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Transcriptomics of C 3 +CAM Peperomia species reveals potential initial steps toward a CAM pathway

G Young Kim

gykim@uconn.edu

University of Connecticut

David Wickell

University of Connecticut

Cassidy Downs

University of Hawai□i at Mānoa

Chase Kane

University of Hawai□i at Mānoa

Karolina Heyduk

University of Connecticut

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Abstract

Background

The genus *Peperomia* (family Piperaceae) is the only known lineage with Crassulacean Acid Metabolism (CAM) in the Magnoliids.. Previous studies have demonstrated C_3+CAM in multiple *Peperomia* species, yet there are ~ 1,600 species in the genus and very few have been assessed for CAM. Further, no studies have used genomics to understand CAM evolution in the Magnoliids. This study not only aims to find evidence of CAM in Hawaiian *Peperomia*, but also seeks to reveal genomic changes associated with C_3+CAM evolution via transcriptomics and gene regulatory network analysis.

Results

While carbon isotope ratios suggested the majority of Hawaiian *Peperomia* species were C_3, our detailed physiological measurements on a subset of species unveiled two two C_3+CAM species. Transcriptomic analysis showed typical CAM-like expression of a number of genes in the core CAM pathway in both C_3 and C_3+CAM species studied, but with increasing levels of expression in the C_3+CAM species. One core CAM gene, phosphoenolpyruvate carboxylase kinase (*PPCK*), had clear upregulation of expression in the C_3+CAM species, and also had induced expression under drought. Cluster and network analysis of *PPCK* expression indicated that circadian rhythm factors are also highly co-expressed with *PPCK* in C_3+CAM species.

Conclusions

Our findings not only reveal the existence of the C_3+CAM pathway in Hawaiian *Peperomia* species, but highlights *PPCK* as a regulatory bridge that possibly connects the evolution between C_3 and C_3+CAM. With further analysis, understanding functional regulators of PPCK will be a key to understanding how CAM evolved from ancestral C_3.

Background

In photosynthetic organisms, photorespiratory stress due to the biochemistry of carbon fixing enzyme, Rubisco, can limit overall productivity [1]. Rubisco has affinities for both O_2 and CO_2; the former increases under high temperatures or conditions that promote low CO_2 concentrations, such as changes to atmospheric CO_2 over time, or more acute environmental conditions like drought stress. Drought reduces available CO_2 in plant leaves through stomatal closure, which effectively reduces water loss to the atmosphere, but restricts availability of CO_2 and leads to O_2 fixation by Rubisco. Rubisco binding O_2 leads to a process called photorespiration, which uses cellular energy to remove toxic byproducts and free the substrate for Rubisco activity. Photorespiration, while a critical pathway for nitrogen metabolism,

also can result in up to a 36% reduction in productivity, and thus represents a major stress for photosynthetic organisms [1].

To minimize photorespiratory stress, plants evolved modified forms of the common C_3 photosynthetic pathway called carbon concentrating mechanisms (CCMs) that increase carbon concentrations around Rubisco. The two main types of CCMs are C_4 and Crassulacean Acid Metabolism (CAM), which share the same biochemical pathways yet separate atmospheric CO_2 uptake from sugar production spatially and temporally, respectively. Both C_4 and CAM species use phosphoenolpyruvate carboxylase (PEPC) instead of Rubisco as the initial carboxylating enzyme, which converts incoming CO_2 into a four-carbon organic acid. While C_4 plants take up CO_2 during the day and shuttle the fixed CO_2 as an organic acid between adjacent mesophyll and bundle sheath cells, CAM plants open their stomata at night for gas exchange. Nocturnally acquired CO_2 in CAM plants is stored as malate in the vacuole overnight, then moved to the cytosol for decarboxylation during the day, when CAM plants close their stomata. The concentrated CO_2 of both CCMs reduces photorespiration by limiting interaction between Rubisco and O_2. CAM plants, in particular, achieve high water use efficiencies by limiting their stomatal opening to the nighttime, when temperatures are often cooler and transpiration rates are overall lower. Unsurprisingly, CAM is often associated with plants inhabiting arid environments, including cacti, agave, and euphorbs, but is also present in tropical epiphytes that have no access to soil moisture or those that experience seasonal rains [2].

The nocturnal nature of the CAM pathway has led to diagnostic temporal patterns of expression in many of the core biochemical pathway genes of CAM. For example, the main carboxylating enzyme PEPC shows an increase in mRNA expression just before the onset of the dark period [3]. PEPC is inhibited by its own product, malate (a vestige of its role in the citric acid cycle in all plants). In CAM plants, however, this negative feedback loop would limit overall nocturnal CO_2 fixation. To limit malate inhibition, CAM plants use a dedicated kinase, PEPC kinase (PPCK), that phosphorylates PEPC only at night, rendering it insensitive to malate inhibition. *PPCK* likewise has a striking increase in expression only in the dark in CAM plants and is thought to be a central regulator of CAM activity [4]. On the other hand, a number of genes suggested to be important for CAM have unclear temporal expression patterns. At night, atmospheric CO_2 is thought to be converted to bicarbonate by beta-carbonic anhydrase (β-CA) in the cytosol, but expression of *β-CA* does not always have a night-specific expression pattern in all CAM species [5–8]. Decarboxylation pathways in CAM plants can come in two main forms: NAD(P)-malic enzyme (NAD(P)-ME) converts malate to pyruvate, then ortho phosphoenolpyruvate dikinase (PPDK) regenerates the PEP substrate from pyruvate, or NAD(P)-malate dehydrogenase (NAD(P)-MDH) generates oxaloacetate (OAA) from malate, which is further converted to PEP by PEP carboxykinase (PEPCK). The enzymes used in the CAM photosynthesis are present in all plant species, regardless of photosynthetic pathway, and have various roles associated with the citric acid cycle. PEPC, for example, facilitates the same biochemical reaction in all plants as a way to provide the citric acid cycle with OAA. It is thought, therefore, that the evolution of CAM requires shifts in the timing and magnitude of gene expression of existing genes, rather than *de novo* evolution of gene copies or entire pathways.

Recent studies have suggested that CAM is found in nearly 7% of all vascular plants [2] and has evolved at least 66 times independently [2]. The strength of CAM varies across lineages and species but can be generally categorized into two groups: strong CAM and weak CAM (C_3+CAM). Strong CAM plants, also known as obligate CAM plants, are highly dependent on CAM for carbon assimilation [9]. In contrast, C_3+CAM plants use both C_3 and CAM for carbon assimilation simultaneously or can alter the extent of pathway usage under different environmental conditions. For example, facultative CAM is a specific stress-induced phenotype whereby plants use the C_3 pathway under stable conditions but employ CAM when exposed to abiotic stress [9]. C_3+CAM plants also include those that can reuse respired nocturnal CO_2 for CAM activity. Determining the level of CAM activity requires measuring multiple metrics, including gas exchange patterns under nominal and stressful conditions, acid accumulation in the photosynthetic tissues, and, more recently, gene expression of canonical CAM genes.

Among CAM species, the genus *Peperomia* is the only CAM lineage documented in subclass Magnoliids [2]. The genus *Peperomia* is the second most speciose genus in the family Piperaceae, consisting of nearly 1,600 documented species [10, 11]. *Peperomia* has diversified in pantropical, neotropical, and Pacific regions [12] is thought to have independently arrived in Hawai□i four to five times, approximately between the Pliocene and Pleistocene periods [13]. Lineages of *Peperomia* (ʻalaʻala wai nui in ʻŌlelo Hawaiʻi) in Hawai□i are divided into four clades: Hawaiian radiation A, blanda clade, Hawaiian radiation B, and tetraphylla clade [12] (Fig. 1). Most species belong to the Hawaiian radiation A and Hawaiian radiation B, which together further diversified after arrival to the islands into 25 species native (23 endemic) to Hawai□i [12]. Recent evidence suggests that the Hawaiian radiation originated from either neotropical or Pacific regions [12, 14]. Most species of Hawaiian *Peperomia* are epiphytes in the tropical forests, with the exception of *P. tetraphylla*, which is terrestrial [12].

Epiphytic *Peperomia* species experience frequent drought stress from poorly developed root systems [15], and multiple studies have reported CAM and C_3+CAM in *Peperomia*, including in the Hawaiian species *P. blanda* [16]. While multiple studies have described CAM in *Peperomia* [17–19], few of these included transcriptomic analysis to complement physiological data, and very few have focused on species native to Hawai□i [16]. The majority of prior genomic studies on CAM species have focused on strong CAM and changes in expression of core CAM genes or circadian regulation relative to C_3 species, with far fewer studies focusing on C_3+CAM or facultative species [6, 20, 21]. Thus, our understanding of the initial genomic changes from ancestral C_3 to CAM is limited. Moreover, prior genomic work has largely focused on eudicot or monocot lineages, with a few notable studies outside of the angiosperms [22–24]. Here we undertake a comprehensive study of CAM evolution in the genus *Peperomia*, which represents a unique lineage of CAM species in the Magnoliids, and one where C_3+CAM has been extensively documented [25]. We first broadly characterize CAM diversity in the Hawaiian *Peperomia* species with carbon isotope ratios, then further explore a subset of species for CAM activity and induction under drought through both physiological measurements and transcriptomic analyses. Our results show that *Peperomia blanda* and *P. sandwicensis* are C_3+CAM, but we identify very few transcriptomic changes relative to a C_3 species, *P. mauiensis*; instead, we show that induction and

circadian co-regulation of *PPCK* appears to be an initial transcriptomic switch for acquiring low level CAM activity in *Peperomia*.

Methods

Plant materials —Dried herbarium samples of 25 native Hawaiian *Peperomia* species were collected from J.F. Rock Herbarium (HAW) and Bishop Museum Herbarium (BISH) for evaluating stable isotopes (Supplementary Table 1). For physiological studies, 3–4 replicates of five native species (*P. blanda, P. cookiana, P. hypoleuca, P. mauiensis* and *P. sandwicensis*) were obtained from Lyon Arboretum. All five species were used for titratable acidity and leaf gas exchange measurements. Based on physiological traits measured, the five species were narrowed down to three (*P. blanda, P. sandwicensis*, and *P. mauiensis*,) for RNA-sequencing.

δ^{13}C stable isotope analysis —Using herbarium specimens from the J.F. Rock Herbarium and the Bishop Museum Herbarium, we collected 1–2 mg of dried leaf samples from 1–2 separate specimens for each of the 25 native species. Samples were dried for up to two weeks to remove all water in the tissue, then were hand-ground with mortar and pestle. Fine hand-ground samples were sent to the Biogeochemical Stable Isotope Facility at University of Hawai'i at Mānoa, where 0.5–0.8 mg of tissue was run on an Exeter Analytical CE 440 Elemental Analyzer for obtaining delta δ^{13}C ratios.

Leaf gas exchange — Eighteen plants (3–4 replicates per species: *P. blanda*, *P. cookiana*, *P. hypoleuca*, *P. mauiensis*, and *P. sandwicensis*) were stem-cut and grown to maturity in the Lyon Arboretum as part of their standard plant propagation protocol. Plants were all grown in a standard potting mix, except for the four replicates of *P. blanda*, which were planted in volcanic soil. Plants were placed in an environmentally controlled room at the University of Hawai'i at Mānoa for a two week acclimation period at 24°C and 60–70% humidity. Each plant was watered every 3–4 days and exposed to 10 hours of artificial light at 350 μmol $m^{-2}s^{-1}$ daily (lights were turned on at 8 AM local and turned off at 6 PM local). Our time metric follows the Zeitgeber-Time (hereafter called ZT), which counts the time after one hour of the start at the light period. Replicates across all three species were randomly sorted into two groups in order to allow us to measure photosynthesis for all plants at a timepoint within 1 hour of each other. Each group had 9 plants that were a random representation of the five species. After the two week acclimation period, a LI-6800 (LI- COR) Portable Photosynthesis Photosystem was used to measure gas exchange on leaves, with measurements taken every four hours over a 24 hour period. System settings were at 350 μmol m^{-2} s^{-1} PAR, 420 μmol mol^{-1} CO_2, and 60–70% humidity with flow rates of 500 μmol sec^{-1}. Water content in the soil was recorded with a soil moisture meter every sampling day at the first time point (8 AM). Under well-watered conditions, each plant was given supplemental water one hour prior to the first time point measurement. After 7 days of withholding water, gas exchange was measured again as described above for well-watered plants. The two randomized groups of plants were offset by two days: the first group was measured for gas exchange under well-watered, then the second group two days later; the first group had drought stress initiated, then the second group did two days later. Leaf area was measured by

taking photos of leaves with a ruler in frame, then calculations of leaf area were made in ImageJ (Schneider *et al.*, 2012) and used to adjust A_{max} values to a per-area basis. Normalized data was filtered by removing measurements that had low (< 100 μmol mol^{-1}) or high (> 800 μmol mol^{-1}) intercellular CO_2 concentration (C_i), or those with a leak above 40%.

Titratable acidity — Leaf samples were collected from each experimental plant at two time points: dusk (ZT 9; 4 PM local) and dawn (ZT 21; 4 AM local) in both well-watered and drought-stressed conditions. Samples were harvested onto ice then immediately stored in a -20°C freezer. Samples were then weighed and boiled in 60 mL of 20% ethanol in a beaker until it reached half volume (30 mL). An additional 30 mL of deionized water was added and re-boiled until it again reached half volume (30 mL). Another 30 mL of deionized water was added and the beaker was left in ice to cool down. Once the beaker cooled down to room temperature, leaf samples were removed and the solution was titrated with 2 mM NaOH until the pH level reached pH 7.0. Acid accumulation was calculated for μmol H+ equivalents using the equation: $\Delta H+ (\mu mol\ H^+\ g^{-1})$ = {mL titrated (pH7.0) x (mM of solution used)} ÷ weight (g). We used R 4.2.2 for paired t-tests within each species in both treatments to test if acid accumulation (dawn value - dusk value) was significantly different from zero.

RNA isolation —RNA-sequencing was conducted to compare gene expression between a C_3 (*P. mauiensis*) and two C_3+CAM species (*P. blanda* and *P. sandwicensis*), as determined by our physiological measurements. Leaf tissue samples were collected at the same six time points as the leaf gas exchange measurements under both well-watered and drought-stress conditions. Samples were preserved in RNA-Later solution (Invitrogen) and stored in a -20°C freezer. For RNA isolation, samples in RNA-Later were thawed, the tissue weighed, and then finely ground with mortar and pestle in liquid nitrogen. RNA was extracted using the Qiagen RNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. Isolated RNA was cleaned with the Turbo DNA-free Kit (Invitrogen) and quantified with Qubit RNA High Sensitivity Kit (Thermo Fisher Scientific). RNA extracts were further checked for RNA integrity through gel electrophoresis and finally stored in a -70°C freezer. For RNA library preparation, we used the KAPA HyperPlus mRNA Kit (Roche) at half reaction but otherwise followed the manufacturer's protocol. We used dual-indexed barcodes purchased from the BadDNA lab at the University of Georgia. Amplified library samples were assessed with a Qubit DNA High Sensitivity Kit (Invitrogen) and were sent to the University of Connecticut's Center for Genome Innovation for sequencing on a NovaSeq 6000 using 150 bp paired reads, targeting 15 million read pairs per library. Raw sequencing reads are deposited on NCBI's Short Read Archive under BioProject PRJNA1468765.

Data Processing –Raw read files were quality controlled with Trimmomatic v0.38 [26] to filter out low quality reads and Illumina primer adapters. Sequences were then mapped against annotated coding sequences from a draft version of the South American *Peperomia trollii* draft reference genome (CoGe genome ID: 70004 [27]) with Kallisto 0.46.1 [28]. We used sleuth 2.0 [29] to obtain normalized counts (CPM) and transcripts mapped per million (TPM) values for each transcript. PCA was used for pattern detection across species and time, as well as outlier removal (3 replicates from *Peperomia blanda* and 2

replicates from *P. sandwicensis* were removed, Supplementary Fig. 1) via the ade4 package in R 4.3.1. Normalized count data were used for maSigPro [30] for cluster and time-structure analysis of transcripts. maSigPro was run separately for each species with treatment (watered vs. drought) tested as a factor affecting gene expression. Each species was fitted to find time-structured genes with adjusted epsilon parameters to promote convergence (*P. blanda* = 0.05, *P. mauiensis* = 0.1, *P. sandwicensis* = 0.05). We used the wss() function to determine the optimal number of clusters (k = 8 for each species) and used a modified see.genes() function from maSigPro (https://github.com/kheyduk) to assign genes to clusters in each species based on expression profiles (Supplementary Fig. 2). Finally, to assign a putative function to transcripts, we used genome annotation of the *P. trollii* reference genome, which was annotated based on orthogroup assignment with Orthofinder 2.5.1 [31], using the Arabidopsis gene IDs as the basis for annotation.

To further explore how gene regulation differs between C_3 and C_3+CAM species, we generated gene regulatory networks focused on the key CAM gene, *PPCK*, for each species. Using only genes in each species' *PPCK* cluster, we generated network statistics using ARACNe-AP [32] (*P. blanda*: cluster 8, *P. mauiensis*: cluster 5, *P. sandwicensis*: cluster 8; Supplementary Fig. 2). Gene networks were analyzed via Cytoscape 3.10.4 [33].

Results

Carbon isotope analysis

Our carbon isotope analysis of 25 native Hawaiian *Peperomia* species showed that most species are likely C_3 or C_3+CAM species. Isotope ratios were all less than − 22‰, placing the *Peperomia* isotope values within the range of C_3 to C_3+CAM ratios [34]. Mean values ranged from the lowest in *Peperomia ligustrina* as -30.7‰, to the highest values of *Peperomia pellucida* as -23.8‰ (Table 1).

Physiology: gas exchange measurements and titratable acidity

Our gas exchange measurements showed plants were primarily using respired CO_2 over uptaking atmospheric CO_2 to sustain CAM activity. In overall, daytime carbon assimilation rates were generally low across all species and only reached above 5 umol m^{-2} s^{-1} in replicates of *P. blanda* (Fig. 2A). Similarly, carbon assimilation values at night were below or at zero for all five species (Fig. 2B). Consistently low assimilation rates in day and night in nearly all species indicate that these plants may use cellular respired CO_2 rather than actively uptaking atmospheric CO_2 in order to keep stomata closed and conserve water [34, 35].

Titratable acidity was consistent with the CO_2 assimilation rates measured; we observed significant acid accumulation as an indicator of weak CAM in two species: *P. blanda* and *P. sandwicensis*. The average titratable acidity of *P. blanda* ($t = 3.117$, $df = 7$, $p = 0.017$) and *P. sandwicensis* ($t = 2.941$, $df = 7$, $p = 0.0217$)

were significantly greater than zero (Fig. 2C). Further, *P. blanda* had significant acid accumulation only under drought stress (t = 6.1478, df = 3, p = 0.0087).This suggests weak CAM only in *P. blanda* and *P. sandwicensis*, as other species measured had non-significant leaf acid accumulation; *P. blanda* also appeared to have slight facultative behavior, with CAM up-regulated under drought stress. Based on gas exchange and titratable acidity patterns, we concluded that *P. blanda* and *P. sandwicensis* are both C_3+CAM species.

Gene expression differences between species

From a total of 46,848 transcripts in the reference genome we used for mapping, 11,223 genes in *Peperomia blanda*, 7,496 genes of *P. sandwicensis*, and 7,569 genes in *P. mauiensis* were time structured. Of the time structured transcripts, 505 transcripts of *P. blanda*, 283 transcripts of *P. sandwicensis*, and 385 transcripts of *P. mauiensis* were differentially expressed between watered and drought treatments.

Peperomia trollii genome had 6 copies of plant-type *PEPC*, 1 copy of bacterial-type *PEPC*, 10 copies of *PPCK*, 1 copy of *PPDK* and *PEPCK*, 3 copies of *β-CA*, 2 copies of *MDH*, and 3 copies of *ME*. From these gene copies, there were 4 copies of plant-type *PEPC* that were time-structured in *P. blanda* and *P. sandwicensis*, and 3 copies that were time-structured in *P. mauiensis*. *PPCK* had the highest number of time-structured copies among the species —9 copies in *P. blanda*, 6 copies in *P. sandwicensis*, and 5 copies in *P. mauiensis*. Within the time structured *PPCK* copies, 5 copies in *P. blanda* and 1 copy in *P. mauiensis* were differentially expressed between watered and drought treatments. Bacterial-type *PEPC* was time-structured across all species. Two copies of *β-CA* were time-structured in *P. blanda* and *P. sandwicensis*, and one copy was time-structured in *P. mauiensis.* MDH had two copies of time-structured and one copy that is differentially expressed in *P. blanda*, while there was only one time-structured copy in both *P. sandwicensis* and *P. mauiensis*. One copy of *PPDK* was both time-structured in *P. sandwicensis* and *P. mauiensis*, but not in *P. blanda*. One copy of *PEPCK*, on the other hand, was time-structured in *P. blanda* and *P. sandwicensis*, but not in *P. mauiensis*. Two different copies of *ME* were time-structured in *P. blanda* and *P. mauiensis*, and one copy was time-structured in *P. sandwicensis*.

Gene expression profiles of nocturnal carboxylation genes were different between C_3+CAM (*P. blanda* and *P. sandwicensis*) vs. C_3 (*P. mauiensis*) species. For example, while all three species showed an increase in expression of plant-type *PEPC* during the nighttime, *P. blanda* had higher expression at nighttime compared to other two species (Fig. 3A-C). Bacterial-type *PEPC*, which is recruited for CAM in certain lineages [22, 36], did not show CAM-like expression in *Peperomia*, suggesting it is not part of the CAM pathway in this lineage (Fig. 3D-F). Among nocturnal carboxylating genes, 5 out of 9 time-structured copies of *PPCK* displayed a clear induction of nocturnal expression in *P. blanda* (Fig. 3G-I) (Supplementary Fig. 3). While *PPCK* was lowly expressed compared to other genes, the stark increase in expression in the dark period is diagnostic of CAM. One copy of *MDH* showed peak expression at night (Fig. 3J-L) and is likely involved in conversion of oxaloacetate into malate. *β-CA* expression was completely inverted in *P. blanda* and *P. sandwicensis* relative to *P. mauiensis* (Fig. 3M-O), with an increase

in expression at night in the former, and peak expression during the day in the latter C_3 species, consistent with β-CA's role in generating HCO_3^- for the CAM pathway at night.

Daytime decarboxylating gene expression profiles indicate *Peperomia* uses the PEPCK decarboxylation pathway, rather than through the PPDK route (Fig. 4). In *P. blanda* and *P. sandwicensis*, *PEPCK* (Fig. 4A-C) and a copy of *MDH* (Fig. 4D-F) had peak expression during the daytime; neither gene was time-structured in *P. mauiensis.* Moreover, *PEPCK* had much higher expression in *P. blanda* than in either of the two other species. On the other hand, *PPDK* and *ME* both had low and inconsistent expression patterns (Fig. 4G-I, J-L), which supported our hypothesis of PEPCK as a main decarboxylating enzyme in these *Peperomia* species.

Gene regulatory networks of PPCK

Based on the number of time-structured and differentially expressed copies with clear nighttime induction under drought treatment in *P. blanda*, we focused on *PPCK* for gene network analysis to identify genes co-expressed with *PPCK* across the three species (Supplementary Table 4–6). We used the time-structured and differentially expressed *PPCK* gene, Ptro.C010G33374. Our gene network analysis yielded 81 genes of *P. blanda*, 183 genes of *P. sandwicensis*, and 97 genes of *P. mauiensis* that showed strong ($p < 0.05$) association to *PPCK* in the same co-expression cluster (*P. blanda*: cluster 8, *P. sandwicensis*: cluster 8, *P. mauiensis*: cluster 5)(Supplementary Tables 4–6). The majority of other *PPCK* copies were also found in these clusters. Of note, in both *P. blanda* and *P. sandwicensis*, REVEILLE *(RVE*, Ptro.C003G7359.t1, Table 2) had a significant ($p < 0.001$) co-expression with *PPCK*. *RVE* is a MYB transcription factor involved in regulating circadian expression of other genes [37, 38], and similar to our *PPCK* gene expression, *RVE* gene expression showed a clear increase at night (Fig. 5). *RVE* displayed the highest expression in *P. blanda*, and expressed in similar levels in *P. sandwicensis* and *P. mauiensis*. *RVE* was also time-structured in all species, yet was not differentially expressed in well-watered vs. drought treated. Neither *RVE* nor any other circadian transcription factors were detected in the *P. mauiensis PPCK* cluster.

Discussion

Peperomia is a particularly powerful system to interrogate the early changes in gene expression associated with CAM evolution, as the majority of species appear to be C_3+CAM, including two of the species studied here. Such weak CAM phenotypes, while evolutionary endpoints in their own right, are also representative of the early changes that occurred in the evolutionary trajectory from C_3 to CAM and thus can be used to understand the shifts in gene expression associated with CAM. Our transcriptomic data show many canonical CAM genes have shared diel expression patterns across all three *Peperomia* species studied here, regardless of their photosynthetic phenotype. Instead, shifts in the magnitude of expression of CAM genes and their association with the circadian clock may underlie the transition to a CAM phenotype in *Peperomia*.

CAM in Peperomia

While carbon isotope data on its own was not informative with regard to CAM activity in *Peperomia*, our gas exchange and titration data before and after 7 days of drought clearly indicate C_3+CAM activity in *P. blanda* and *P. sandwicensis*. In *P. blanda*, the induction of acid accumulation under drought further suggests this species may be weakly facultatively CAM. Net assimilation rates were low in all species measured, both during day and nighttime hours, consistent with prior work measuring gas exchange in *Peperomia*. For example, previous research measured net CO_2 exchange in *P. blanda* across a cycle of hydration-drought-rehydration [16], and maximal CO_2 assimilation per shoot reached a little over 12 nmol s^{-1} during the day, and only barely went above zero at night. In the same experiment, drought stress reduced the amount of respired nocturnal CO_2 until net assimilation became positive after 7 days of drought stress [16]. Prioritizing use of respired CO_2 over fixing atmospheric CO_2 enables the maintenance of a CAM cycle without continuous supply of atmospheric carbon through stomatal opening. It remains a mystery why *Peperomia*, which in Hawai'i inhabits wet understories, would use CAM at all, though its epiphytic habit or growth on rocks may limit water availability enough that even low level CAM is beneficial for growth [16].

Our study illustrates the difficulty of detecting weak CAM species like those in *Peperomia*; it is likely researchers are vastly underestimating the number of species capable of C_3+CAM cycle. In *Peperomia*, a genus of nearly 1,600 species, only a handful of studies have thoroughly examined the extent of CAM photosynthesis (48/92 species studied showed CAM activity)[25]. While this has been extrapolated to indicate that ~ 50% of *Peperomia* uses CAM [2], the phylogenetic placement of species with direct measurements could drastically change this estimate (e.g., if all species measured were in a single clade). As a result, the evolutionary history of CAM in the lineage remains unknown. Our study reiterates that carbon isotope ratios alone are not informative for assessing CAM activity in *Peperomia*, meaning that time-consuming physiological experiments are needed to fully characterize the genus. Combined with improved phylogenetic resolution, mapping photosynthetic pathway phenotypes onto the lineage's evolutionary history would clarify whether CAM has been gained multiple times, lost multiple times, or a combination of both.

CAM gene expression

The CAM pathway has diagnostic gene expression patterns that help separate C_3 from CAM and C_3+CAM species—for example, a high level of nocturnal *PEPC* expression is expected to be found only in CAM species. Our gene expression profiles matched the typical indicators of CAM. For example, *P. blanda* had the highest nocturnal expression of plant-type *PEPC*; high nocturnal plant-type *PEPC* is diagnostic in strong CAM species [3, 39] and has even been used to demonstrate the presence of C_3+CAM [40, 41]. Similarly, *PPCK* also had nocturnally upregulated expression, with a clear induction in expression under drought in *P. blanda* (Fig. 3G-I). *PPCK* is thought to be a key regulator of CAM activity

through its phosphorylation of PEPC [4, 42]. Some copies of *MDH* also showed a clear nocturnal increase in expression in the C_3+CAM species (Fig. 3J-L), in line with its role in converting oxaloacetate to malic acid for storage in the vacuole.

Of the canonical CAM genes mentioned above, nearly all had similar expression profiles in all three species, regardless of photosynthetic pathway. One notable exception was the expression pattern of daytime decarboxylation genes (Fig. 4); in *P. blanda*, *PEPCK* expression was nearly 10 times the level of expression in *P. sandwicensis* and *P. mauiensis*, and the gene was only time structured in the two C_3+CAM species (Fig. 4A-C). Similarly, expression of the daytime *MDH* copy was only time structured in the C_3+CAM species (Fig. 4D-F). While the decarboxylation pathway used by CAM species is not always clear, increased and time-structured expression of genes associated with decarboxylation such as *PEPCK* or *PPDK* can provide evidence of their relative importance of different pathways [5, 22]. Thus, expression patterns observed in *Peperomia* suggest preferential use of the PEPCK + MDH pathway, rather than the PPDK associated pathway. Moreover, expression of daytime decarboxylation genes may provide another useful diagnostic for distinguishing C_3 and CAM species in *Peperomia* and a key to understanding the evolution of CAM. In *Kalanchoe fedtschenkoi*, for example, knockdown lines of either decarboxylation gene (*PPDK* or *NAD-ME*, in this case) led to a lack of nocturnal CO_2 assimilation and thus an overall reduction in CAM activity [43]. *PEPCK* expression in *Peperomia* C_3+CAM species may therefore be important not only for remobilizing malate, but may also provide circadian or metabolic feedback loops for the remainder of the CAM cycle.

In contrast to prior studies that posit a large number of changes occur in the lineage that evolves CAM [44, 45], our results instead suggest that many of the expression patterns required for CAM are present in the C_3 relatives. As such, the changes required for the emergence of CAM physiology may be relatively subtle. A similar pattern has been seen in other CAM lineages, including *Yucca* [5] and orchid species [46]. In *Peperomia*, our finding of shared expression patterns could be a result of extreme lability in the evolution of CAM in the genus. Because of the large size of *Peperomia*, phylogenetic analyses are often restricted to certain geographic regions (e.g., [12]) or to the use of limited-information genetic markers [11]. As a result of limited phylogenetic information, combined with an overall small proportion of the total species with measurements on CAM activity, the evolutionary history of CAM in *Peperomia* is unknown. It's possible that the ancestor of the Hawaiian clade used CAM photosynthesis, a trait which was subsequently lost as species in Hawai'i radiated into more mesic environments; in Hawai'i, species are both epiphytic and terrestrial, but nearly all are found in wet valleys or forests [47]. In this scenario, *P. mauiensis* may retain remnants of the ancestral time-structured expression of CAM genes despite the loss of CAM physiology. More generally, CAM may be a labile trait in *Peperomia*, with frequent gains and losses driven by an ancestral suite of genes expressed such that they facilitate recurrent evolution of CAM. Finally, it's possible *P. mauiensis* does have low level CAM activity, but only under prolonged drought conditions, or perhaps under other environmental stresses. Recent work on CAM plants has demonstrated the myriad conditions that affect the strength of CAM outside of water availability, including light intensity, duration of light, temperature, and CO_2 concentration [48–51]. Our experimental

growth conditions may have precluded CAM induction in this species to a degree that is measurable by titratable acidity or gas exchange.

PPCK: *changes to co-regulation underpin C_3+CAM activity*

Multiple studies have identified PPCK as an essential component of a functional CAM pathway. Nocturnal phosphorylation of PEPC by PPCK reduces its sensitivity to inhibition by malate, allowing sustained carbon fixation and malate accumulation during the night (Taybi et al. 2000). Similarly, disruption of *PPCK* expression has been shown to substantially reduce nocturnal carbon assimilation and malate production in CAM species (Boxall et al. 2017). Finally, *PPCK* is tightly coupled with the circadian regulation of the CAM pathway (Hartwell et al. 1996, Taybi et al. 2000), with reduction in its rhythmic expression disrupting core circadian clock function in *Kalanchoe fedtschenkoi* (Boxall et al. 2017).

Our transcriptomic data corroborates the role of *PPCK* as a regulatory bridge between C_3 and CAM lineages in *Peperomia* through increased nighttime expression (particularly under drought), through the presence of numerous of time-structured and differentially expressed gene copies (Supplemental Fig. 3), and by co-regulation with the circadian clock associated gene, *RVE*. Though expression of *PPCK* is relatively low in all three species of *Peperomia*, this pattern is consistent with other CAM species in which *PPCK* is expressed at low levels but shows a distinct increase in the nighttime [22, 52]. In addition, expression of multiple copies of *PPCK* in each species could reflect both functional redundancy or specialization among gene copies. Finally, co-expression of *RVE* and *PPCK* indicates the potential significance of *PPCK* as a circadian regulator of CAM in *Peperomia*.

RVE, a MYB-family transcription factor involved in circadian regulation [37, 53, 54], generally tracked expression of *PPCK* (Fig. 5), with highest expression in *P. blanda* and showed a steady increase throughout the night, peaking shortly before dawn, in all three species. The nocturnal expression represents a distinct shift compared to C_3 species, where *RVE* shows highest expression at or immediately after dawn [55]. A similar though more pronounced shift was described in *Agave* relative to *Arabidopsis* (Yin et al., 2018), suggesting that shifts in timing of *RVE* expression may be tied to regulatory rewiring in other CAM species. However, because the *Agave* study compared species millions of years diverged, the results were difficult to generalize to the evolution of CAM specifically. While our results do show differences in clustering of *RVE* with the CAM gene *PPCK*, *RVE* in all three species peaked at night (Fig. 5). Shared expression of *RVE* across both C_3+CAM and C_3 species supports the different hypotheses on CAM evolution in *Peperomia*: RVE could be exhibiting shared, ancestral expression in all three species that facilitated recurrent CAM evolution, it could be shared due to ancestral CAM activity that was lost in *P. mauiensis*, or it could be that all three species are C_3+CAM but at undetectable levels in *P. mauiensis*. However, that *RVE* does not appear to be night-expressed in Arabidopsis does suggest its nocturnal expression may be related to CAM evolution, perhaps particularly associated with *PPCK* circadian regulation. To date, *PPCK's* transcriptional regulators have not been identified. Additional studies focusing on *RVE* and the broader suite of circadian genes in closely related C_3 and CAM species

is needed to fully understand which circadian gene expression changes are truly associated with CAM evolution.

Conclusions

Our integrated physiological and transcriptomic study on C_3+CAM in *Peperomia* provides key evidence to understand the evolutionary trajectory from C_3 to CAM: we observed shared expression patterns in *PEPC* and *PPCK* across all three species regardless of photosynthetic pathway and showed *PPCK* co-expression with a circadian regulator gene in the C_3+CAM species. Our data highlights the potential significance of *PPCK* as a regulatory bridge between C_3 and C_3+CAM. However, our expression results also generate new questions on the broader evolution of CAM in *Peperomia*. Additional work to resolve phylogenetic relationships in the genus and to better classify the broad diversity of species into photosynthetic categories could help resolve the evolution of CAM in the clade. Because it appears that many *Peperomia* species are likely to use C_3+CAM, a resolved evolutionary history would be helpful in future work to determine what the initial steps of CAM evolution may look like from a physiological and genomic perspective. More broadly, our analyses on the photosynthetic physiology and transcriptomics of *Peperomia* are one of only a handful of studies of C_3+CAM species [6, 40, 56, 57] and provide evidence on what early transcriptomic changes are associated with the evolution of CAM. Finally, our results show the importance of interrogating the genomic shifts in phenotypically intermediate species for understanding the evolution of complex traits.

Declarations

Ethics approval and consent to participate -

Not applicable

Consent for publication -

Not applicable

Competing interests -

The authors declare that they have no competing interests.

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Author Contribution

GYK conceptualized the research, collected and analyzed the data, and wrote the manuscript. DW assisted with data analysis and writing. CD and CK helped isolate RNA and measure titratable acidity. KH helped conceptualize the research, assisted with data collection and analysis, and helped in writing the manuscript. All authors read and approved the final manuscript.

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Data Availability

Physiology data, including carbon isotope values, LiCor gas exchange data, and titratable acidity measurements, are included in this published article [and its supplementary information files]. RNA-sequencing generated during the current study is available in NCBI's Sequencing Read Archive under BioProject PRJNA1468765. Metadata for RNA-libraries and resulting count and TPM matrices are available at Github (https://github.com/kheyduk/CAM_Transcriptomics/).

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Tables

Table 1 and 2 are available in the Supplementary Files section.

Figures

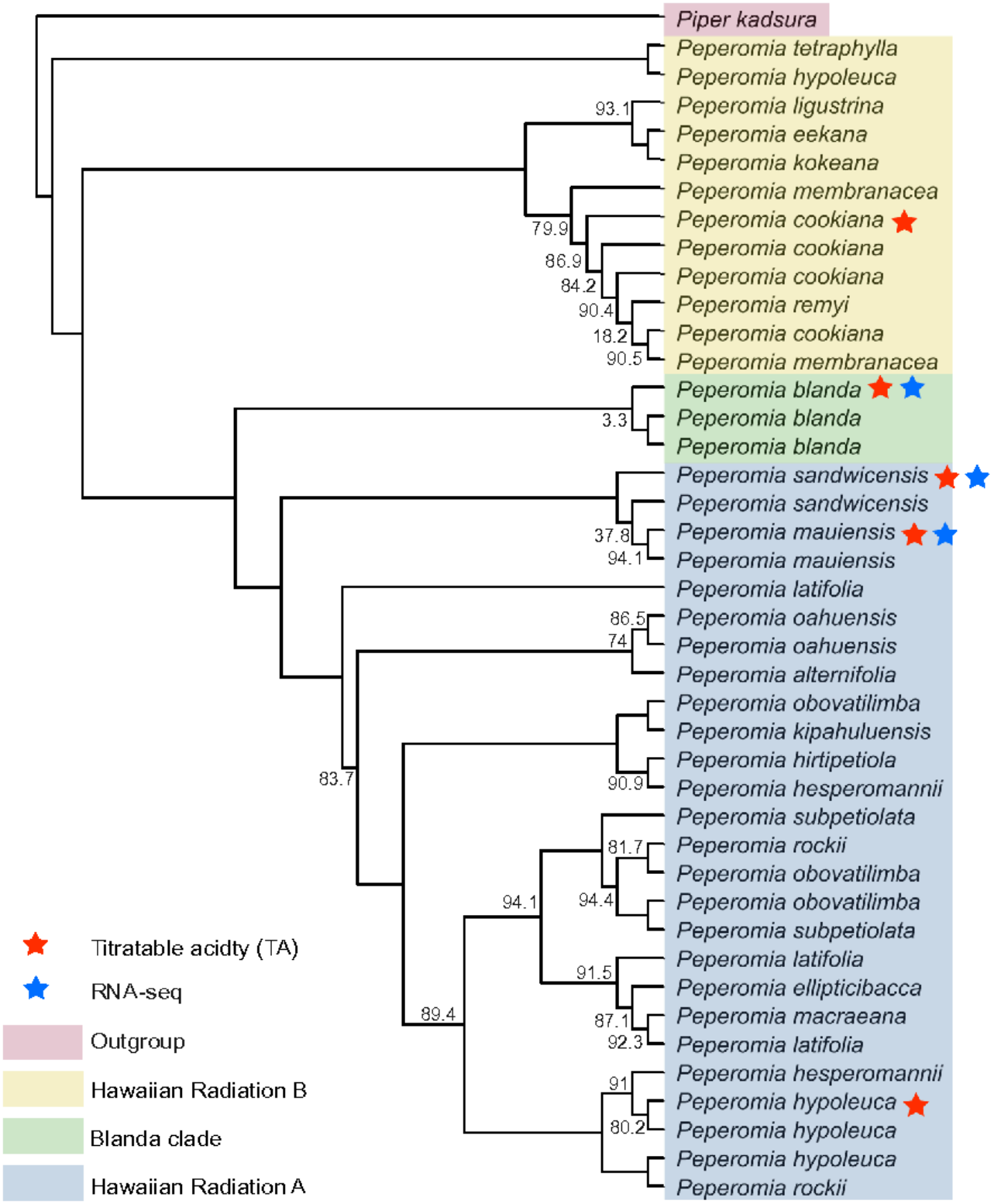


Figure 1

Hawaiian *Peperomia* phylogenetic tree adapted from Lim et al., 2019 [14]. Red-colored stars indicate species that were used as samples for titratable acidity, and blue-colored stars are the ones that are used for RNA-sequencing. Maximum likelihood bootstrap support values >95% are masked.

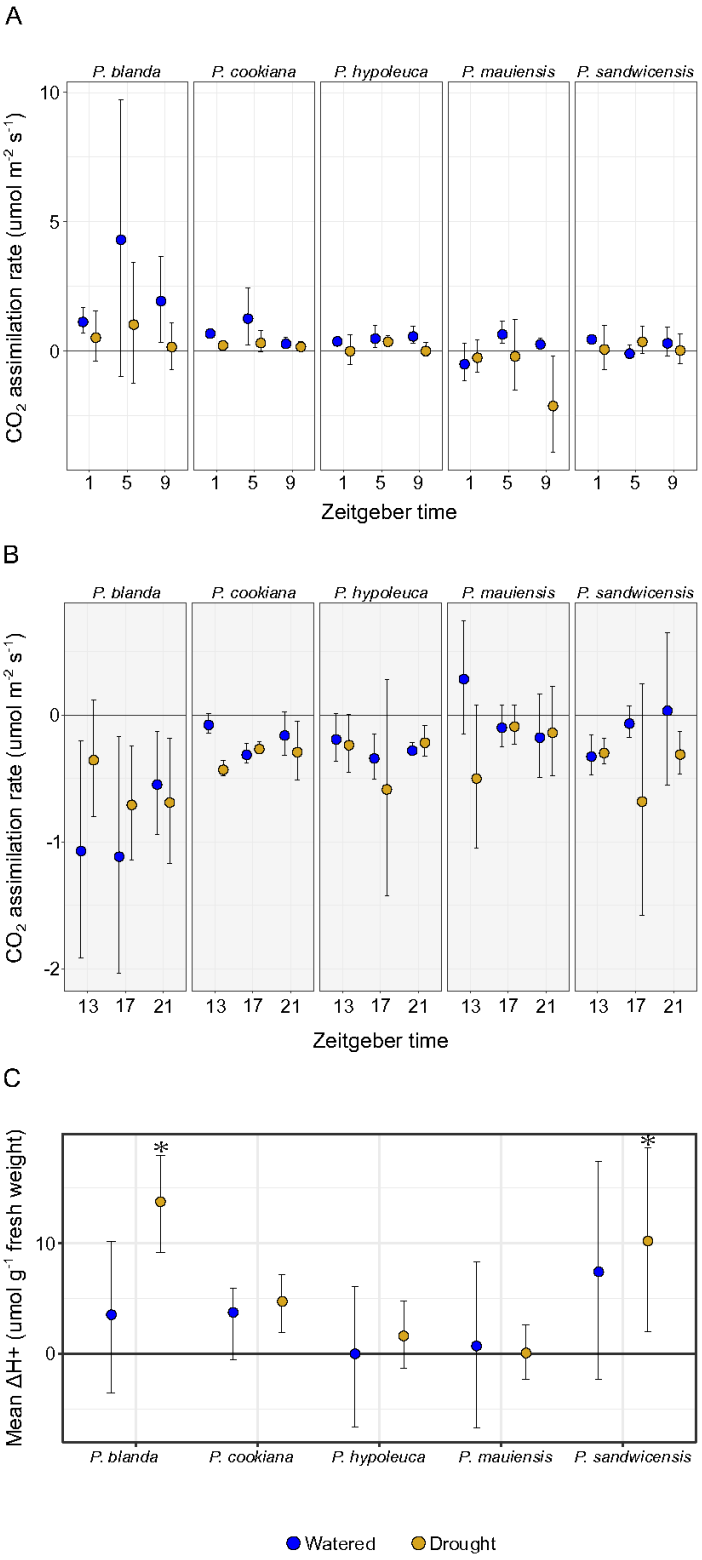


Figure 2

Gas exchange and titratable acidity data. A) Daytime gas exchange from time point 1 (ZT 1; 8 AM local) to time point 3 (ZT 9; 4 PM local). Error bars indicate standard deviation. B) Nighttime gas exchange from time point 4 (ZT 13; 8 PM local) to time point 6 (ZT 21; 4 AM local). C) Leaf titratable acidity at dusk (ZT 9; 4 PM local) and at dawn (ZT 21; 4 AM local) with error bars indicating standard deviation. Asterisk indicates a significant difference from zero ($p < 0.05$).

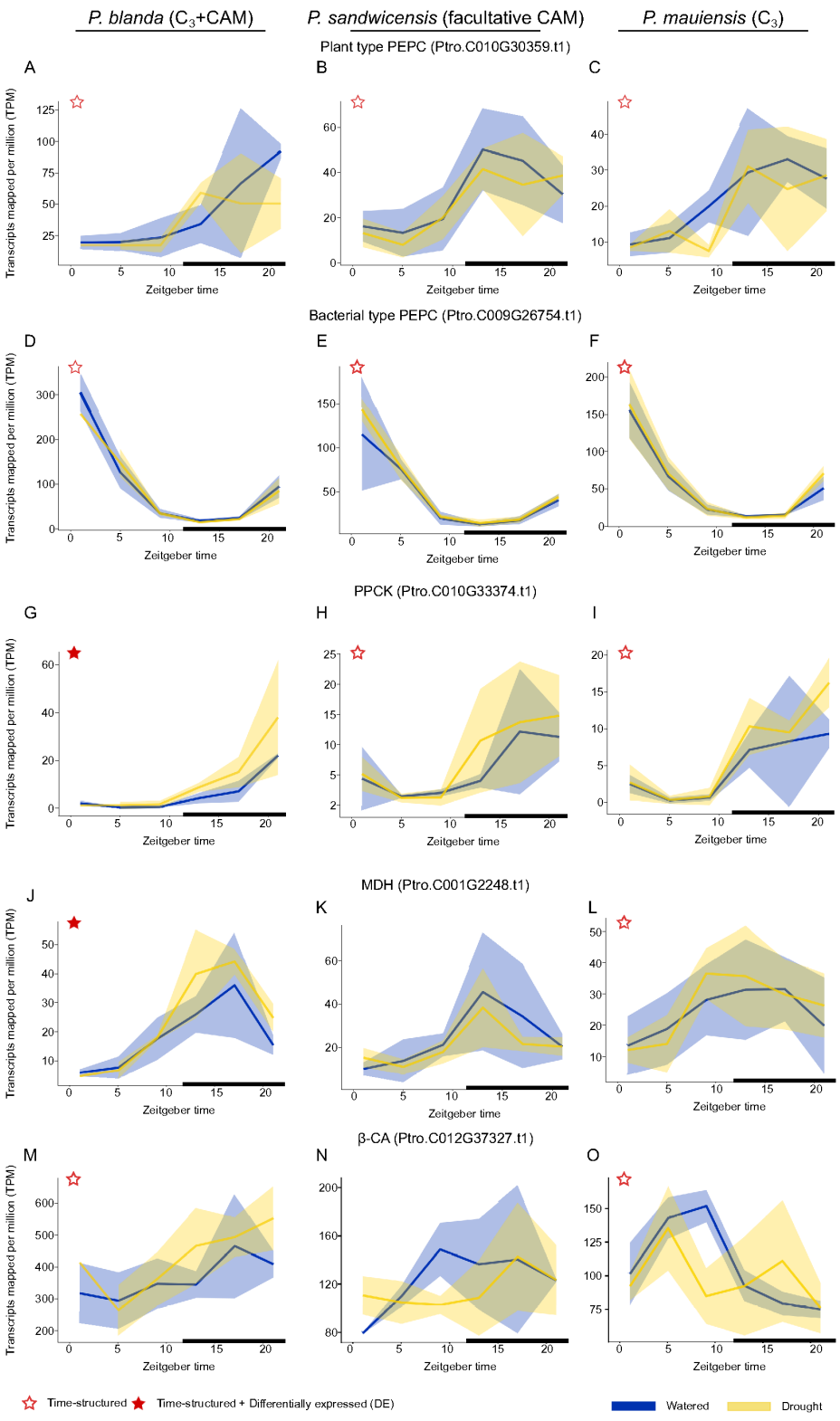


Figure 3

Nighttime gene expression in *Peperomia blanda* (A,D,G,J), *P. sandwicensis*(B,E,H,K), and *P. mauiensis* (C,F,I,L). Nighttime gene expression includes plant-type phosphoenolpyruvate carboxylase (*PEPC*; A-C), bacterial-type phosphoenolpyruvate carboxylase (bacterial-type *PEPC*; D-F), phosphoenolpyruvate carboxylase kinase (*PPCK*; G-I), NAD(P)-malate dehydrogenase (*MDH*; J-L), and beta-carbonic anhydrase (β-CA; M-O). Shaded regions around the fitted line indicate standard error. An unfilled red star indicates

that the gene is time-structured, and filled red-star indicates that the gene is both time-structured and differentially expressed (DE) between well-watered (blue) and drought (yellow) treatments. Black bar indicates the night zeitgeber times.

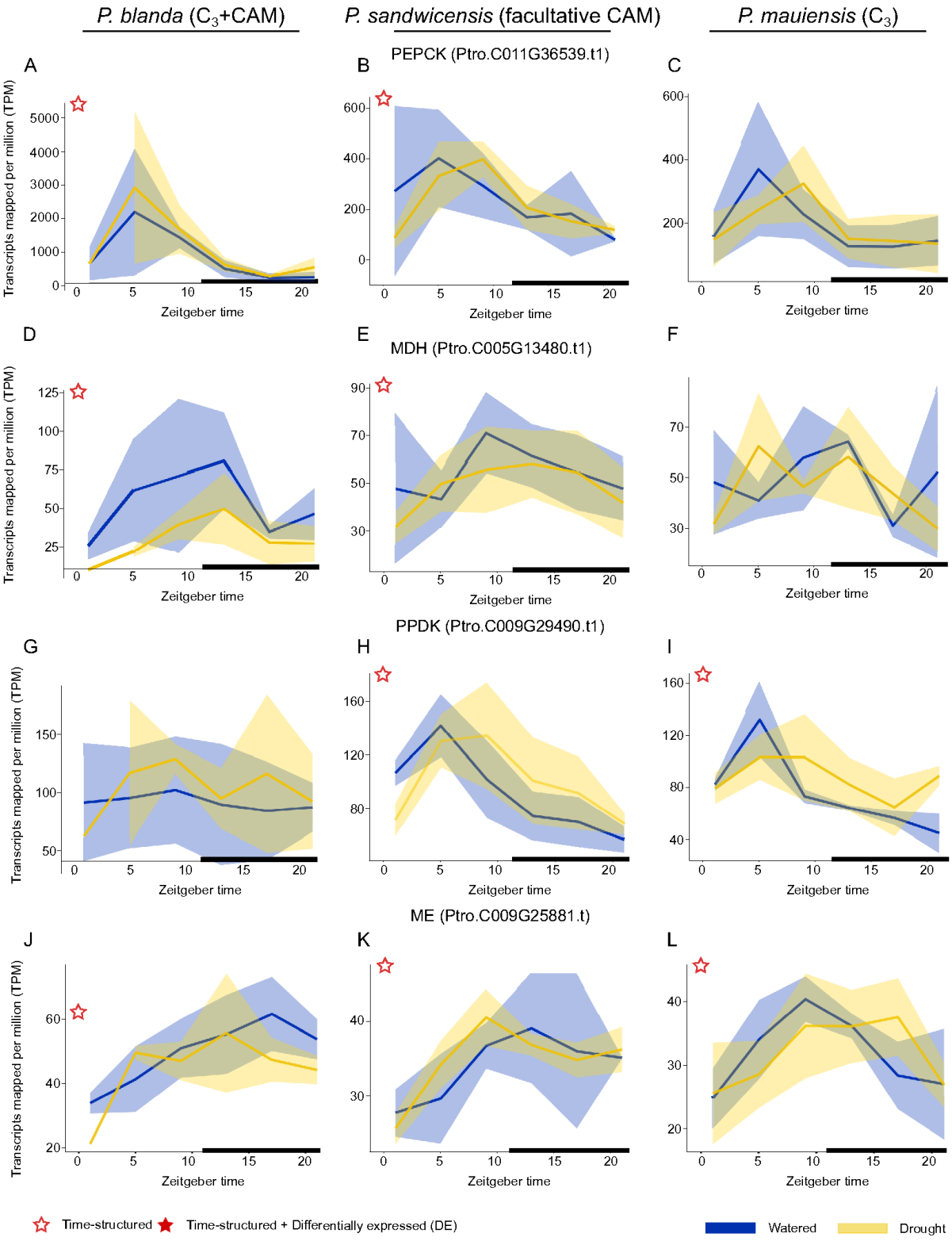


Figure 4

Daytime gene expression in *Peperomia blanda* (A,D,G,J), *P. sandwicensis*(B,E,H,K), and *P. mauiensis* (C,F,I,L). Daytime gene expression include phosphoenolpyruvate carboxykinase (*PEPCK*; A-C), NAD(P)-malate dehydrogenase (*MDH*; D-F), phosphoenolpyruvate pyruvate dikinase (*PPDK*; G-I), and NAD(P)-malic enzyme (*ME*; J-L). Shaded regions around the fitted line indicate standard error. An unfilled red star indicates that the gene is time-structured, and filled red-star indicates that the gene is both time-structured and differentially expressed (DE) between well-watered (blue) and drought (yellow) treatments. Black bar indicates the night zeitgeber times.

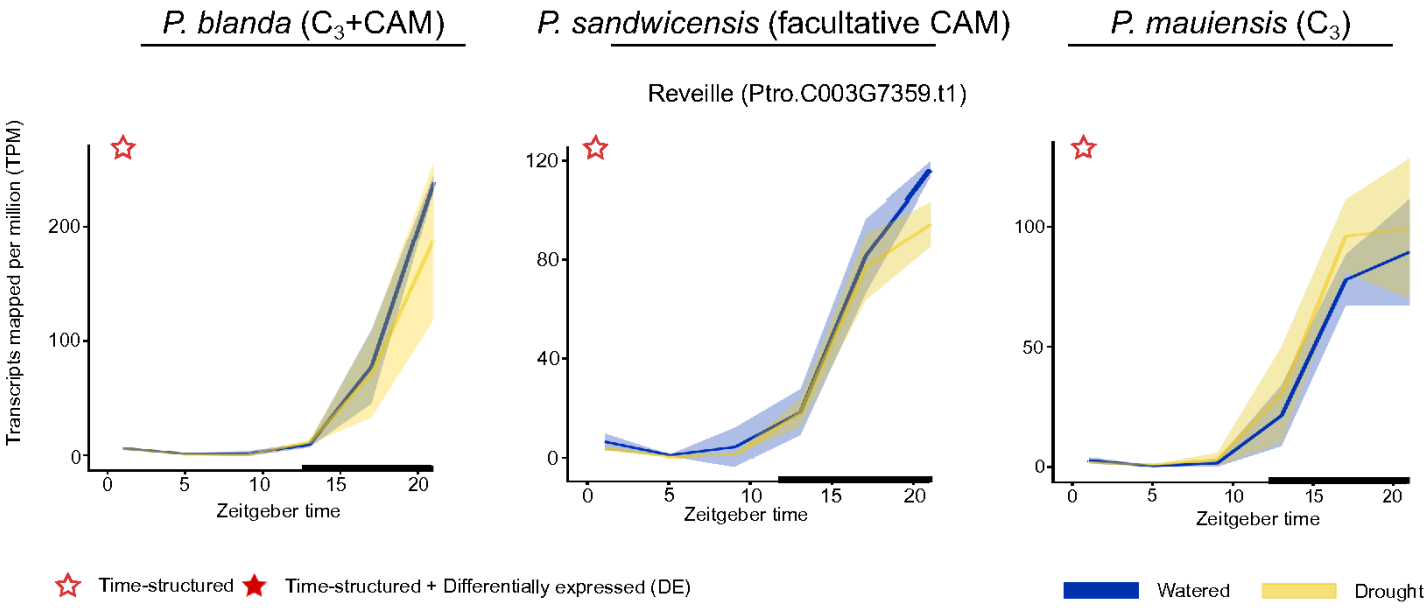


Figure 5

Gene expression of REVEILLE (*RVE*) over a 24-hour time course in *Peperomia blanda* (A), *P. sandwicensis* (B), and*P. mauiensis* (C). Shaded regions around the fitted line indicate standard error. An unfilled red star indicates that the gene is time-structured, and filled red-star indicates that the gene is both time-structured and differentially expressed (DE) between well-watered (blue) and drought (yellow) treatments. Black bar indicates the night zeitgeber times.

Supplementary Files

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- SupplementaryTable4.csv
- SupplementaryTable5.csv
- SupplementaryTable6.csv