wild type in rPPC1-B (Figure 8F). OPEN STOMATA1 (OST1), encoding a protein kinase that acts downstream of HT1 (Imes et al., 2013), was up-regulated in rPPC1-B both at 8h into the dark period and at dawn, the time of the daily trough in the wild type (Figure 8G).

Genes involved in ion transport across the plasma membrane and tonoplast play a crucial role in the changes in cell turgor pressure that drive stomatal movements (Jezek and Blatt, 2017). SLOW ACTIVATED ANION CHANNEL1 (SLAC1) is a key player in the closure of stomata in response to high CO₂ concentrations (Hedrich and Geiger, 2017). SLAC1 is regulated by ABA signalling, which requires de-phosphorylation steps catalysed by protein phosphatase type 2C.
In rPPC1-B, SLAC1 was up-regulated and peaked during the light (Figure 8H), and PP2C was up-regulated relative to the wild type during the dark period (Figure 8I). ABA is recognized and bound by the REGULATORY COMPONENT OF ABA RECEPTORS (RCARs)/PYRABACTIN RESISTANCE1 (PYR1/PYL), which interact with PP2C to stimulate ABA signalling (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009). In rPPC1-B epidermal peels, RCAR3 transcripts were induced spanning dusk and the first half of the dark period, and peaked at least 4 h earlier than the wild type (Figure 8J).

In rPPC1-B, the transcript level of GUARD CELL OUTWARD RECTIFYING K⁺ CHANNEL (GORK), which is involved in regulating stomatal movement according to water status (Ache et al., 2000), peaked 4 h earlier than wild type (Figure 8K).

STELAR K⁺ OUTSIDE RECTIFIER (SKOR) encodes a selective outward-rectifying potassium channel (Gaymard et al., 1998). SKOR transcript levels peaked at dawn in the wild type, whereas in rPPC1-B, SKOR transcript levels reached their daily minimum at dawn (Figure 8L). The transcript level of plasma-membrane localised ALUMINIUM ACTIVATED MALATE TRANSPORTER12 (ALMT12), which is involved in dark-, CO₂-, ABA- and water deficit-induced stomatal closure, was elevated relative to the wild type at dawn in rPPC1-B (Figure 8M). The E3 UBIQUITIN-PROTEIN LIGASE RMA1 promotes the ubiquitination and proteasomal degradation of the aquaporin PIP2:1, which plays a role in GC regulation (Grondin et al., 2015). RMA1 was induced 3-fold at dawn in rPPC1-B compared to the wild type (Figure 8N). EMBRYO SAC DEVELOPMENT ARREST39 (EDA39) is a calmodulin binding protein that promotes stomatal opening (Zhou et al., 2012). In rPPC1-B, EDA39 was up-regulated relative to the wild type and peaked at dusk (Figure 8O).

SALT OVERLY SENSITIVE2 (SOS2) is a Calcineurin B-Like (CBL)-interacting protein kinase involved in the regulatory pathway for intracellular Na⁺ and K⁺ homeostasis and salt tolerance (Liu et al., 2000). SOS2 interacts with and activates the vacuolar H⁺/Ca²⁺ antiporter CAX1, thereby functioning in cellular Ca²⁺ homeostasis, an important function during stomatal opening and closing (Cheng et al., 2004). SOS2 transcript levels were elevated relative to the wild type at all time points, and, in particular, it was induced ~5-fold at its peak 4 h into the dark period in epidermal peels of rPPC1-B relative to the wild type (Figure 8P). Ca²⁺-ATPase2 (ACA2) and ENDOMEMBRANE-TYPE Ca²⁺-ATPASE4 (ECA4) catalyze the hydrolysis of ATP coupled with the translocation of calcium from the cytosol into the
endoplasmic reticulum and/or an endomembrane compartment (Jezek and Blatt, 2017). ACA2 and ECA4 were induced by ~4-fold to ~8-fold in rPPC1-B epidermal peels relative to the wild type, particularly when they reached peak levels at 4 h into the 12-h-dark period (Figure 8Q and 8R).

Finally, the transcription factor MYB60 is involved in stomatal opening in response to light and promotes GC deflation in response to water deficit (Cominelli et al., 2005). In rPPC1-B, MYB60 was induced ~5-fold relative to the wild type at dawn, although it also maintained a dusk-phased peak of transcript abundance like the wild type (Figure 8S). MYB61 functions as a transcriptional regulator of stomatal closure (Liang et al., 2005). MYB61 was down-regulated in rPPC1-B relative to wild type and peaked 8 h later in rPPC1-B, 4 h into the dark period, whereas in the wild type, MYB61 peaked in the light 4 h before dusk (Figure 8T).

**Diel Regulation of GC-Metabolism Genes in GC-Enriched Epidermal Peels of rPPC1-B**

PPC1 and PPC2 transcript levels were low in epidermal peels (Supplemental Figure 5A and 5B) compared to whole leaves (Figure 1A). PPC3 and PPC4 transcripts were more abundant in wild type epidermal peels (Supplemental Figure 5C and 5D) relative to whole leaves (Figure 1C and 1D). Furthermore, PPC3 and PPC4 were up-regulated in epidermal peels of rPPC1-B compared to the wild type, and their transcript abundance peaked 4 h into the dark period, whereas in the wild type, the abundance of both transcripts peaked at the end of the dark period or dawn (Supplemental Figures 5A to 5D).

CAM-specific PPCK1 was up-regulated in rPPC1-B epidermal peels compared to wild type, whereas the expression of PPCK2, which does not currently have a proposed role in the regulation of CAM-specific PPC1 in the mesophyll, was unchanged (Supplemental Figure 5E and 5F). Both PPCK2 and PPCK3 transcripts were more abundant than PPCK1 in epidermal peels, suggesting their encoded proteins might regulate the activity of the GC PPC(s) (Supplemental Figure 5F and 5G). Most strikingly, PPCK3 was up-regulated ~5-fold at 4 h after lights-on in rPPC1-B epidermal peels relative to the wild type (Supplemental Figure 5G).

In C3 plants, starch is degraded before dawn to fuel stomatal opening (Blatt, 2016). In Arabidopsis GCs, BAM1 encodes the major starch-degrading enzyme (Valerio et al., 2010; Prasch et al., 2015; Horrer et al., 2016; Santelia and Lunn,
that functions with AMY3 to mobilize starch at dawn, releasing maltose in the chloroplasts (Horrer et al., 2016). In rPPC1-B epidermal peels, GWD transcript levels were halved relative to wild type at its peak, i.e., 8 h into the 12 h light period (Supplemental Figure 5H). AMY3a and AMY3b were up-regulated at dusk and in the second half of the light period, respectively (Supplemental Figure 5I and 5J). BAM1 was up-regulated at dawn compared to wild type, and peaked with a very similar transcript abundance to wild type at 20:00 h, 8 h into the dark period (Supplemental Figure 5K), whereas BAM3 transcript levels were ~3-fold lower in rPPC1-B relative to the wild type at the time of its nocturnal peak, which also occurred 8 h into the dark (Supplemental Figure 5L). BAM9 expression in rPPC1-B rose later than the wild type, lagging behind the wild type at dusk and 4 h into the dark, and it stayed ~5-fold higher at dawn and 4 h into the light period, when the wild type reached its daily trough (Supplemental Figure 5M). BAM9 in Arabidopsis is predicted to be catalytically inactive and has no known function to date (Monroe and Storm, 2018).

The peak of PHS1 was delayed by 4 h, peaking at the light/dark transition (Supplemental Figure 5N), and MEX1 was down-regulated in epidermal peels of rPPC1-B, peaking at 08:00 in the light, 4 h after the wild type peak (Supplemental Figure 5O).

**Diel Regulation of Core Circadian Clock genes in Epidermal Peels of rPPC1-B**

The circadian clock genes PRR3/7 and PRR7 were up-regulated in epidermal peels of rPPC1-B relative to wild type (Supplemental Figure 5P and 5Q), with a similar diel pattern to that measured in whole CAM leaves (Figure 5S and 5T). PRR3/7 peaked 4 h earlier in rPPC1-B, whereas PRR7 transcript levels were greater throughout the light period in rPPC1-B (Supplemental Figure 5P and 5Q). LUX is a component of the morning transcriptional feedback circuit within the clock, encoding a transcription factor that directly regulates the expression of PRR9 by binding to specific sites in its promoter (Helfer et al., 2011). In epidermal peels, LUX was downregulated >7-fold in rPPC1-B compared to the wild type at its peak, 4 h into the light period, and also peaked 4 h later than the wild type (Supplemental Figure 5R).

**Diel Regulation of Additional GC-Signalling Genes in GC-Enriched Epidermal Peels of rPPC1-B**
GCs perceive CO₂ and regulate stomatal aperture via ABA and reactive oxygen species signaling, with low CO₂ levels mediating stomatal opening and high CO₂ levels causing closure (Chater et al., 2015). NADP OXIDOREDUCTASE encodes an NADP-binding Rossmann-fold super-family protein that we only detected in epidermal peels in Kalanchoë. The enzyme encoded by this gene might generate the H₂O₂ burst induced by ABA as part of the stomatal closure signalling pathway (Daszkowska-Golec and Szarejko, 2013). This transcript was up-regulated ~3-fold in rPPC1-B at dawn (Supplemental Figure 5S).

In Arabidopsis, the transcription factors MYB94 and MYB96 function together in the activation of cuticular wax biosynthesis under drought stress (Lee et al., 2016). MYB96 may also be involved in the response to drought stress in Arabidopsis through ABA signalling that mediates stomatal closure via the RD22 pathway (Seo et al., 2011). Both MYB94 and MYB96 were induced by as much as 3-fold in rPPC1-B compared to wild type (Supplemental Figure 5T and 5U). Specifically, MYB94 transcript levels rose 8 h earlier than they did in the wild type and were already close to peak levels by the light-to-dark transition, whereas in the wild type, MYB94 levels peaked sharply 8 h into the dark period (Supplemental Figure 5T). MYB96 transcription was induced relative to the wild type at both dawn and dusk, but the peak of MYB96 transcription at dawn represented the largest fold-change relative to the wild type (Supplemental Figure 5U).

DISCUSSION

CAM Provides a Selective Advantage in the Face of Drought

The data presented here for CO₂ fixation during progressive drought demonstrate very clearly the importance of a fully functional CAM system for continued atmospheric CO₂ fixation throughout a period of drought lasting just over 3-weeks (Figure 4D). Although the intermediate line rPPC1-A continued to perform atmospheric CO₂ fixation and malate accumulation in the dark and stomatal closure in the light period, it was less able to adapt to drought by inducing CAM compared to the wild type. Thus, by comparing the impacts of the different levels of PPC1 silencing in these transgenic lines of Kalanchoë, it is possible to conclude that the
wild type level of *PPC1* transcript enables the plant to adapt to drought more rapidly by increasing nocturnal CO$_2$ fixation and malate accumulation and by decreasing the magnitude and duration of phases II and IV of CAM in the light. These swift adaptations allow the wild type to curtail excessive water-loss more rapidly than either of the transgenic lines with reduced *PPC1* transcript levels, suggesting that the high level of *PPC1* transcript in the wild type provides a genuine adaptive advantage in terms of allowing the plant to prevent excessive water loss during gradual soil drying.

Upon re-watering on day 23, both wild type and *rPPC1*-A plants returned to performing strong, four-phase CAM, including pronounced phase II and IV at the start and end of the light period, respectively (Figure 4D). Line *rPPC1*-B also bounced back to its normal, pre-drought physiology after re-watering, which is consistent with the re-watering response reported previously for a range of facultative, weak-CAM species, including *M. crystallinum*, purslane (*Portulaca oleracea*), *P. umbraticola*, *T. triangulare*, and various *Calandrinia* species (Winter and Holtum, 2014; Holtum et al., 2017; Winter, 2019). Overall, the response of *rPPC1*-B to progressive drought stress and subsequent re-watering is reminiscent of the physiological response of facultative CAM species such as *T. triangulare* and *M. crystallinum*, although both of these true facultative CAM species did achieve net atmospheric CO$_2$ fixation in the dark period following approximately 12 to 13 days of drought (Winter and Holtum, 2014).

Although the *rPPC1*-B plants fixed all of their CO$_2$ in the light, especially during a pronounced and extended phase II in the hours after dawn, the CO$_2$ uptake pattern was not constant over the light period (Figure 4A). For example, in LP6 of a well-watered plant, CO$_2$ fixation dropped from ~8 µmol m$^{-2}$ s$^{-1}$ during phase II after dawn to ~2 to 3 µmol m$^{-2}$ s$^{-1}$ around 4 h after lights on (Figure 4A), and stomatal conductance decreased to a similar extent (Figure 4B). The observed stomatal closure could not have been due to a high malate concentration and associated internal release of CO$_2$ in the mesophyll (Figure 3A). These data are consistent with the current hypothesis that a signal from the core circadian clock drives the closure of CAM stomata in the light and the associated decline in atmospheric CO$_2$ fixation, even when a CAM leaf has not produced malate during the preceding dark period (Von Caemmerer and Griffiths, 2009).
Overall, with respect to CAM physiology, the data presented here provide strong support for the long-held view that the ability to use CAM, and to use it increasingly in response to drought stress, provides a genuine adaptive advantage in terms of prolonging net atmospheric CO$_2$ fixation during drought progression (Figure 4D) (Kluge and Fischer, 1967; Osmond, 1978). The wild type and line rPPC1-A achieved, respectively, net atmospheric CO$_2$ fixation of 18,568 and 19,071 μmoles m$^{-2}$ over the 22-day drought progression, whereas line rPPC1-B only achieved 13,983 μmoles m$^{-2}$ net atmospheric CO$_2$ fixation (Supplemental Table 4). Thus, the wild type, with its fully functional CAM system, was able to fix 33% more CO$_2$ over the entire drought treatment period than the mutant.

**Insights into the Evolutionary Trajectory from C$_3$ to CAM**

The discovery that the very low level of PPC activity in the rPPC1-B transgenic line was associated with nocturnal refixation of respiratory CO$_2$ revealed that even a small increase in PPC activity in the dark period may be sufficient to support a minimal, but functional, CAM cycle. Thus, during CAM evolution from ancestral C$_3$ species, small increases in nocturnal PPC activity in photosynthetic mesophyll cells behind closed stomata may have been sufficient to support the refixation of respiratory CO$_2$ to stored vacuolar malic acid, allowing its subsequent decarboxylation in the light, leading to partial stomatal closure, enhanced WUE, and improved RuBisCO carboxylation efficiency relative to the oxygenase (Edwards, 2019). However, despite our data for Kalanchoë supporting this evolutionary perspective on the progression to CAM, initial attempts to increase the expression of core CAM pathway enzymes in the C$_3$ model species *Arabidopsis thaliana* have not reciprocated the phenotype of even the most strongly silenced rPPC1-B line reported here. Constitutive over-expression of the CAM-specific PPC isogene from *M. crystallinum* in *A. thaliana* led to increased stomatal conductance and titratable acidity (Lim et al., 2019). However, the increased stomatal conductance was measured in the middle of the light period, and the timing of the titratable acidity measurements relative to the light/dark cycle was not mentioned (Lim et al., 2019). Therefore, it is difficult to compare these results with the results presented here documenting alterations in nocturnal stomatal opening and atmospheric CO$_2$ fixation and dawn-phased malate accumulation in the rPPC1 transgenic lines of *Kalanchoë*. 
It should be noted, however, that the *K. laxiflora* lines with reduced CAM-specific *PPC1* transcript levels still possessed succulent leaves with large mesophyll cells, plus wild type levels of key CAM transcripts and enzymes such as PPDK. Thus, the *rPPC1* plants were by no means directly comparable to the transgenic *A. thaliana* lines reported by Lim et al. (2019).

**Impact of *PPC1* Silencing on the CAM Circadian Rhythm of CO₂ Fixation**

The circadian rhythm of CO₂ fixation observed for CAM species of *Kalanchoë* under constant free-running conditions is a classic example of a plant clock output rhythm driven by the underlying multi-gene-loop, autoregulatory core oscillator mechanism in the nucleus that sets the time (Hartwell, 2006). Under continuous light and temperature (LL) conditions, the CAM-associated circadian rhythm of CO₂ fixation dampened towards almost complete arrhythmia in detached leaves and whole, young plants of *rPPC1*-B, and CO₂ was fixed continuously (Figure 6A and 6B). The collapse of the CO₂ circadian rhythm suggests that CO₂ fixation by RuBisCO in *rPPC1*-B was not subject to robust and high amplitude circadian control, certainly not in comparison to the rhythm of atmospheric CO₂ fixation during CAM in the wild type (Figure 6B). In *K. daigremontiana*, RuBisCO is thought to make a large contribution to the observed rhythm of CO₂ assimilation under LL conditions, as the level of malate does not oscillate (Wyka and Lüttge, 2003). By contrast, online carbon isotope discrimination measurements demonstrated that the LL CO₂ rhythm of CAM leaves of *M. crystallinum* had a RuBisCO carbon isotope discrimination early in each subjective dark period, which then transitioned to a PPC carbon isotope ratio later in the subjective dark (Davies and Griffiths, 2012). In *rPPC1*-B (with no detectable PPC), the remaining RuBisCO-mediated rhythm displayed, at best, only a very weak rhythm of CO₂ assimilation (Figure 6A). This suggests that C₃ carboxylation via RuBisCO only makes a small contribution to the LL rhythm in *K. laxiflora* CAM leaves, or perhaps the robust, high amplitude CO₂ rhythm of the wild type depends on interplay between high PPC activity and RuBisCO, which ceases in the absence of *PPC1*. Overall, the weakly rhythmic to arrhythmic pattern of CO₂ assimilation in *rPPC1*-B under LL was very similar to that of C₃ *M. crystallinum* (Davies and Griffiths, 2012).
Regulation of Core Clock Genes in \(rPPC1-B\)

Previous reports for other CAM species have emphasised that most core circadian clock genes share their pattern and phasing of diel transcript regulation between Arabidopsis and, for example, Agave, pineapple (\textit{Ananas comosus}), and white stonecrop (\textit{Sedum album}) (Sharma et al., 2017; Yin et al., 2018; Wai et al., 2019). These reports therefore emphasise that the timing of core clock genes is conserved between widely divergent species, regardless of the underlying mechanism of photosynthetic CO\(_2\) fixation, which is consistent with the ancestral role of the core circadian clock in the temporal control and optimisation of many biological processes in plants. However, individual studies have identified specific core clock genes, or genes closely associated with the core clock, that were differentially expressed and/or temporally phased in certain CAM species relative to Arabidopsis. For example, RNA-seq based transcriptome analysis in \textit{A. americana} identified a gene related to the clock-associated output pathway gene \(RVE1\) (Rawat et al., 2009), which was rescheduled to the dark period in \textit{Agave}, but peaked phased to dawn in Arabidopsis (Yin et al., 2018). The \textit{K. laxiflora} \(RVE1\)-like gene measured here reached its 24 h transcript peak phased to dawn in LD cycles (Figure 5) and was repressed in \(rPPC1-B\) relative to wild type. Under LL, \(RVE1\)-like was induced in \(rPPC1-B\) relative to wild type, and its transcript abundance oscillated robustly, with a higher amplitude oscillation than the wild type (Figure 7). These findings suggest that the \(RVE1\)-like gene measured here does not likely share a role in the circadian coordination of the daily CAM cycle, as has been hypothesised for \textit{Agave} \(RVE1\). In fact, based on our current data for the \(rPPC1\) lines, it is possible that \(RVE1\)-like might function in coupling the core clock to the robust rhythm of DF observed in the \(rPPC1\) transgenic lines (Figure 6C).

In pineapple, the core clock genes \(PRR5\) and \(PRR9\) are rhythmic specifically in the green leaf tip tissue that performs CAM (Sharma et al., 2017). By contrast, the \(PRR7\), \(PRR3/7\) and \(PRR9\) transcripts measured in the current study were rhythmic over the LD cycle in wild type, \(rPPC1-A\) and \(rPPC1-B\), although the levels of all three \(PRR\) transcripts were higher in \(rPPC1-B\) relative to the wild type (Figure 5). However, the abundance and rhythmicity of \(PRR3/7\) and \(PRR9\) collapsed in \(rPPC1-B\) under LL conditions, whereas \(PRR7\) was induced and rhythmic (Figure 7). In \textit{S. album}, a member of the Crassulaceae along with \textit{Kalanchoë}, the induction of weak
CAM-cycling in response to drought stress led to the repression of transcripts related to \textit{GI} and \textit{FKF1} and the induction of transcripts related to \textit{CCA1} and \textit{TOC1} (Wai et al., 2019). However, the timing of peak transcript abundance for core clock gene orthologs was largely consistent between C\textsubscript{3} and CAM-cycling leaf samples of \textit{S. album}, with phase delays of 2 to 4 h at most for \textit{PRR1\textsubscript{A}}, \textit{PRR5\textsubscript{A}}, \textit{PRR5\textsubscript{B}} and \textit{PRR5\textsubscript{D}} (Wai et al., 2019). In \textit{rPPC1-B} under LD cycles, only changes in the transcript peaks of \textit{CCA1-2} (down), \textit{TOC1-1} (up) and \textit{TOC1-2} (up) were detected (Figure 5). The timing of the peaks and troughs of these transcripts remained the same between wild type and both \textit{rPPC1} lines. \textit{GI} and \textit{FKF1} transcript oscillations and peak abundance were remarkably similar between wild type and the two \textit{rPPC1} lines under LD cycles (Figure 5). However, under LL conditions, \textit{CCA1-2}, \textit{TOC1-1}, \textit{TOC1-2}, \textit{GI} and \textit{FKF1} were all induced relative to the wild type and oscillated more robustly, with lengthening periods as the LL time course progressed (Figure 7).

Overall, there were no clear and consistent correlations between the transcript abundance cycles of core clock gene transcripts reported here for \textit{K. laxiflora} wild type and RNAi lines with reduced \textit{PPC1} levels and the published RNA-seq data for the LD cycles of transcript abundance of the orthologous genes in \textit{Agave}, pineapple and \textit{S. album}. Furthermore, it was not possible to detect any temporal patterns of regulation of core clock gene transcripts between different species or between C\textsubscript{3} and CAM tissues in individual species, which would suggest that conserved alterations in the core clock have accompanied the evolution of CAM in these diverse CAM lineages.

**Contrasting Core Clock Responses Between \textit{rPPC1} and \textit{rPPCK1} RNAi Lines**

Similar to the LL \textit{CO\textsubscript{2}} fixation phenotype reported here for \textit{rPPC1-B}, transgenic \textit{K. fedtschenkoi} lines lacking \textit{PPCK1} also lost the \textit{CO\textsubscript{2}} fixation rhythm, and the transcript oscillations of many core clock genes were altered (Boxall et al., 2017). However, different core clock genes were perturbed in \textit{rPPC1-B} compared to those whose rhythmic regulation changed in the \textit{rPPCK1} lines of \textit{K. fedtschenkoi} (Boxall et al., 2017 cf. Figure 7). In \textit{rPPC1-B}, \textit{CCA1-1} transcript oscillations dampened, and both \textit{TOC1-1} and \textit{TOC1-2} transcripts were up-regulated and oscillated with a delayed peak phase relative to the wild type (Figure 7H, 7J and 7K). By contrast, in line \textit{rPPCK1-3}, the transcription of clock genes \textit{CCA1-1}, \textit{CCA1-2} and \textit{TOC1-2}
dampened rapidly towards arrhythmia under LL free-running conditions, whereas *TOC1-1* was up-regulated and rhythmic (Boxall et al., 2017). Furthermore, whilst *PRR7* was up-regulated and rhythmic in *rPPC1-B* (Figure 7), the same gene dampened to arrhythmia in *rPPCK1-3* after an initial induced peak during the first 6 h of LL (Boxall et al., 2017). The evening phased genes *GI* and *FKF1* were induced and rhythmic under LL conditions in both *rPPC1-B* and *rPPCK1-3*, but the level of transcript induction and the rhythm amplitude were much greater in *rPPC1-B* (Figure 7U and 7V), suggesting that these two clock genes are more important for robust oscillations in C3 leaves.

Overall, these differences between the rhythms of core clock genes in *rPPC1-B* and *rPPCK1-3* revealed that the clock responded very differently to the silencing of two interconnected genes that lie at the heart of the circadian control of nocturnal CO2 fixation. Elucidating the mechanistic basis for these differences should be a fruitful avenue for further investigation, especially in light of the proposed cross-talk between CAM-associated metabolites and regulation within the core clock (Boxall et al., 2017), which was further supported by the clock gene phenotypes reported here (Figure 7).

**Interactions Between Sugars Linked to CAM and the Core Circadian Clock**

In Arabidopsis, sugars associated with primary photosynthetic metabolism play a role in the entrainment of the core circadian oscillator in the nucleus (Haydon et al., 2013). In particular, *PRR7* is thought to be required for sensing metabolic status and coordinating the clock with photosynthesis (Haydon et al., 2013). In *rPPC1-B*, the low sucrose levels at 2 h after dawn (Figure 3C) may be sensed via a mechanism involving *PRR7* (Boxall et al., 2017). However, in *Kalanchoë*, a second *PRR7*-related gene, *PRR3/7*, produced the more abundant transcript and displayed a transcript peak 2 h before dusk under LD (Figure 5T). In the wild type, *PRR3/7* may function as part of a signal transduction pathway that senses metabolic status at dusk when primary CO2 fixation begins (Haydon et al., 2013; Boxall et al., 2017).

**rPPC1 and rPPCK1 Mutants Display Contrasting Levels of Delayed Fluorescence Circadian Rhythms**
Delayed fluorescence (DF) circadian rhythms were more robust than the wild type in rPPC1-A and rPPC1-B (Figure 6C). This result represents one of the most striking contrasts to the previous finding of arrhythmic DF in the K. fedtschenkoi rPPCK1-3 line (Boxall et al., 2017). This fundamental difference provides important insights into the regulatory signal transduction network underpinning the DF rhythm, which originates from inside the chloroplasts due to the changing redox state of the plastoquinone pool (Gould et al., 2009). In terms of core clock gene regulation under both LD and LL, CCA1-2, TOC1-2, PRR7 and the clock-controlled gene CDF2 were all induced and rhythmic in rPPC1-B. Thus, CCA1-2, TOC1-2, PRR7 and CDF2 are the most likely candidates for driving the induction and robust LL rhythmicity of DF in rPPC1-B. Conversely, the decline in the amount and rhythmicity of these transcripts in rPPCK1-3 may play a role in the dampening of the DF rhythms in the absence of nocturnal phosphorylation of PPC1 (Boxall et al., 2017).

These results also allow us to propose that robust rhythmicity of the CAM-associated CO₂ fixation rhythm in wild type Kalanchoë is most likely driven by an underlying oscillator consisting of CCA1-1, PRR3/7, PRR9, and LNK3-like. This hypothesis is supported by the finding that the rhythmicity of these core clock components responded in the same way in both rPPC1-B and the previously reported rPPCK1-3 transgenic lines of Kalanchoë (Boxall et al., 2017). However, only a limited set of core clock and clock-associated genes has been profiled under LL in rPPC1-B and rPPCK1-3, making it likely that other clock-associated genes are also involved in driving robust CAM CO₂ fixation rhythms and robust DF rhythms.

**Perturbation of diel rhythms of gene transcript oscillations in GCs**

A key gap in the current understanding of the molecular genetics underpinning the physiology associated with CAM centres on the cell signalling mechanisms that mediate the inverse pattern, relative to C₃, of stomatal opening and closing (Borland et al., 2014; Males and Griffiths, 2017). The GCs of CAM leaves and stems are thought to respond directly to the internal supply of CO₂. This theory has recently led several groups to use whole leaf RNA-seq datasets to investigate alterations, relative to C₃ leaves, in the temporal phasing of known GC regulatory genes and membrane transporters (Abraham et al., 2016; Wai and VanBuren, 2018; Yin et al., 2018; Heyduk et al., 2019; Moseley et al., 2019). However, these studies did not use
enriched GCs as the source of RNA, and therefore the data that were mined represented all leaf cell types, including palisade and spongy mesophyll, GCs, subsidiary cells, phloem, phloem companion cells, xylem, bundle sheath, and water storage parenchyma in *Agave* and pineapple. Thus, the re-scheduling of the temporal patterns of candidate GC genes in these datasets may have been complicated by transcripts from the same genes that were functional in other leaf cell types.

Separated epidermal peels from *Kalanchoë* CAM leaves are enriched for intact GCs. We leveraged this feature in order to compare the temporal pattern of transcript regulation between GC-enriched epidermal peels of the wild type and *rPPC1-B* (Figure 8). Relative to the wild type, a wide range of genes known to be involved in stomatal opening and closing displayed alterations in transcript abundance and/or the timing of the daily transcript peak (Figure 8A to 8U). These results support the theory that the measured changes in transcript abundance and temporal patterns for a range of guard cell regulatory genes were connected to the measured changes in stomatal opening and closing (Figure 4B).

Thus, the up-regulation of *HT1*, *SKOR* and *MYB61* in the wild type relative to *rPPC1-B*, and the down-regulation of a wide range of genes in the wild type, including *SLAC1*, *PP2C*, *SOS2*, *ACA2*, *ECA4*, and *MYB60*, are likely important regulatory changes that facilitate nocturnal stomatal opening and light period closure (Figure 4 and 8). As *MYB60* is required for stomatal opening in response to light in *C₃* Arabidopsis (Cominelli et al., 2005), it is particularly noteworthy that its transcript abundance in wild type *K. laxiflora* GC-enriched epidermal peels peaked at dusk, when stomata open in the wild type (Figure 8LL). Furthermore, in *rPPC1-B*, *MYB60* also had a dramatic 3- to 4-fold induction at dawn relative to the wild type, suggesting that high *MYB60* transcript levels at the start of the light period may play a key role in the observed light period stomatal opening of the *rPPC1-B* line (Figure 4B). It was also notable that *rPPC1-B* continued to have a peak of *MYB60* transcripts phased to dusk, as observed for the wild type, which is in agreement with the observation that *rPPC1-B* did open its stomata slightly throughout the dark period (Figure 4B). However, this nocturnal stomatal opening was futile in terms of atmospheric CO₂ fixation, as *rPPC1-B* released respired CO₂ from its leaves throughout the dark period (Figure 4A). Overall, *MYB60*, as well as several other mis-regulated guard cell signalling, ion channel, and metabolite transporter genes
(Figure 8 and Supplemental Figure 5), represent key targets for future genetic manipulation experiments in transgenic *Kalanchoë* aimed at understanding the important regulators underpinning the inverse stomatal control associated with CAM.

**Informing Biodesign Strategies for Engineering CAM into C₃ crops**

Efforts are underway to engineer CAM and its associated increased WUE into C₃ species as a means to develop more climate-resilient crop varieties that can continue to fix CO₂ and grow in the face of drought, whilst using water more wisely than C₃ varieties (Borland et al., 2014; Borland et al., 2015; Lim et al., 2019). The data presented here for the *rPPC1* loss-of-function lines of *K. laxiflora* confirm experimentally the proposed core role of *PPC1* for efficient and optimised CAM. Our results also provide encouragement that the level of over-expression of a CAM-recruited *PPC1* gene introduced into an engineered C₃ species may not need to be as high as the level found in extant obligate CAM species, because reducing *PPC1* levels to only 43% of wild type activity in line *rPPC1*-A led to plants that were still capable of full CAM and fixed more CO₂ than the wild type over 3 weeks of drought (Figure 4D).

The results presented here also emphasise that only certain sub-components of the core circadian clock are essential for the temporal optimisation of CAM in *Kalanchoë*, which in turn further simplifies the challenge of achieving correct temporal control of an engineered CAM pathway introduced into a C₃ species. It is clear from our current RT-qPCR data that the transgenic manipulation of the expression and regulation of *CCA1-1*, *PRR3/7*, *PRR9* and *LNK3-like* in *Kalanchoë* using RNAi, over-expression and/or CRISPR-Cas mediated gene editing, will allow the further refinement of the evolving model for the subset of core clock genes that form the transcription-translation feedback loop that underpins the temporal optimisation of CAM. However, in addition, transcriptome-wide analysis of the light/dark regulation of all detectable transcripts in LP6 of *rPPC1-B* compared to the wild type will allow the identification of other candidate core clock genes that were not profiled in the targeted RT-qPCR analysis presented here.

**METHODS**


**Plant Materials**

*Kalanchoë laxiflora* plants were propagated clonally from adventitious plantlets in the leaf margin using the same clonal stock originally obtained from the Royal Botanic Gardens (RBG), Kew in 2008: accession number 1982-6028, which was kindly provided by the RBG-Kew Living Collection but mis-identified under the name *K.*, *fedtschenkoi*. Plants were initially grown in a heated transgenic greenhouse with supplementary lighting (Gavita reflector lamps HPS-R Agro 600 W S-281) providing year round 16-h-light/ 8-h-dark, minimum light intensity at plant height of 300–400 μmoles photons m$^2$ s$^{-1}$, and a minimum temperature of 20°C. Prior to all experiments, clonal populations of developmentally synchronised plants raised under greenhouse conditions were entrained in a Snijders Microclima MC-1000 growth cabinet for 7-days under 12-h-light/ 12-h-dark and/ or subsequently released into constant light and temperature conditions according to Boxall et al. (2017).

**Time Course Experiments**

For both light/dark and LL time course experiments, opposite pairs of leaf pair 6 (LP6) were collected every 4 h over a 12-h-light/12-h-dark cycle (12:12 LD), starting 2 h (02:00) after the lights came on at 00:00 h. Plants were entrained for 7-days in a Snijders Microclima MC-1000 growth cabinet with fluorescent lamps (20 Phillips TL5-HO and 3 Phillips TLD-90 providing a mixed spectrum from 400 – 650 nm) set to 12-h-light (450 μmoles photons m$^2$ s$^{-1}$), 25°C, 60% humidity/12-h-dark, 15°C, 70% humidity. For constant light, constant temperature, constant humidity (LL) free-running circadian time course experiments, plants were entrained under 12-h-light/ 12-h-dark (LD) conditions as above and switched to LL after a dark period. For LL, the constant conditions were: light 100 μmoles photons m$^2$ s$^{-1}$, temperature 15°C and humidity 70%. For both LD and LL experiments, LP6 were sampled every 4 h from three individual (clonal) plants, starting at 02:00 (2 h after lights on). All leaf samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

**Generation of Transgenic *K. laxiflora* Lines**
An intron-containing hairpin RNAi construct was designed to target the silencing of both copies of the CAM-associated *PPC1* gene in the tetraploid *K. laxiflora* genome (JGI Phytozome accession numbers: Kalax.0018s0056.1 and Kalax.0021s0061.1, which are the *K. laxiflora* orthologues of the previously characterised *K. fedtschenkoi* CAM-associated *PPC1*, GenBank accession: KM078709). A 323 bp fragment was amplified from CAM leaf cDNA using high fidelity PCR with KOD Hot Start DNA Polymerase (Merck, Germany). The amplified fragment spanned the 3' end of the *PPC1* coding sequence and extended into the 3' untranslated region to ensure specificity of the silencing to both of the aforementioned CAM-associated *PPC1* gene copies. Alignment of the 323 bp region with the homologous regions from the three other plant-type *PPC* genes, and their homeologs, in the *K. laxiflora* genome demonstrated that none of the other *PPC* genes shared any 21 nucleotide stretches that were an exact match for the 323 bp *PPC1* RNAi fragment. Thus, the RNAi construct in the hairpin RNA binary vector used to generate the stable transgenic lines was predicted to silence only the two homeologous copies of the CAM-associated *PPC1*, and to be equally specific and efficient at silencing both copies. This specificity and equality of silencing efficiency was confirmed by the RT-qPCR data in Figure 1, as the qPCR primers targeted both copies of the CAM-associated *PPC1* (Supplemental Figure 1), and so the quantitative signal is representative of the averaged signal for the transcript abundance of the two gene copies.

The primers used for the amplification of the *PPC1* gene fragment cloned to generate the hairpin RNAi binary construct were: *PPC1 RNAi F* 5’ CACCAAGCTACCAAGTGCCGGTG 3’, and *PPC1 RNAi R* 5’ CCCTCCTGCTGCTGCTGCTGC 3’. The PCR product was cloned into the pENTR/D Gateway-compatible entry vector, as described previously (Dever et al., 2015). Following confirmation of the correct sequence and orientation of the *PPC1* fragment in the ENTRY vector using Sanger sequencing, the pENTR/D *PPC1* RNAi clone was recombined into intron-containing hairpin RNAi binary vector pK7GWIWG2(II) (Karimi et al., 2002) using LR Clonase II enzyme mix (Life Technologies). *Agrobacterium*-mediated stable transformation of *K. laxiflora* was achieved using a method described previously for the very closely related species *K. fedtschenkoi* (Dever et al., 2015), with the following changes. For *K. laxiflora* transformation, the initial sterile leaf explants used for dipping in *Agrobacterium tumefaciens* GV3101 carrying the engineered binary vector were generated by germinating surface-
sterilised *K. laxiflora* seeds on Murashige and Skoog medium with Gamborgs B-5 vitamins (Murashige and Skoog, 1962; Gamborg et al., 1968) and 3% sucrose. 4- to 6-week-old seedlings grown by tissue culture were chopped into explants for transformation in a sterile laminar flow bench using a sterile scalpel. The explants were dipped in the *Agrobacterium* suspension carrying the PPC1 RNAi binary vector, and co-cultivated and regenerated as described previously (Dever et al., 2015).

**High-Throughput Leaf Acidity and Starch Content Screens**

Leaf acidity (as a proxy for leaf malate content) and leaf starch content were screened in stained leaf discs using chlorophenol red and iodine solution at both dawn and dusk as described by Cushman et al. (2008). For each transgenic line, leaf discs were sampled from LP6 in triplicate at 1 hour before dawn and 1 hour before dusk and stained in a 96 well plate format.

**Net CO$_2$ Exchange Measurement Using Whole Young Plants**

Whole plant gas exchange measurements were collected over a period of 27-days using 2-month-old plants at the 9-leaf-pairs stage (results shown in Figure 4D). Four individual clones per line were potted in 150 ml beakers of compost mix, which was initially watered to field capacity, and the beakers were sealed with Parafilm around the rim of the beaker and the base of the stem. Beakers with potted plants were lowered into the lower half of each of the 12 gas exchange cuvettes. Lids were sealed over each plant using bulldog clips to create a gas tight seal due to compression of foam rubber that lined the joint between the lower and upper half of each gas exchange cuvette. During the 27-day time period, the progression into drought of young, initially well-watered, wild type plants was documented as they transitioned from displaying all four phases of CAM with a relatively low proportion of dark period CO$_2$ fixation in phase I on day 1, through to full CAM (only phase I dark CO$_2$ fixation and phase III CO$_2$ refixation behind closed stomata throughout the light) from day 12, followed by recovery of all 4 phases of CAM after re-watering on day 22. Measurements were made using a custom-built, 12-channel IRGA system (PP Systems, Hitchin, UK), which allowed for individual environmental control (CO$_2$/H$_2$O) and measurement of the rates of CO$_2$ uptake for each of 12 gas exchange cuvettes,
with measurements collected every ~18 min. The system was described in full by Dever et al. (2015), but was expanded here with the addition of 6 gas exchange cuvettes, thereby doubling the scope for replication and throughput. The experiment was performed using four individual clonal young plants (2 months old, 9-leaf-pairs) per line: wild type, $rPPC1$-A and $rPPC1$-B. The four-fold replicated wild type and $rPPC1$ lines were compared in neighbouring gas exchange cuvettes during each experimental run, such that the data are directly comparable between each line. As the entire gas exchange system was housed in a Snijders Microclima MC-1000 growth cabinet, all 12 gas exchange cuvettes were under identical conditions in terms of light intensity and temperature.

**Measurement of Circadian Rhythms of Net CO$_2$ Exchange Under Constant Light and Temperature Free-Running Conditions**

Various *Kalanchoë* species display a persistent and robust circadian rhythm of CO$_2$ exchange when detached CAM leaves are measured with their petiole in distilled water (Wilkins, 1959). This discovery led to the current understanding that the endogenous circadian clock underpins the optimised temporal control of CAM in *Kalanchoë* (Hartwell, 2006; Boxall et al., 2017). The circadian rhythm of CO$_2$ exchange was measured for wild type, $rPPC1$-A and $rPPC1$-B using both detached LP6 sampled from 4-month-old mature plants and intact young plants at the 9-leaf-pairs stage (2 months old). The 12-cuvette gas-switching CIRAS-DC based gas exchange system (Dever et al., 2015) was used to measure both the detached LP6 and the intact young plants with their roots in compost mix. For detached LP6, leaves were detached from each line at the base of their petiole using a scalpel, and the cut petiole was immediately immersed in distilled water by pushing the petiole through a small hole cut in Parafilm covering a 150 ml beaker of distilled water. The gas exchange cuvettes were designed to hold a 150 ml beaker in the lower half of the cuvette such that the leaves were enclosed in the clear, upside-down 1 l beaker that formed the upper section of the cuvette in which gas exchange was measured. After the upper section of each cuvette was placed over the leaves or intact young plants, the upper and lower sections of the cuvettes were sealed using bulldog clips to clamp the join, which was coated in foam-rubber sealing material to generate a gas tight seal. To measure intact young plants, individual clones of each line at the 9-leaf pairs stage were potted in compost mix in 150 ml pots that fit into the base of the gas
exchange cuvettes, and Parafilm was used to seal around the upper rim of the beaker and the central stem of the young plant to prevent soil and root respiration from interfering with the gas exchange measured for the above ground leaf pairs (LP1 to LP9). Plants were pre-entrained to 12-h-light/12-h-dark cycles in a Snijders Microclima MC-1000 growth cabinet for 7-days as described above prior to release into LL free-running conditions at the end of the last dark period. For LL, the constant conditions were: light 100 μmoles photons m^{-2} s^{-1}, temperature 15°C and humidity 70%. The entire gas exchange system was housed inside a Snijders Microclima MC-1000 growth cabinet, so all 12 cuvettes were under identical conditions.

**Measuring Net CO₂ Exchange in Attached Leaf Pair 6 Using the LI-COR 6400XT System**

The gas exchange of mature CAM leaves (LP6) was measured over a 12-h-light, 25°C, 60% humidity: 12-h-dark, 15°C, 70% humidity cycle using an infra-red gas analyser (LI-6400XT, LI-COR, Inc.) attached to a large CO₂ gas cylinder. Data were logged every 10 minutes using an auto-program that tracked the light and temperature regimes of the growth cabinet.

**Measuring Leaf Malate, Starch and Sucrose Contents**

LP6 (full CAM in wild type) from mature plants were sampled into liquid nitrogen every 4 hours and at dawn and dusk and stored at -80°C until use. Biological triplicates were sampled, where each replicate was a pair of leaves of the designated developmental age from a separate, developmentally synchronised, clonal plant of each line. The frozen leaf samples were prepared and assayed for malate and starch as described by Dever et al. (2015) using the published methods for assaying malate in an enzyme-linked spectrophotometric assay (Möllering, 1985) and starch (Smith and Zeeman, 2006). Sucrose, glucose, and fructose were assayed according to the manufacturer’s protocol (Megazyme Technologies).

**Chlorophyll Assays**
Chlorophyll was assayed from mature greenhouse-grown plants from leaf pairs 2-7. Biological triplicates were sampled, where each replicate was a pair of leaves of the designated developmental age from a separate, developmentally synchronised, clonal plant of each line. Chlorophyll was extracted twice from 0.5 cm diameter leaf discs in 2 ml 80% (v/v) acetone and homogenised in a bead beater (PowerLyzer 24; Mo-Bio, Inc). The tubes were centrifuged at full speed in a bench top microfuge at 4°C for 2 min, and the supernatants were combined and transferred to a new tube and protected from the light. Absorbance was read at 663 nm and 645 nm, and chlorophyll contents were calculated according to the published method (Arnon, 1949).

**Total RNA Isolation and RT-qPCR**

Leaf samples (LP6) for RNA isolation were collected from wild type, rPPC1-A and rPPC1-B plants every 4 h starting at 2 h after dawn for both 12-h-light/12-h-dark cycles, and continuous light (LL) experiments. Biological triplicates were sampled, where each replicate was a pair of leaves of the designated developmental age from a separate, developmentally synchronised, clonal plant of each line. Total RNA was isolated from 100 mg of frozen, ground leaf tissue using a Qiagen RNeasy kit (Qiagen, Germany) following the manufacturer’s protocol with the addition of 13.5 µL 50 mg mL\(^{-1}\) PEG 20000 to the 450 µL RLC buffer used for each extraction. cDNA was synthesised from the total RNA using a Qiagen Quantitect RT kit according to the manufacturer’s instructions (Qiagen, Germany). The resulting cDNA was diluted 1:4 with molecular biology grade water prior to use in RT-qPCR. Transcript levels were determined using a SensiFAST SYBR No Rox kit (Bioline) in an Agilent MX3005P qPCR System Cycler. The results for each target gene transcript of interest were normalized to the reference gene **THIOESTERASE/THIOL ESTER DEHYDRASE-ISOMERASE SUPERFAMILY PROTEIN (TED)**; Kalax.0134s0055.1 and Kalax.1110s0007.1; Arabidopsis orthologue AT2G30720.1). Gene expression in a pool of RNA generated from LP6 samples collected every 4 hours over a 12-h-light/12-h-dark cycle was set to 1. Primers used for RT-qPCR analyses are listed in Supplemental Data Set 2 and were designed to target both homeologs of each gene present in the tetraploid genome. It should be noted that primers that were specific to both homeologs of **CCA1-1** did not target the two **CCA1-2** homeologs in the *K.*
laxiflora genome. Likewise, the primers that we used to quantify transcripts from both homeologs of TOC1-1 did not target the two TOC1-2 homeologs.

Immunoblotting

Total protein extracts of K. laxiflora leaves were prepared according to Dever et al. (2015). One-dimensional SDS-PAGE and immunoblotting of leaf proteins was carried out following standard methods. Blots were developed using the ECL system (GE Healthcare, UK). Immunoblot analysis was carried out using antisera to PPC raised against purified CAM leaf PPC from K. fedtschenkoi (diluted 1:5000), kindly supplied by Prof. Hugh G. Nimmo, University of Glasgow (Nimmo et al., 1986), and the phosphorylated form of PPC (diluted 1:1000) raised against a phospho-PPC peptide from wheat, and kindly supplied by Prof. Cristina Echevarría, Universidad de Sevilla, Spain (González et al., 2002; Feria et al., 2008). The PPDK antibody (diluted 1:1000) was raised against maize (Zea mays) C₄ PPDK, and the phospho-PPDK antibody (diluted 1:3000) was raised against a synthetic phospho-peptide spanning the PPDK phosphorylation site in the Z. mays C₄ PPDK. Both PPDK antibodies were kindly provided by Prof. Chris J. Chastain, University of Minnesota, Moorhead, USA (Chastain et al., 2000; Chastain et al., 2002). The specificity of the antibodies used and, in the case of the phospho-peptide specific antibodies for PPC and PPDK, their target phospho-site amino acid sequences are described in the Supplemental Methods.

Growth Measurements

Mature plants of wild type and the two rPPC1 lines were grown from developmentally synchronized clonal leaf plantlets in greenhouse conditions for 4 months. At the start of the drought treatment, all the plants were watered to full capacity. Water was withheld from at least six replicate plants of each line for 28-days, and at least six plants were maintained well-watered over the same period. The plants were harvested as separated above-ground (shoot) and below-ground (root) tissues, weighed to determine fresh weight, and dried in an oven at 60°C until they reached a constant dry weight.
PPC Assays

LP6 samples were taken at 6 h into the light period and frozen in liquid nitrogen. Biological triplicates were sampled, where each replicate was LP6 from a separate, developmentally synchronised, clonal plant of each line. Frozen leaf tissue was ground in liquid nitrogen with a small quantity of acid washed sand and the relevant enzyme specific extraction buffer (approximately 1 g tissue to 3 ml of extraction buffer). Extracts were prepared and PPC assays were performed according to the extraction, desalting and assay buffer conditions described previously (Dever et al., 2015).

Determination of the Apparent $K_i$ of PPC for L-Malate in Rapidly Desalted Leaf Extracts

The apparent $K_i$ of PPC for L-malate was determined using leaf extracts that were rapidly desalted as described by Carter et al. (1991). LP6 were collected at 10:00 (2 h before the end of the 12-h-light period) and 18:00 (middle of the 12-h-dark period) from three biological replicates of wild type, rPPC1-A and rPPC1-B. Each biological replicate was LP6 from a separate, developmentally synchronised, clonal plant of each line. The apparent $K_i$ of PPC activity for feedback inhibition by L-malate was determined as described by Nimmo et al. (1984), with the modifications to the range of L-malate concentrations added to the assays as described by Boxall et al. (2017).

PPDK Assays

PPDK assays were performed using the extraction and assay buffers described previously (Kondo et al., 2000; Dever et al., 2015), with the addition of NADH and G6P (Salahas et al., 1990), and Cibercron Blue (Burnell and Hatch, 1986). Biological triplicates were sampled, where each replicate was LP6 from a separate, developmentally synchronised, clonal plant of each line. Briefly, 0.3 g of powdered leaf tissue that had previously been ground to a fine powder in liquid nitrogen was extracted in 1 ml of ice-cold extraction buffer (containing 100 mM Tris pH 8.0, 10 mM DTT, 1 mM EDTA, 1% Triton, 2.5% w/v PVPP, 2% PEG-20000, 10 mM MgCl$_2$, 1 mM PMSF, 2 µM orthovanadate, and 10 µM Cibercron Blue) by grinding with a small
quantity of acid washed sand in a pestle and mortar. Extracts were vortexed for 30 s and the pH was adjusted to pH 8.0. Extracts were then placed on ice for 10 min before spinning them at full speed in a benchtop microfuge at 4°C. The supernatant (500 µl) was desalted using PD minitrap Sephadex-G25 columns (GE Healthcare). The desalting buffer contained: 100 mM Tris-HCl pH 8.0, 10 mM MgCl$_2$, 10 mM DTT, 0.1 mM EDTA, 10 µM Cibercron Blue. The desalted extracts were assayed in a plate reader at 340 nm using the following assay buffer: 100 mM Tris pH 8.0, 5 mM DTT, 10 mM MgCl$_2$, 1.25 mM Pyruvic acid, 2.5 mM NaHCO$_3$, 2.5 mM K$_2$HPO$_4$, 0.25 mM NADH, 2 U Malate dehydrogenase, and 6 mM glucose 6-phosphate. The reactions were started by the addition of 0.2 U of phosphoenolpyruvate carboxylase followed by 1.25 mM ATP (pH 8.0).

**Delayed Fluorescence (DF) Measurements**

The imaging system for DF was identical to the luciferase and delayed fluorescence imaging system described previously (Gould et al., 2009) with the exception of the CCD camera (Retiga LUMO™ CCD Camera; Qimaging). DF was quantified using Imaris image analysis software (Bitplane) to measure mean intensity for specific regions within each image. Background intensities were calculated for each image and subtracted to calculate a final DF value for each image (Gould et al., 2009).

**DF Rhythm Analysis**

*K. laxiflora* plants were grown in greenhouse conditions for 4 months and then entrained in 12-h light (450 µmoles m$^{-2}$ s$^{-1}$), 25°C, 60% humidity/12-h dark, 15°C, 70% humidity cycles in a Snijders Microclima MC-1000 (Snijders Scientific) growth cabinet for 7-days as described previously (Dever et al., 2015). At dawn on the 8th day, 1.5-cm leaf discs were punched from each LP6 for three biological replicates (i.e., 7 leaf discs from each biological replicate, totalling 21 leaf discs per line) and placed on 0.3% Phytoagar (Duchefa Biochemie) on a 10-cm square Petri dish. The Petri dish was left under 12-h light/25°C: 12-h dark/15°C for a further 24 h to synchronise the leaf discs. At subjective dawn, the Petri dish was placed in the imaging system at 14°C in constant red-blue light (LL). DF images were collected every hour for 120 h as described previously (Gould et al., 2009; Boxall et al., 2017).
The DF images were processed as described by Gould et al. (2009). The luminescence was normalized by subtracting the Y value of the best straight line from the raw Y value. Biodare was used to carry out fast Fourier transform (nonlinear least square) analysis and spectral resampling on each DF time-course series using the time window from 24 to 120 h in order to generate period estimates and calculate the associated relative amplitude error (RAE) (Moore et al., 2014; Zielinski et al., 2014).

**Accession Numbers**

Sequence data associated with this article are available via the JGI Phytozome portal for the *K. laxiflora* genome: [https://phytozome.jgi.doe.gov](https://phytozome.jgi.doe.gov) *Kalanchoë laxiflora* v1.1. The specific gene IDs for each gene measured in this work are provided in Supplemental Data Set 2.

**Supplemental Data**

- **Supplemental Figure 1.** Loading control for immunoblot analyses and PPC malate sensitivity assays.
- **Supplemental Figure 2.** Impact of loss of PPC activity on vegetative yield during growth under well-watered and drought-stressed conditions.
- **Supplemental Figure 3.** Photographs demonstrating the visual appearance of small plants after 22 d drought prior to re-watering.
- **Supplemental Figure 4.** Impact of leaf age on the development of the characteristic 24 h light/dark pattern of CAM-associated CO₂ exchange.
- **Supplemental Figure 5.** Impact of the loss of PPC1 on the light/dark regulation of the transcript abundance of CAM-, starch-, and circadian clock-associated genes in the CAM leaf epidermis.
- **Supplemental Table 1.** Student’s t-test analysis of PPC activity.
- **Supplemental Table 2.** Student’s t-test analysis of PPDK activity.
- **Supplemental Table 3.** Student’s t-tests for chlorophyll a and b.
- **Supplemental Table 4.** Calculations for the cumulative total CO₂ fixation (area under the curve) by each line during the 27-day drought-stress and re-watering experiment.
- **Supplemental Methods.**
Supplemental Data Set 1. Student's t-test results for relative growth measurements between wild type (WT) and \(rPPC1-B\).

Supplemental Data Set 2. Primers used for RT-qPCR.

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AUTHOR CONTRIBUTIONS

J.H. and S.F.B. designed the research. S.F.B. performed all of the experiments except some of the tissue culture work for the regeneration of the transgenic lines, which was performed by N.K., and the statistical analysis of the delayed fluorescence data, which was performed by P.J.D.G. J.K. generated the RNAi binary construct for \(PPC1\). L.V.D. performed the PPDK assays. J.L.W. grew and maintained the plants and helped with grinding and processing of leaf samples. P.J.D.G. helped S.F.B. with the delayed fluorescence experiments and the analysis and interpretation of the associated data. S.F.B. and J.H. wrote the manuscript.

Table 1. Analysis of \(\text{CO}_2\) uptake in wild type (WT), \(rPPC1-A\) and \(rPPC1-B\) during 22-d of progressive drought-stress and for 4-days following re-watering.

|                  | Night 1 | Day 1 | Total | Night 7 | Day 7 | Total | Night 13 | Day 13 | Total | Night 22 | Day 22 | Total | Night 27 | Day 27 | Total |
|------------------|---------|-------|-------|---------|-------|-------|----------|--------|-------|----------|--------|-------|----------|--------|-------|
| **WT (% of diet \(\text{CO}_2\) uptake)** | 7       | 93    | 100   | 52      | 48    | 100   | 111      | -11    | 100   | 133      | -33    | 100   | 56       | 44     | 100   |
| **WT \(\text{CO}_2\) uptake (µmol m\(^{-2}\))** | 78      | 1015  | 1093  | 698     | 647   | 1345  | 908      | -113   | 795   | 152      | -76    | 76    | 931      | 735    | 1666  |
| **\(rPPC1-A\) (% of diet \(\text{CO}_2\) uptake)** | 8       | 94    | 100   | 40      | 60    | 100   | 112      | -12    | 100   | 128      | -28    | 100   | 46       | 54     | 100   |
Table Footnote: Data are presented for selected days (1, 7, 13, 22 and 27) throughout the 27-day drought-stress and re-watering experiment. Results expressed as percentages were calculated separately for the 12-h-light and 12-h-dark periods by determining the percentage of the total diel/ 24 h CO₂ uptake that occurred in that period. The actual CO₂ uptake over the 12-h-light and 12-h-dark period is also presented for each line.

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| rPPC1-A CO₂ uptake (µmol m⁻²) | 73 | 1081 | 1008 | 553 | 614 | 1367 | 922 | 120 | 802 | 209 | -81 | 130 | 768 | 889 | 1657 |
|-------------------------------|----|------|------|-----|-----|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| rPPC1-B (% of diel CO₂ uptake) | -26 | 126 | 100 | -14 | 114 | 100 | -6  | 106 | 100 | -27 | -73 | 100 | -17 | 117 | 100 |
| rPPC1-B CO₂ uptake (µmol m⁻²) | -575 | 1653 | 1078 | -254 | 1759 | 1506 | -17 | 161 | 145 | -8  | -22 | -30 | -385 | 1825 | 1440 |
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Figure 1. Confirmation of Target Gene Silencing in Transgenic *K. laxiflora* RNAi Lines *rPPC1*-A and *rPPC1*-B.

Gene transcript abundance in leaf pair 6 (LP6) was measured using RT-qPCR for target genes: **(A)** PPC1; **(B)** PPC2; **(C)** PPC3; **(D)** PPC4; **(E)** PPCK1; **(F)** PPCK2; and **(G)** PPCK3. LP6 were sampled every 4 h across the 12-h-light/12-h-dark cycle. A *THIOESTERASE/THIOL ESTER DEHYDRASE-ISOMERASE* superfamily gene (*TEDI*) was amplified from the same cDNAs as a reference gene. Gene transcript abundance data represents the mean of 3 technical replicates for biological triplicates and were normalized to loading control gene (*TEDI*); error bars represent the standard error. In all cases, plants were entrained under 12-h-light/12-h-dark cycles for 7-days prior to sampling. Black data are for the wild type, blue data *rPPC1*-A, and red data *rPPC1*-B.
Figure 2. Loss of PPC1 Transcripts Leads to Loss of PPC Protein and Activity and Failure to Activate PPDK in the Light.

Total leaf protein from leaf pair 6 (LP6) was isolated from leaves sampled at dawn and dusk plus every 4 h, starting at 2 h into the light period, across the 12-h-light/12-h-dark cycle, separated using SDS-PAGE and used for immunoblot analyses with antibodies raised to PPC (A), and phospho-PPC (B). (C) PPC activity was measured in rapidly desalted extracts from LP6, ** = rPPC1-A p= 0.0054, *** = rPPC1-B, p=0.00016. (D) Anti-PPDK immunoblot. (E) Anti-phospho-PPDK immunoblot. (F) PPDK activity was measured in leaf extracts prepared from both dark leaves and illuminated leaves, ** = rPPC1-B, p=0.003. The activities of PPC, measured 6 h into the light period, and PPDK, measured at both 6h into the dark, and 6h into the light periods, were the mean of 3 technical replicates of 3 biological replicates and the error bars represent the standard error. The white bar below each panel represents the 12-h-light period and the black bar below each panel represents the 12-h-dark period. Black data are for the wild type, blue data rPPC1-A, and red data rPPC1-B. Asterisks indicate significant difference from the wild type based on the students t test.*. All Student’s t-test parameters are presented in Supplemental Table 1 and 2.
Figure 3

A) Malate

B) Starch

C) Sucrose

D) Glucose

E) Fructose

F) Shoot DW

G) Plant images
Figure 3. Impact of silencing PPC1 on malate, starch, soluble sugars and growth. (A) The contents of malate, (B) starch, (C) sucrose, (D) glucose and (E) fructose were determined from leaf pair 6 (LP6) samples collected every 4 h using plants entrained under 12-h-light/12-h-dark cycles. Methanol extracts were prepared from the leaves from wild type, rPPC1-A and rPPC1-B and used for malate and soluble sugar determination, whereas starch was measured in the insoluble pellet. (F) Dry weight of 148-day-old greenhouse-grown plants, either well-watered (w) or drought-stressed for the last 28-days (d). * denotes significant difference between wild type and rPPC1-B based on the Student's t-test. Well-watered, rPPC1-B * p = 0.017; drought-stressed rPPC1-B * p = 0.011, n = 6 to 9, developmentally synchronised, clonal plants per line. (G), 4-month-old plants raised in a greenhouse under 16-h-light/ 8-h-dark. In all graphs, black data are for the wild type, blue data rPPC1-A, and red data rPPC1-B. Error bars represent the standard error. Asterisks indicate significant difference from the wild type:* . See Supplemental Data Set 1 for full parameters from the Student's t-test results for the relative growth data.
Figure 4. Impact of silencing PPC1 on 24 h light/ dark gas exchange profiles under well-watered and drought-stressed conditions, and on chlorophyll content.

(A), CO₂ uptake profile showing the 4 phases of CAM, (B), stomatal conductance (gₛ) profile and (C), the calculated internal partial pressure of CO₂ inside the leaf (Cᵢ) profile for CAM leaves (leaf pair 6; LP6) using plants pre-entrained for 7-days under 12-h-light/ 12-h-dark cycles. (D), Impact of drought on K. laxiflora wild type and transgenic lines with reduced PPC1. Gas exchange profile for shoots of whole young plants (9-leaf-pair stage) measured throughout 27-days. The plants were watered to full capacity on day 0 and allowed to progress into drought until day 23 when they were re-watered to full capacity (black arrow). The means for each of the four gas exchange traces for each line are shown, with error bars showing the standard error of the mean (SEM) in the paler-coloured extensions that extend above and below each of the data points. (E) and (F) Impact of silencing PPC1 on chlorophyll a (E) and chlorophyll b (F) content in leaf pairs 2 through 7. In (E) and (F) the error bars represent the standard error of the mean calculated for the three biological replicates, which were individual clones of each line. Asterisks indicate significant difference from the wild type based on the Student’s t-test: * (E), Chlorophyll a in rPPC1-A: LP2, p = 0.000160; rPPC1-B: LP2, p = 0.043676; LP3, p = 0.000003; LP4, p = 0.000001; LP5, p = 0.000094; LP6, p = 0.000012; LP7, p = 0.010883. * (F), Chlorophyll b in rPPC1-A: LP2, p = 0.000304; rPPC1-B: LP3, p = 0.000019; LP4, p = 0.000016; LP5 p = 0.000653; LP6, p=0.000951. All Student’s t-test parameters are presented in Supplemental Table 3. Black data are for the wild type, blue data rPPC1-A, and red data rPPC1-B.
Figure 5. Impact of the Loss of PPC1 Transcripts on the Light/Dark Regulation of the Transcript Abundance of CAM- and Central Circadian Clock-Associated Genes in RNAi Lines rPPC1-A and rPPC1-B.

Gene transcript abundance was measured using RT-qPCR for target genes: (A) PPDK; (B) PPDK-RP; (C) β-NADME; (D) GWD; (E) AMY3a; (F) AMY3b; (G) BAM1; (H) BAM3; (I) BAM9; (J) PHS1; (K) GPT2; (L) MEX1; (M) pGlcT; (N) CAB; (O) TPS7; (P) CCA1-1; (Q) CCA1-2; (R) TOC1-1; (S) TOC1-2; (T) PRR7; (U) PRR3/7; (V) PRR9; (W) JMJ30I; JMJ5; (X) ELF3; (Y) CDF2; (Z) RVE1-like; (AA) EPR1; (BB) LNK3; (CC) FKF1 and (DD) GI. Mature leaves (leaf pair 6; LP6) were sampled every 4 h across the 12-h-light/12-h-dark cycle. A thioesterase/thiol ester dehydrase-isomerase superfamily gene (TEDI) was amplified from the same cDNAs as a reference gene. Gene transcript abundance data represents the mean of 3 technical replicates for biological triplicates and were normalized to loading control gene (TEDI); error bars represent the standard error. In all cases, plants were entrained under 12-h-light/12-h-dark cycles for 7-days prior to sampling. Black data are for the wild type, blue data rPPC1-A, and red data rPPC1-B.
Figure 6. Effects of silencing *PPC1* on CAM CO₂ exchange rhythms measured under constant light and temperature (LL) conditions.

(A), Gas exchange profile for detached CAM leaves (leaf pair 6; LP6) was measured using leaves entrained under a 12-h-light/12-h-dark cycle followed by release into LL constant conditions (100 µmol m⁻² s⁻¹ at 15 °C). (B), Gas exchange profile for well-watered whole young plants (9-leaf-pairs stage) using plants entrained under 12-h-light/12-h-dark cycles followed by release into constant LL conditions (100 µmol m⁻² s⁻¹ at 15 °C). The data represents the mean CO₂ uptake of 3 individual plants. (C), Delayed Fluorescence (DF) circadian rhythms became more robust in lines *rPPC1*-A and *rPPC1*-B. Plants were entrained under 12-h-light/12-h-dark cycles before being transferred to constant red/blue light (35 µmol m⁻² s⁻¹) under the CCD imaging camera system. DF was assayed with a 1-h time resolution for 120 h. The plots represent normalized averages for DF measured for 21 leaf discs sampled from 3 biological replicates of LP6 from each line. Error bars indicate SE of the mean calculated from three biological replicates. (D), Relative Amplitude Error (RAE) plot for the DF rhythms. (E), Mean period length plot, and (F), Mean RAE plot; the plotted values were calculated using the Biodare package for circadian rhythm analysis. FFT = fast Fourier transform-nonlinear least squares analysis, or SR = spectral resampling analysis. Black data are for the wild type, blue data *rPPC1*-A, and red data *rPPC1*-B. LL begins at 0h in Figures 6A-C.
Figure 7. Impact of the Loss of PPC1 Transcripts on Circadian Clock Controlled Gene Transcript Abundance during Constant Light and Temperature (LL) Free Running Conditions.

Mature leaves (leaf pair 6), which performed CAM in the wild type, were sampled every 4 h under constant conditions (100 μmol m⁻² s⁻¹ at 15 °C) for wild type and rPPC1-B. RNA was isolated and used for real-time RT-qPCR. A thioesterase/thiol ester dehydrase-isomerase superfamily gene (TEDI) was amplified as a reference gene from the same cDNAs. Gene transcript abundance data represents the mean of 3 technical replicates for biological triplicates and were normalized to loading control gene (TEDI); error bars represent the standard error. In all cases, plants were entrained under 12-h-light/12-h-dark cycles prior to release into LL free-running conditions at 0h. Black data are for the wild type and red data rPPC1-B. The grey bars behind the data represent the subjective dark period, the time when it would have been dark under the previous entrainment conditions. Likewise, the white regions represent the subjective light period.

(A), Circadian rhythm of PPC1 transcript abundance under constant LL conditions (100 μmol m⁻² s⁻¹ at 15 °C) for wild type and rPPC1-B. (B), PPC2; (C), PPCK1; (D), PPCK2; (E), PPCK3; (F), GPT2; (G), CAB; (H), CCA1-1 ; (I), CCA1-2; (J), TOC1-1; (K), TOC1-2; (L), PRR7; (M), PRR37; (N), PRR9; (O), LNK3-like; (P), JMJ30/JMJD5; (Q), ELF3; (R), CDF2; (S), RVE1-like; (T), EPR1; (U), FKF1 and (V), GI.
Figure 8

Figure 8. Impact of the Loss of PPC Activity on the Light/Dark Regulation of the Transcript Abundance of Stomatal Control Genes in the Epidermis.

Plants were entrained under 12-h-light/12-h-dark for 7 days prior to sampling. Epidermal peel samples were separated from leaf pairs 6, 7 and 8, with samples collected every 4h starting at 02:00, 2 h into the 12-h-light period. Each biological sample represents a pool of epidermal peels taken from 6 leaf pair 6, 7 and 8 leaves from 3 clonal stems of each line. Each peel was frozen in liquid nitrogen immediately after it was taken and pooled later. RNA was isolated and used in RT-qPCR. A thioesterase/thiol ester dehydrase-isomerase superfamily gene (TEDI) was amplified as a reference gene from the same cDNAs. Gene transcript abundance data represent the mean of 3 technical replicates for biological triplicates, and were normalized to the reference gene (TEDI); error bars represent the standard error. In all cases, plants were entrained under 12-h-light/12-h-dark cycles for 7 days prior to sampling. Black data are for the wild type and red data rPPC1-B. (A), PHOT1; (B), CRY2; (C), βCA5; (D), PATROL1; (E), CBC1/2; (F), HT1; (G), OST1; (H), SLAC1; (I), PP2C; (J), RCAR3; (K), GORK; (L), SKOR; (M), ALMT12; (N), RMA1; (O), EDA39; (P), SOS2; (Q), ACA2; (R), ECA4; (S), MYB60 and (T), MYB61.
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Kalanchoë PPC1 is Essential for Crassulacean Acid Metabolism and the Regulation of Core Circadian Clock and Guard Cell Signaling Genes
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