A matter of time: regulatory events behind the synchronization of C_4 and crassulacean acid metabolism in *Portulaca oleracea*

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Highlights

A dynamic interplay between phytohormones, transcription factors and the circadian clock provides strict temporal coordination of C_4 and crassulacean acid metabolism gene expression in purslane under changing environmental conditions.

Abstract

Portulaca species can switch between C_4 and crassulacean acid metabolism (CAM) depending on environmental conditions. However, the regulatory mechanisms behind this rare photosynthetic adaptation remain elusive. Using *Portulaca oleracea* as a model system, here we investigated the involvement of the circadian clock, plant hormones and transcription factors in coordinating C_4 and CAM gene expression. Free-running experiments in constant conditions suggested that C_4 and CAM gene expression are intrinsically connected to the circadian clock. Detailed time-course, drought and rewatering experiments revealed distinct timeframes for CAM induction and reversion (days *versus* hours, respectively), which were accompanied by changes in abscisic acid (ABA) and cytokinin metabolism and signaling. Exogenous ABA and cytokinins were shown to promote and repress CAM expression in *P. oleracea*, respectively. Moreover, the drought-induced decline in C_4-transcript levels was completely recovered upon cytokinin treatment. The ABA-regulated transcription factors *HB7*, *NFYA7*, *NFYC9, TT8* and *ARR12* were identified as likely candidate regulators of CAM induction following this approach, whereas *NFYC4* and *ARR9* were connected to C_4 expression patterns. Therefore, we provide insights into the signaling events controlling C_4-CAM transitions in response to water availability and over the day/night cycle, highlighting candidate genes for future functional studies in the context of facultative C_4-CAM photosynthesis.

Keywords

Abscisic acid; Circadian clock; Crassulacean acid metabolism; C_4 photosynthesis; Cytokinin; Drought; Facultative CAM; Portulacaceae; Purslane; Transcription factors; *Portulaca oleracea*

Introduction

C_4 photosynthesis and the crassulacean acid metabolism (CAM) are two carbon concentrating mechanisms (CCMs) that have evolved multiple times, each of them causing a rewiring of the plant primary metabolism and conferring adaptative advantages for species occupying particularly arid and light-intense environments (Edwards and Ogburn, 2012). In both C_4 and CAM, the cycle starts with a temporary CO_2 fixation step carried out by phospho*enol*pyruvate carboxylase (PPC), leading to the formation of a 4-carbon acid, commonly malate. Subsequently, malate is decarboxylated, generating CO_2 to sustain Rubisco activity (Kanai and Edwards, 1999; Dodd *et al.*, 2002). Although similar enzymes are employed to concentrate CO_2 in the vicinity of Rubisco, C_4 relies on a spatial specialization of cell types within the leaf, whereas CAM leverages temporary CO_2 concentration at night, preceding daytime Rubisco activity under closed stomata (Winter and Smith, 1996; Sage, 2004).

In both the C_4 and CAM systems, PPC is phosphorylated by PPC kinase (PPCK), which requires transcription and *de novo* translation to generate the active kinase (Hartwell *et al.*, 1996, 1999; Hibberd and Covshoff, 2010). PPCK is induced by light in C_4 species (e.g. Shenton *et al*., 2006), and by the circadian clock with a dark period phased peak in CAM species (e.g. Hartwell *et al*., 1999). Furthermore, PPCK temporal control of PPC in the dark is mediated by the core circadian clock and plays a pivotal role in the temporal optimization of CAM (Hartwell, 2005; Hartwell, 2006; Boxall *et al*., 2017). Phosphorylated PPC is less sensitive to feedback inhibition by malate, which allows PPC to remain active for longer into the dark period during CO_2 fixation and associated malate accumulation (Boxall *et al*., 2017).

As opposed to the known compatibility of C_3 and CAM in the same photosynthetic mesophyll cells, C_4 and CAM were believed to be incompatible since they are rarely found occurring in the same species (Sage 2002), one exception being the *Portulaca* lineage (Winter *et al.*, 2019). In leaves of *P. oleracea* L., the C_4-CAM transition is triggered by reduced water availability (Koch and Kennedy, 1980, 1982; Winter and Holtum, 2014). Under drought stress, CAM genes are induced, the diel regulation of C_4/ CAM-shared genes is rescheduled, and the mRNA levels of many of the key C_4 genes are significantly reduced (Ferrari *et al.*, 2020*b*). The recruitment of specific members of the *PPC* gene family to function in C_4 and CAM (*PPC-1E1a'* and *PPC-1E1c*, respectively) was confirmed across multiple *P. oleracea* accessions and under distinct experimental designs (Christin *et al.*, 2014; Ferrari *et al.*, 2020*c*,*a*). In contrast, *PPCK-1E* has been proposed to function in association with both CCM pathways (Ferrari *et al.*, 2020*b*). Additional C_4-marker genes have also been recently described for *P. oleracea*, including genes involved in carboxylation (beta-carbonic anhydrase - *βCA-2E3*), acid formation (aspartate

aminotransferase - *ASPAT-1E1;* alanine aminotransferase - *ALAAT-1E1*), decarboxylation (NAD-malic enzyme - *NADME-2E.1*) and PEP regeneration reactions (adenylate kinase - *AK-1;* pyruvate orthophosphate dikinase - *PPDK-1C1b.1*), as well as plasma membrane-localized malate transporter (aluminum-activated malate transporter - *ALMT-12E.2*) (Christin *et al.*, 2014; Ferrari *et al.*, 2020*b*). This pattern is completely reversed upon rewatering, highlighting the flexibility of this photosynthetic transition (Ferrari *et al.*, 2020*c*).

Hence, *P. oleracea* serves as a natural blueprint for how C_4 and CAM are connected within the leaves of a single plant, and may facilitate the identification of the regulatory, physiological, and biochemical requirements for C_4 and CAM to co-occur. However, given the facultative nature of the C_4-CAM transition, a regulatory network fine-tuning the expression of each or both CCMs in response to environmental cues would be expected in *P. oleracea*. In addition, clock-controlled expression of components of each CCM might also be involved. CAM-associated CO_2 fixation is tightly coupled to the core circadian clock (Hartwell, 2005; Hartwell, 2006; Boxall et al., 2017; Boxall et al., 2020), and circadian oscillation of photosynthesis-related genes is observed in both C_3 and C_4 species (Khan et al., 2010). In Arabidopsis, the central circadian oscillator consists of an interlocked feedback loop of genes and their products, with a typical 24 h circadian cycle initiating in the nucleus with the single MYB-repeat transcription factors *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY*) and *REVEILLE 1 (RVE1*) that peak around dawn (Alabadi *et al.*, 2002; Rawat *et al.*, 2009). Sequentially throughout the light period, a series of pseudo-response regulators (*PRR*s) accumulate (*PRR9, PRR7, PRR5*), repressing *CCA1/ LHY/ RVE1* (Pokhilko *et al.*, 2012). These steps precede *TIMING OF CAB EXPRESSION1* (*TOC1,* also known as *PRR1*) accumulation around dusk (Matsushika *et al.*, 2000). *FLAVIN-BINDING, KELCH REPEAT, F BOX 1 (FKF1*) represses *TOC1* and *PRR5,* and usually peaks 8 h after the start of illumination (Baudry *et al.*, 2010). Finally, *TOC1* peaks early in the dark period, and is down-regulated at the end of the night by targeted degradation via interaction with its E3 ubiquitin ligase ZEITLUPE (ZTL). The degradation of *TOC1* leads to the up-regulation of *CCA1/ LHY/ RVE1*, restarting the cycle (Pokhilko *et al.*, 2010, 2012). *GIGANTEA (GI*) typically peaks 8-10 h after dawn, when one of its functions involves acting as a chaperone for *ZTL*. *GI* thus delays the interaction of *ZTL* with *TOC1*, and so inputs a time delay into *TOC1* degradation via the 26S proteasome (Cha *et al*., 2017). Although a broadly similar core clock system has been demonstrated in both facultative and obligate CAM species that do no possess C_4, which is vital for the daily rhythm of CAM-associated CO_2 fixation (e.g. Boxall et al., 2005; Boxall et al., 2017), limited information is

available regarding circadian clock operation and its role in coordinating the metabolic pathways associated with CO_2 fixation in C_4-CAM plants.

Plant hormones have also been implicated in mediating C_4 and CAM expression in response to environmental cues (Taybi *et al.*, 2002; Freschi and Mercier, 2012; Ferrari and Freschi, 2019). In C_3-CAM facultative species, accumulating evidence indicates abscisic acid (ABA) as a major endogenous signal connecting the plant's water status to the regulation of CAM expression (Taybi *et al.*, 2002). Compared to ABA, less is known about the involvement of other hormones in the regulation of CAM induction (Taybi *et al.*, 2002). A repressive, and still controversial, role of cytokinins (CK) in CAM was described in both facultative C_3-CAM and constitutive CAM species (Schmitt and Piepenbrock, 1992; Thomas *et al.*, 1992; Thomas and Bohnert, 1993; Dai *et al.*, 1994; Peters *et al.*, 1997). In *M. crystallinum*, whereas a decrease in CK content was reported for CAM-induced plants under salt stress (Schmitt and Piepenbrock, 1992; Peters *et al.*, 1997), the exogenous application of benzylaminopurine (BA) and *trans*-zeatin (Z) also resulted in either increase or repression of the CAM-specific *PPC* transcript abundance (Thomas *et al.*, 1992; Thomas and Bohnert, 1993).

In addition to hormones, plant stress responses also commonly involve the action of multiple transcription factors (TFs), many responsible for connecting and coordinating signaling cascades (Li *et al.*, 2004; Abuqamar *et al.*, 2009; Seo and Park, 2010; Kohli *et al.*, 2013). As such, the identification of novel candidate TFs via bioinformatic analyses has been an important source of information (Mitsuda and Ohme-Takagi, 2009; Wang *et al.*, 2016). Through this approach, several TFs have been proposed as regulators of CAM in obligate CAM *Kalanchöe fedtschenkoi* and facultative C_3-CAM *Mesembryanthemum crystallinum* and *Talinum triangulare* (Brilhaus *et al.*, 2016; Amin *et al.*, 2019), even though their connections with hormonal signals, including ABA and CKs, remain unknown.

Whether similar regulatory networks regulate inducible CAM in a C_3 or C_4 background remains elusive. In addition, whether the circadian molecular clock plays equivalent roles in controlling the C_4 and CAM-related gene expression also remains to be elucidated. Given the opposite impacts of water availability on CCM expression in *P. oleracea* (Ferrari *et al.*, 2020*b*,*c*), we hypothesized that drought-induced temporal changes in leaf cell signaling may be as important to promote CAM expression as to limit the transcription of C_4 genes in this species. Here, we provide the first insights into the circadian control and the molecular signaling events behind the synchronization of C_4 and CAM pathways in *P. oleracea* plants facing contrasting watering regimes. Moreover, candidate regulators were identified as likely involved in this

158 remarkable photosynthetic transition, ranging from drought- and ABA-regulated transcription
159 factors to ABA-cytokinin crosstalk elements.
160
161 ## Material and methods
162 *Plant material, growth conditions and sampling*
163 Seeds from a commercial cultivar of *Portulaca oleracea* were germinated and grown in 300-ml
164 square pots containing commercial substrate (Plantmax HT) 2 : 1 vermiculite. Comprehensive
165 morphological and biochemical characterization of this *P. oleracea* accession is provided in
166 Ferrari et al. (2020*c*). Unless otherwise specified, plants were grown in a climate chamber using
167 a 12 h photoperiod (light period from 06:00 to 18:00, local time), 27 ± 1°C day : 22 ± 1°C night
168 temperature, 65 ± 10 % day : 80 ± 10% night air humidity, and a photosynthetic photon flux
169 density (PPFD) of 400 µmol m^{-2} s^{-1}.
170 For all analyses and experiments in this work, four biological replicates were sampled, and each
171 replicate was composed of all fully expanded, non-senescent leaves from three plants.
172 Following sampling, leaves were immediately frozen in liquid nitrogen (N_2), and stored at -80°C
173 until powdered and used in analyses.
174
175 *Light/dark and free-running circadian time-course experiments*
176 For free-running circadian time-course experiments (constant light, temperature, and humidity;
177 LL), plants that were 30-days-old were grown under the standard 12-h-light/ 12-h-dark
178 conditions (LD), and either subjected to complete water withholding (drought-stressed), or
179 watered daily (well-watered), for 21-days. Two days before sampling, plants were switched to
180 LL free-running conditions at the end of the dark period, or maintained under LD conditions as a
181 control. For LL, the constant conditions were as follows: light 100 µmol m^{-2} s^{-1}, temperature 22 ±
182 1°C and humidity 70 ± 10 %. Mature leaves were sampled every three hours for 24 h, starting
183 48h into the LL treatment (Zeitgeber (ZT) 48 until ZT 72), which configures sufficient time
184 without the plant receiving entrainment signals of light/ hot, or dark/ cold, and allows for
185 monitoring the circadian clock control of biological functions. Clock genes were selected based
186 on previous studies with CAM plants (Boxall *et al.*, 2017, 2020) (Table S2). This was performed
187 to ensure any time-of-day dependent changes observed in the subsequent analyses of the
188 samples were entirely due to temporal control mediated by the endogenous circadian clock.
189
190 *Drought and rewatering treatments*

To investigate the drought-induced C_4-to-CAM signaling, plants that were 30-days-old were either subjected to complete water withholding (drought-stressed) or watered daily as a control (well-watered). Leaves were sampled after 3-, 6-, 9-, 12-, 15-, 18-, 21- and 22-days of treatment at dawn (07:00, 1 h after the onset of illumination lights) and dusk (17h:00, 1 h before the end of the light period). To analyze the CAM-to-C_4 signaling, plants were drought-stressed for 21-days and subsequently rewatered for 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 12 h before sampling. As most CCM genes display a marked diel oscillation of their transcript abundance (Ferrari *et al.*, 2020*b*), rewatering was started at distinct moments of the day or night to ensure the simultaneous sampling of all rewatering treatments in a single moment of the diel cycle. For genes with a nocturnal transcript peak, sampling occurred at 20:00 (2 h after lights were off), with rewatering events starting at 19:30, 19:00, 18:30, 18:00, 17:00, 16:00, 15:00, 14:00, 12:00 and 8:00. For genes whose transcript levels peaked at dawn, sampling took place at 04:00 (2 h before lights on), with rewatering events starting at 3:30, 3:00, 2:30, 2:00, 1:00, 24:00, 23:00, 22:00, 20:00 and 16:00. Plant and soil water status were monitored as described in Ferrari et al. (2020*b*).

Plant hormone treatments

To examine the influence of ABA and CKs on C_4 and CAM regulation, short- and long-term treatments with these hormones were performed at the concentrations specified in each experiment. In all cases, hormones were dissolved in ultra-pure water with 0.001% (v/v) Tween-20. For the short-term treatments, plants that were 60-days-old, either maintained well-watered, or drought-stressed for 21-days, were sprayed with 500 µM ABA or 6-benzylaminopurine (BA) at the start or the end of the light period, and sampled approximately 12 h later. For the long-term treatment of well-watered plants, plants that were 30-days-old were sprayed after three hours into the light period with 0, 25, 100 and 500 µM ABA for four consecutive days, and sampled on the fifth day. For the long-term treatment of drought-stressed plants, plants that were 30-days-old were sprayed with 0, 5, 10, 20 µM of BA or *trans*-zeatin (Z) three hours after the start of the light period every 5-days for over 20-days, and sampled on the 21st day. Sampling for both the ABA and CKs long-term treatments occurred at dawn (07:00, 1 h after light on) and dusk (17:00, 1 h before lights off).

Titratable acidity analysis

Leaf titratable acidity was determined as described in Ferrari et al. (2020*b*). Briefly, frozen leaf samples (200 mg fresh weight – FW) were extracted in 1 ml 80 % (v/v) methanol for 10 min at 80°C, and the supernatants were recovered by centrifugation (15,000 g, 10 min, 25ºC). The

pellets were re-extracted three times, and all supernatants were combined before the analysis. Aliquots of the supernatant fraction were titrated with 0.2 M NaOH to pH 8.0 using phenolphthalein as an indicator. Dawn and dusk values were subtracted to calculate diel acidity changes (ΔH^+), with positive values indicating nocturnal accumulation. Standard errors were calculated as follows: $SE_{\Delta H+} = \sqrt{((\text{standard error}_{\text{well-watered}})^2 + (\text{standard error}_{\text{droughted}})^2}$ (Popp *et al.*, 2003).

Abscisic acid (ABA) quantification

Endogenous ABA levels were determined by gas chromatography-tandem mass spectrometry-selective ion monitoring. Briefly, frozen leaf samples (~100 mg FW) were extracted with an isopropanol: acetic acid (95:5 v/v) solution containing 0.5 µg of the labeled ABA standard ([$^2H_6$]-ABA, Olchemin Ltd) and maintained under constant shaking for 2 h at 4 °C. The mixture was centrifuged at 13,000 *g,* and the supernatant was recovered and concentrated to 50 µl under nitrogen gas flow. Thereafter, 200 µl of ultra-pure water was added, and phase partition was performed with 500 µl of ethyl acetate. The separated organic phase was transferred to a new tube and dried under a nitrogen gas flow. Samples were methylated with trimethylsilyldiazomethane, dried under nitrogen gas flow, and re-suspended in ethyl acetate. The material was analyzed by gas chromatography (model 6890) coupled to mass spectrometry (Shimadzu model: GCMS-QP2010 SE) using an HP-1701 column (30 m, 0.25mm ID, internal film 0.50mm thick). Helium was used as the carrier gas at a flow rate of 4 ml min^{-1} in the following program: 3 min at 150 °C, followed by a ramp by 5 °C min^{-1} to 210 °C and 15 °C min^{-1} to 260 °C. Ions with (m/*z*) 134, 162 and 190 (corresponding to endogenous ABA) and 138, 166 and 194 (corresponding to [$^2H_6$]-ABA) were monitored.

RNA extraction and reverse transcriptase quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from approximately 80 mg of frozen leaves using the ReliaPrep RNA Tissue Miniprep System (Promega) for fibrous tissues, and following the protocol described in Ferrari *et al.* (2020*a*). Complementary DNA synthesis used SuperScript IV Reverse Transcriptase kit (Thermo Fisher Scientific), and RT-qPCR reactions were performed in a QuantStudio Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific), using 10 µl reaction volume and run conditions as described in Ferrari et al. (2020*b*). The relative transcript abundance was calculated by applying the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All primer sequences used are listed in Table S1. Reference genes were chosen according to Ferrari *et al.* (2020*a*).

Statistical Analysis

All statistical analyses were performed using R (version 3.6.1; R Core Team, 2021) via RStudio (version 1.2.1335). The data were checked for normality and variance, and appropriate tests were applied (Ferrari *et al.*, 2020*b*). Correlation indexes were generated using R package "corrr".

Bioinformatic analysis: transcriptomic data, co-expression analysis and data mining

Each of the 32,306 contigs assembled from *de novo* transcriptome sequencing of leaves from well-watered and drought-stressed *P. oleracea* plants by Ferrari *et al.* (2020*b*) was associated with an *A. thaliana* gene identifier (AGI) based on sequence similarity analysis compared to the Arabidopsis transcriptome (Araport11 Official Release dataset) (Berardini *et al.*, 2015) using BLASTX (Camacho *et al.*, 2009) with e-value = 10^{-6} and the -max_hsps = 1 flag. This provided a more straightforward alternative for cross-referencing our findings to the current literature on plant molecular stress signaling networks. Subsequently, *P. oleracea* transcripts were identified based on AGI numbers of genes involved in key steps of ABA and CK metabolism and the molecular circadian clock. For hormone-related genes, the differential expression (DE) analysis data described in Ferrari et al. (2020*b*) was used to filter the most strongly differentially regulated contigs ($\log_2$ fold-change (logFC) > |1.5| with adjusted p-value (false discovery rate - FDR) < 0.05 when comparing well-watered and drought-stressed leaves).

For TF identification, peptide sequences identified as *A. thaliana* TFs were downloaded from the online plant transcription factor database by Jin *et al.* (2014), version 3.0, and used as query sequences for BLAST searches against the *P. oleracea* transcriptome.

For the co-expression analysis, low abundance transcripts (total TPM < 5) were filtered from well-watered and drought-stressed leaf RNA-seq libraries (Ferrari *et al.*, 2020*b*), and the remaining sequences were used to construct two co-expression networks, one for each water availability condition, with the WGCNA R-package (Langfelder and Horvath, 2008). The following parameters were used for both sample sets: power = 12, corType = "bicor", networkType = "signed hybrid", TOMType = "signed", minModuleSize = 30, maxPOutliers = 0.05, reassignThreshold = 0, mergeCutHeight = 0.20, pamRespectsDendro = FALSE, maxBlockSize = 40000. DE data for these genes were retrieved and filtered for TF homologs presenting logFC > |1.5|; FDR < 0.05 when comparing well-watered and drought-stressed leaves.

Results

Impacts of free-running conditions on the temporal oscillation of transcripts of genes involved in clock, C_4, and CAM functions

To explore the circadian coordination and optimization of CCM-associated genes in leaves of *P. oleracea*, C_4- and CAM-performing adult plants under well-watered or drought-stress conditions, respectively, were pre-entrained in 12-h-light/ 12-h-dark cycle conditions (LD) for 18 days. On the 19th day, plants from both water availability groups were either kept in LD or switched to constant light, temperature, and humidity free-running conditions (LL). Leaf sampling occurred every three hours between ZT48 and ZT72 (Fig. 1A).

Well-watered *P. oleracea* leaves under LD showed consistent diel cycles of *CCA1*, *LHY* and *RVE1* transcripts, with mRNA levels rising at the end of the dark period and peaking at dawn (Fig. 1B). On the third day under LL, the amplitude of the oscillations of all three transcripts was reduced when compared to LD in both water availability conditions, revealing that the free-running rhythms of these circadian clock components dampened as LL progressed (Fig. 1B). Their mRNA levels were also down-regulated in drought-stressed leaves in LD compared to well-watered counterparts (Fig. S1A). *GI*, *PRR7* and *FKF1* transcript levels peaked in the late afternoon (ZT57) under LD in both well-watered and drought-stressed plants (Fig. 1B). This preceded the accumulation of *TOC1* transcripts in the dark period, which peaked at ZT60 – ZT63 in LD (Fig. 1B). When comparing LL and LD, the amplitude of the oscillations of *GI* and *PRR7* mRNA levels was reduced regardless of water availability treatments, and their transcripts were still abundant when *TOC1* transcripts increased at ZT57 in LL (Fig. 1B). *FKF1* under LL displayed a low amplitude rhythm for the drought-stressed samples when compared to the oscillation in LD. *GI* and *PRR7* displayed an additional peak at ZT63 when compared to LD treatment (Fig. 1B). The amplitude of the *TOC1* oscillation was down-regulated in LL compared to LD in both well-watered and drought-stressed leaves, but the transcript abundance was still clearly rhythmic between ZT48 and ZT72 under LL (Fig. 1B).

The transcript abundance of C_4- and CAM-marker genes was measured in LD and LL to explore the extent to which the core circadian clock modulates both CCMs in *P. oleracea*. In well-watered plants, C_4-markers (*PPC-1E1a', βCA-2E3, ASPAT-1E1, NADME-2e.1, ALAAT-1E1, PPDK-1C1b.1, AK-1, ALMT-12E.2)* exhibited similar circadian transcript oscillations under either LD or LL (Fig. 1C). After water was withheld for 21-days under LD, *P. oleracea* leaves displayed significant nocturnal acid accumulation (ΔH^+) (Fig. S1C), and the CAM-marker *PPC-1E1c* gene was up-regulated at least 100-fold, with its transcript peak phased to the end of the light period and first half of the dark period (Fig. 1C). However, a less conspicuous ΔH^+ was observed in *P.*

oleracea leaves on day 3 in LL during the subjective night (Fig. S1C). *PPC-1E1c*, however, oscillated in abundance on day 3 of LL in the drought-stressed plants (Fig. 1C). *PPCK-1E* transcripts under LD conditions peaked at the start of the light period in well-watered control plants performing C_4, whereas the timing of peak shifted to the dark period in the drought-stressed plants (Fig. 1C).

Temporal synchronization of C_4 and CAM gene expression in P. oleracea *under varying water availability*

After exploring the diel and circadian rhythms of CCM genes in C_4- and CAM-performing plants, it was important to further dissect the temporal synchronization of C_4 and CAM genes during drought progression, and following rewatering to field capacity. First, a time-course analysis was performed with measurements of ΔH^+ and mRNA levels of key genes of both CCMs in response to the gradual and progressive decline in soil water, and subsequent rewatering. Over a period 22-days of water withholding (Fig. 2A), soil volumetric water content (SVWC) was progressively reduced (Fig. 2B), resulting in a gradual decline in leaf Ψ_S after 12-days (Fig. 2C). However, ΔH^+ and *PPC-1E1c* transcripts revealed that CAM induction occurred after 6-days, thereby preceding any significant change in leaf Ψ_S (Fig. 2D). During the drought treatment, maximum CAM expression was achieved within the 22-day-period of water withholding, as indicated by the peak in ΔH^+ and *PPC-1E1c* transcript levels between 18- and 21-days. Both ΔH^+ and *PPC-1E1c* transcript levels declined on the last day of sampling under drought, probably due to excessive stress. The lowest transcript abundances of C_4-marker genes were also detected after 18-days of drought stress (Fig. 2E), indicating temporal synchronization between the coordinated up- and down-regulation of CAM and C_4, respectively.

Our previous findings indicated that two days after rewatering, CAM was completely abolished and C_4 fully recovered in *P. oleracea* leaves (Ferrari *et al.*, 2020*b*). Here, we refined the monitoring of the temporal dynamics required for this photosynthetic switch, and verified that nocturnal acid accumulation and C_4- and CAM-specific gene regulation were reverted to levels similar to well-watered plants after 24 h (Fig. S2A). In fact, a 12-h-rewatering period proved to be sufficient to revert CCM-related gene transcripts to well-watered levels (Fig. 3B-C). Therefore, a detailed analysis of the rewatering period was performed, focusing on the first hours after the water supply was abundant again. As *PPC-1E1c* and *PPC-1E1a'* transcripts accumulate phased to the light period and the dark period, respectively (Fig. 1C; Ferrari *et al.*, 2020*b*), rewatering events were performed to cover the peak period of transcripts of these two CCM-marker genes (Fig. 3A). When comparing the rewatering-triggered mRNA abundance

changes for the abovementioned *PPC* genes, similar patterns were observed, regardless of whether water was resupplied either in the dark or the light (Fig. 3B). Once the water supply was reestablished, transcript level reprogramming leading to the CAM-to-C_4 reversion in *P. oleracea* occurred in a fast and remarkably synchronized manner. Limited changes were observed within the first 4 h of rewatering, followed by a coordinated change in all CCM mRNA levels from 5 to 8 h after water resupply (Fig. 3B-C).

Monitoring C_4 and CAM-marker transcripts in response to exogenous ABA and CK treatment

Next, we took advantage of the flexible and fast-responding C_4-CAM system in *P. oleracea* leaves to explore the ABA- and CK- related regulatory processes allowing C_4-CAM co-existence in a single leaf. Although orthologies between distantly related species are rarely a perfect overlap, we identified orthologs of the key enzymes involved in ABA and CK metabolism in our *P. oleracea* transcriptome (Ferrari *et al.*, 2020*b*), and monitored their transcript profile under various experimental conditions.

ABA synthesis and conjugation are modulated in response to water deficit primarily due to transcriptional changes (Xiong and Zhu, 2003), with *cis*-epoxycarotenoid dioxygenases (NCEDs) considered as key rate-limiting enzymes (Tan *et al.*, 2003) whereas cytochrome P450 type enzymes, the CYP707As (Okamoto *et al.*, 2011), are regarded as crucial during the prompt ABA inactivation after the water supply is reestablished (Kushiro *et al.*, 2004; Saito *et al.*, 2004). In *P. oleracea*, several ABA-related transcripts were identified as strongly modulated in response to water availability (Table S3, Fig. S4). Monitoring leaf endogenous ABA content and *NCED3* and *CYP707A1* mRNA levels during the water deprivation and subsequent rewatering in *P. oleracea* revealed a fast response in ABA metabolism-related transcripts depending on the water supply (Fig. 4, Fig. S3). ABA accumulation and *NCED3* mRNA levels were significantly promoted as soon as 6-days after water withholding, and both parameters displayed fluctuations as water deficit intensified (Fig. 4A). Drought had no impact on *CYP707A1* mRNA abundance, but it was gradually up-regulated in both well-watered and drought-stressed plants after 15-days of treatment (Fig. 4A). Beyond 12 h after rewatering (i.e., 12 – 24 h), endogenous ABA, as well as ABA metabolism and signaling transcripts, returned to levels similar to those detected in well-watered plants (Figs. S3-S4). *NCED3* was down-regulated significantly within the first hour of rewatering, but the most marked reductions in leaf ABA content were detected between 3 and 8 h after rewatering, coinciding with a transitory peak in *CYP707A1* mRNA levels (Fig. 4B). In drought-stressed and rewatered *P. oleracea* leaves, the temporal oscillations in transcripts encoding the core ABA signaling-related proteins PP2CA (CLADE A PROTEIN

PHOSPHATASES TYPE 2C) and ABF2 (ABA-RESPONSIVE ELEMENT-BINDING FACTOR 2) mirrored the endogenous ABA levels (Fig. 4A-B).

Based on *P. oleracea* transcriptome data (Ferrari *et al.*, 2020*b*), transcripts encoding the CK-activation enzyme LONELY GUY 1 (LOG1) as well as the CK-signal transduction proteins HPT PHOSPHOTRANSMITTER4 (AHP4) and RESPONSE REGULATOR 9 and 12 (ARR9 and ARR12, respectively) were selected for more detailed measurements in this study as they were candidates to be differentially abundant CK-related transcripts in response to water supply (Table S3, Fig. S4). The impacts of water scarcity on *LOG1*, *AHP4*, *ARR9* and *ARR12* mRNA abundance were only detected after 15-days of drought treatment (Fig. 4A), thereby coinciding with the timing of the down-regulation of C_4-related genes (Fig. 2E). On the other hand, most of the *LOG1* and *ARR9* up-regulation and *AHP4* and *ARR12* down-regulation in response to rewatering occurred between 4 and 8 h after water was resupplied (Fig. 4B). Whereas *AHP4*, *ARR12* and *ARR9* transcripts were completely recovered to levels similar to well-watered plants 24 h after rewatering, *LOG1* mRNA levels were only slightly recovered at both 12 and 24 h after rewatering (Fig. S4).

To further elucidate the roles of ABA and CKs on both C_4 and CAM expression in *P. oleracea*, we next conducted a pharmacological approach involving both short- and long-term hormonal treatments (hours *versus* days, respectively). Short-term (i.e., 12 h) treatment with ABA or BA revealed that CK supplementation completely recovered the drought-induced repression of all C_4 genes analyzed (Fig. 5A). Under well-watered conditions, *PPC-1E1c* transcript accumulation was promoted and repressed by ABA and CK supplementation, respectively (Fig. 5A). A small but significant additive effect of ABA on *PPC-1E1c* was also observed in drought-stressed plants (Fig. 5A). In agreement, a dose-dependent up-regulation of *PPC-1E1c* was observed upon long-term (i.e. four consecutive days) treatment with ABA under well-watered conditions (Fig. 5B), and several weeks of treatment with different concentrations of either BA or Z under drought caused a reduction in the abundance of this CAM-marker gene expression (Fig. 5C). Virtually no changes in C_4 gene transcript levels (Fig. 5B-C) were observed under long-term ABA and BA or Z treatment, and there were also no changes in ΔH^+ (Fig. S5). However, short-term CK treatment was shown to completely recover the drought-induced transcriptional repression of core C_4 enzymes in *P. oleracea* (Fig. 5A), and long-term BA or Z treatments led to a dose-dependent down-regulation of *PPC-1E1c* (Fig. 5C).

To gain further insights on the ABA-CK interplay in well-watered and drought-stressed *P. oleracea*, the impacts of exogenous hormone treatments on ABA- and CK-related gene transcript levels were also analyzed (Fig. 6). *NCED3* was the only gene identified as being

repressed by ABA under well-watered conditions, and *PP2CA* and *ABF2* were promoted by exogenous ABA in a dose-dependent manner (Fig. 6A). As additional ABA-CK crosstalk points, *ABF2* and *NCED3* were repressed by the Z treatment, whereas *LOG1*, *AHP4* and *ARR12* were up-regulated by ABA (Fig. 6). Long-term BA treatment increased transcript abundance of *ARR9,* but not *ARR12* (Fig. 6B).

Co-expression network analysis revealed additional candidate regulators of CAM and C_4 genes

Next, we used previously generated transcriptome data (Ferrari *et al.*, 2020*b*) to screen for candidate TFs associated with the drought-induced C_4-to-CAM transition in *P. oleracea*. Based on sequence homology, the transcriptome was annotated against Arabidopsis sequences from a comprehensive online plant transcription factor database (Jin *et al.*, 2014), retrieving 3,996 hits (Table S4). These were then filtered using the criteria logFC > |1.5| and FDR < 0.05 in at least one of the previously three-time points sampled (i.e., early morning, late afternoon and nighttime), and this returned a list of 290 hits (Table S5). Co-expression networks were generated, and modules containing the key CCM genes *PPC-1E1a'*, *PPC-1E1c* and *PPCK-1E* were identified (Fig. S6). TF-encoding transcripts that were found to change significantly in these modules were filtered (Table S6), rendering a list of nine TFs, selected by possessing the greatest fold-change of their transcripts. Of those, five TFs were present in two modules with opposing transcription patterns under the contrasting water regimes. The first of these modules contained *TRANSPARENT TESTA 8 (TT8*)*, EARLY FLOWERING MYB PROTEIN* (*EFM*) and *HOMEOBOX 7* (*HB7*), whereas the second module included *NAC DOMAIN CONTAINING PROTEIN 21/22* (*NAC22*) and *NUCLEAR FACTOR Y, SUBUNIT C4 (NFYC4*). The remaining four selected TFs were chosen from *PPC* and *PPCK* modules showing a pattern of up-regulation after drought, namely *NUCLEAR FACTOR Y, SUBUNIT C9 (NFYC9)*, *WRKY TRANSCRIPTION FACTOR FAMILY PROTEIN 44* (*WRKY44*), *NUCLEAR FACTOR Y, SUBUNIT A7 (NFYA7*) and *MYB DOMAIN PROTEIN 82 (MYB82*) (Table S6). These TFs were subjected to detailed analysis of transcript level regulation during the drought-induced C_4-to-CAM transition in *P. oleracea*, and the subsequent reversion to C_4 following rewatering.

Time-course transcript profiling using RT-qPCR revealed that *EFM*, *HB7, MYB82*, *NFYA7*, *TT8* and *WRKY44* were up-regulated by drought as soon as 6-days (Fig. 7A), whereas *NAC22*, *NFYC9* and *NFYC4* transcript abundances were only impacted after 12-15 days of drought treatment (Fig. 7A). All these transcripts recovered their levels at either 12 h or 24 h after rewatering (Fig. S7). In fact, *EFM, NAC22* and *MYB82* were significantly down-regulated as

soon as 2 h after rewatering, and all other TFs were down-regulated within 4 h of water being resupplied (Fig. 7B).

In order to understand the possible interconnection(s) between these drought-responsive TFs and the ABA- and CK-mediated signaling pathways controlling the expression of C_4 and CAM, the impacts of exogenous hormone applications on the transcript abundance of all nine selected TFs were investigated. *TT8* presented the same pattern as *PPC-1E1c*, being progressively up- and down-regulated by ABA and CK, respectively (Fig. 7C-E). Other TFs, except *EFM* and *NFYC4*, were up-regulated in response to long-term ABA treatment under well-watered conditions (Fig. 7C-D). Finally, drought-stressed plants exposed to long-term CK treatment displayed up- and down-regulation of *NFYC9* and *EFM*, respectively (Fig. 7C,E).

Correlation analysis based on data collected throughout the water deprivation (Fig. 2), rewatering (Fig. 3), and long-term hormonal treatment experiments (Figs. 4-6), revealed a clear connection between the selected TFs, as well as ABA- and CK-related genes, with both the CAM- and C_4-specific *PPC* genes (Table S7). Most drought-induced TFs clustered with *PPC-1E1c*, but presented varying correlation levels. The TF transcripts that were found to correlate most strongly with the regulation of the CAM-marker genes were *NFYA7*, *NFYC9, HB7* and *ARR12* (Fig. 8). In contrast, only *ARR9*, *LOG1* and *NFYC4* correlated positively with the C_4-associated *PPC-1E1a'* (Fig. 8).

Discussion

The unusual occurrence of C_4 and CAM in a single leaf in the species of *Portulaca* remains intriguing even today, more than 40 years after its discovery (Koch and Kennedy, 1980). Among different representatives, *P. oleracea* is the most widely studied species and, being a cosmopolitan species, brings the convenience of being easily accessible and performing CAM regardless of its origin (Ferrari et al. 2020*c*). Here, we sought to monitor transcript abundance changes as a means to initiate the exploration of the signaling events acting during the C_4-CAM transition and reversion by performing a four-stage approach: assessing the circadian clock control; depicting with detailed refinement the induction and reversion processes during drought establishment and rewatering; understanding ABA and CK triggered modulations; and unravelling transcription factors that may be involved in the process.

The molecular clock in C_4-CAM P. oleracea *resonated with the clock characterised in other plant species and* PPCK-1E *transcripts were the most susceptible to circadian control*

Overall, comparing the available evidence for C_3 facultative CAM and obligate CAM plants to the C_4-CAM system in *P. oleracea*, the transcript cycling patterns observed here under well-watered or drought-stressed conditions and the changes to those temporal oscillations that occurred under LL were broadly consistent with the regulation of core clock gene transcripts in LL in other species. Although *CCA1*, *RVE1* and *LHY* transcript levels were consistent with patterns reported in the literature (Alabadi *et al.*, 2002; Rawat *et al.*, 2009), the oscillation dampening of *CCA1* and *LHY* transcripts under LL in C_4-performing *P. oleracea* (Fig. 1B) was reminiscent of the dampening reported for *ZmCCA1* in maize plants under free-running conditions (Wang *et al.*, 2011). On the other hand, when CAM was induced from C_3 photosynthesis in *M. crystallinum*, the core circadian clock genes, including *CCA1/ LHY*, *TOC1*, *GI, ZTL* and *FKF1,* maintained robust rhythmicity under LL (Boxall *et al.*, 2005). Furthermore, in the constitutive CAM species *K. fedtschenkoi*, two *CCA1/ LHY*-related genes, *CCA1-1* and *CCA1-2*, and two *TOC1* genes*, TOC1-1 and TOC1-2*, plus genes for *GI*, *PRR7* and *FKF1*, maintained robust rhythmicity of their transcript oscillations throughout 3-days of an LL time-course (Boxall *et al.*, 2017). Similarly, in the closely related obligate CAM species, *K. laxiflora*, the transcript levels of many core clock genes oscillated under LL for 3-days, but several genes, including orthologs of *CCA1* and *FKF1,* underwent dampening of transcript oscillations as the time under LL progressed (Boxall *et al.*, 2020), corroborating the pattern observed here.

The C_4-related transcript oscillations of *P. oleracea* leaves maintained their circadian rhythmicity under LL even though the morning-phased *CCA1/ LHY/ RVE1* loop of the core clock was found to dampen markedly on day 3 of LL relative to driven LD conditions (Figs. 1B and 1C). In C_4 species, such as maize and sugarcane, a central molecular oscillator similar to that of the C_3 model species *A. thaliana* has been described, and 10 to 30% of the maize or sugarcane leaf transcriptome was reported to oscillate with a circadian rhythm under LL conditions (Khan *et al.*, 2010; Hotta *et al.*, 2013). Moreover, photosynthesis-related transcripts in maize oscillate in a circadian manner as in C_3 plants (Khan *et al.*, 2010). CAM-marker *PPC-1E1c* transcript abundance oscillation on day 3 of drought-stressed plants in LL agrees with findings for the CAM *PPC1* gene in *K. laxiflora* under LL, which also maintained a circadian rhythm of transcript levels under LL (Boxall *et al.*, 2020). However, the minimal variation in ΔH^+ observed here may indicate that the physiology and biochemistry of CAM dampened under LL, as previously reported for *M. crystallinum* (Dodd *et al.*, 2003; Davies and Griffiths, 2012). Many factors may limit CAM functioning under LL, including the disruption in diel carbon cycles, stomatal movements and CAM-related enzymatic activity (Wyka and Lüttge, 2003).

In obligate CAM *K. fedtschenkoi*, the circadian clock-controlled *PPCK1* has been demonstrated to be essential for the temporal optimization of CO_2 fixation to malate in the dark period, followed by malate decarboxylation and secondary CO_2 fixation via Rubisco in the light period (Boxall *et al*., 2017). The change in peak phase for *PPCK-1E* transcripts under LD in C_4- and CAM-performing *P. oleracea* plants observed here is consistent with previous findings (Ferrari et al. 2020*b*), but *PPCK-1E* levels did not display a robust oscillation under LL (Figs. 1C, S1B), resembling the regulation of *GI* and *PRR7* clock gene components on day 3 of LL. Although a 6-h delay in the timing of the *PPCK* transcript abundance peak was reported for CAM-induced *M. crystallinum* under LL (Boxall *et al.*, 2005), in the obligate CAM species *K. fedstchenkoi* and *K. laxiflora, PPCK1* transcript rhythms were easily discernible even after prolonged LL (Dever *et al.*, 2015; Boxall *et al.*, 2017; Boxall et al., 2020). Therefore, amongst all of the CCM-associated genes investigated here in *P. oleracea,* the circadian clock control of *PPCK* was identified as being the most susceptible to previously discussed dampening of the circadian oscillations of the transcript levels of core clock transcripts. In future experiments, it will be important to monitor continuously the circadian rhythms of clock and CCM genes every few hours over multiple days of LL, allowing connections between temporal control of clock output pathways and the regulation of C_4- and CAM-associated transcripts to be made, as well as the subsequent temporal control of the biochemical steps in these CCMs.

Water availability triggers synchronous and parallel modulation of C_4 and CAM

Altogether, our data reveal distinct timeframes for the CAM induction and reversion (days and hours, respectively), in response to changes in soil water availability. The significant changes in ΔH^+ and *PPC-1E1c* transcripts, and their disconnection from the leaf Ψ_S in drought-stressed *P. oleracea* leaves suggest that a reduction in leaf cell turgor was not necessary for triggering CAM induction in *P.* oleracea, as reported previously for the C_3-CAM inducible species, *M. crystallinum* (Eastmond and Ross, 1997). This also implicates the involvement of endogenous signals for interconnecting water deficit perception and the C_4-to-CAM transition. Whereas C_4- and CAM-related gene transcripts co-exist under drought, the complete disappearance of CAM-associated transcripts upon rewatering was fast and synchronized with the recovery of C_4-related gene transcript levels (Fig. 3). In other C_4-CAM facultative species, reductions in nocturnal acid accumulation have also been demonstrated to occur within a few days after rewatering, e.g., up to five days in *P. cyclophylla* and *P. digyna* (Holtum *et al.*, 2017*a*). Similarly, in C_3-CAM facultative species, timescales for recovery to C_3 following rewatering after drought-induced CAM were similarly rapid, e.g., one day in *Portulacaria afra* (Guralnick and Ting, 1986);

two days for *Talinum triangulare* (Brilhaus *et al.*, 2016); four days in several *Calandrinia* species (Holtum *et al.*, 2017*b*). Furthermore, CAM-specific *PPC* mRNA levels in detached leaves of *M. crystallinum* also reverted to C_3 levels within a few hours of water resupply (Piepenbrock and Schmitt, 1991), in a similar fashion to that observed in the CAM to C_4 reversion in leaves of intact *P. oleracea* plants used here. Significant reductions in CAM-related *PPC* transcripts have also been demonstrated in *M. crystallinum* within 2.5 h after salt stress was interrupted (Vernon *et al.*, 1988). Considering the reduced contribution of weak CAM to overall carbon gain in CAM cycling plants such as *P. oleracea*, a quick reversion back to C_4 would ensure the reestablishment of CO_2 assimilation rates favoring growth (Herrera, 2009).

C_4 + CAM hybrid models have been proposed by Lara et al. (2004) and recently modelled using flux balance by Moreno-Villena et al. (2021) in *P. oleracea*, but future studies monitoring protein levels and their biochemical activities at a spatial resolution would be beneficial to this discussion. According to these new lines of evidence, C_4 and CAM within a single leaf are proposed to cooperate instead of compete, and the CCMs feed each other carbon instead of being completely incompatible in *P. oleracea* (Moreno-Villena et al., 2021).

ABA-cytokinin antagonism regulates C_4 and CAM expression in P. oleracea

Our findings indicated that drought-triggered increments in ABA accumulation and signaling took place as soon as 6-days after water withholding, thereby coinciding with the timing of CAM induction and C_4 down-regulation in *P. oleracea* leaves experiencing increasing water deficit. Furthermore, there was a rapid decline in leaf ABA levels that was coincident with the CAM to full C_4 transition upon rewatering. A temporal coincidence between endogenous ABA levels and CAM up-regulation or induction has also been observed in young *A. comosus* plants (Freschi *et al.*, 2010), and mature *M. crystallinum* (Thomas *et al.*, 1992), exposed to water or salt stress, respectively. In addition, ABA accumulation preceded an increase in PPC content in *K. blossfeldiana* (Taybi *et al.*, 1995). Compared to the rapid and completely reversible alterations in ABA accumulation in response to changes in water supply, the impacts of water withholding on CK- related transcripts were significantly slower and only partially reverted by rewatering in *P. oleracea*. Endogenous CK levels have been reported to decrease rapidly under drought in C_3 and C_4 species (Pospíšilová *et al.*, 2005), mainly due to restrictions in its biosynthesis, consequently suppressing CK signaling, and thus being referred to as an ABA antagonist (Hare *et al.*, 1999; Oneto *et al.*, 2016).

Long- and short-term hormonal treatments supported a positive and negative influence of ABA and CKs on CAM-related gene regulation in *P. oleracea*, respectively. In line with our findings,

exogenous ABA has been repeatedly demonstrated to promote CAM-related gene expression in C_3-CAM facultative species (Chu *et al.*, 1990; Dai *et al.*, 1994; Forsthoefel *et al.*, 1995; Tsiantis *et al.*, 1996). For example, ABA can induce short-term transcript level increases in core CAM- and carbohydrate-related genes in *T. triangulare*, a response coupled to increased ΔH^+ and changes in the transcript abundance of ABA signaling components (Maleckova *et al.*, 2019). Even constitutive CAM species such as *Ananas comosus* and *K. blossfeldiana* can respond to exogenous ABA with increments in characteristics of CAM, such as ΔH^+ and gene expression (Taybi *et al.*, 1995; Freschi *et al.*, 2010). *NCED3* was the only hormone-related gene identified here as being repressed by ABA under well-watered conditions. In line with their action downstream to ABA, *PP2CA* and *ABF2* transcript levels were promoted by exogenous ABA in a dose-dependent manner, and, as observed here, were also up-regulated by drought and ABA in Arabidopsis (Matsui *et al.*, 2008), and *T. triangulare* (Brilhaus *et al.*, 2016; Maleckova *et al.*, 2019).

In contrast, very little is known about the influence of ABA on C_4 photosynthesis. ABA has been shown to induce traits of Kranz anatomy and increase C_4-enzyme activities in the C_3-C_4 intermediate and amphibious species *Eleocharis vivipara* and *E. baldwinii* (Ueno, 2001). Also, leaf disks of *Amaranthus hypochondriacus* treated with ABA showed increased PPC protein and mRNA (Aloor *et al.*, 2017). According to our findings, either short- or long-term ABA supplementation had virtually no effect on C_4 gene transcript levels in well-watered *P. oleracea* plants (Fig. 5). This is intriguing, especially considering ABA is widely known to regulate stomatal aperture and leaf physiology across plant species, suggesting that additional investigations into ABA-dependent changes in gas exchange and leaf development in *P. oleracea* may be beneficial in the future.

The unchanged ΔH^+ in *P. oleracea* reported here after long-term ABA treatment indicated that ABA alone is not sufficient to induce all the components required for CAM functioning under well-watered conditions. Nevertheless, CKs promoted C_4 transcript accumulation in *P. oleracea* under drought, which may be due to direct action of these hormones, or via crosstalk with other signaling molecules. A similar effect was observed in detached maize leaves, where C_4-*PPC* and *CA* were up-regulated after Z treatment, but this was dependent on nitrogen availability (Sugiharto *et al.*, 1992; Suzuki *et al.*, 1994; Offermann *et al.*, 2006). In C_3 species such as wheat and rice, exogenous CKs also increased PPC and CA enzyme activity, photosynthetic capacity, and Rubisco content (Ookawa *et al.*, 2004; Lazova and Yonova, 2010). In addition, Arabidopsis seedlings treated with CK showed increased *PPDK* and *NADP-ME* transcript abundances (Brenner *et al.*, 2005). Also, transgenic maize leaves overexpressing the cytokinin biosynthesis-

related gene *ISOPENTENYL TRANSFERASE* (*IPT*) under a senescence-regulated promoter showed increased tolerance to drought and a lower ABA content than control plants (Oneto *et al.*, 2016). Finally, increases in mRNA abundance of *ARR9* but not *ARR12* after CK treatment are consistent with previously reported patterns of differential regulation for type-A and type-B RRs (Kiba *et al.*, 1999), respectively, and thus further support a repressive influence of water scarcity on CK signaling in *P. oleracea*.

Screening of expression profiles allowed the identification of TFs possibly mediating the C_4-CAM alternation in P. oleracea

A range of TFs have been previously hypothesized to have the potential to be involved in CAM induction in *K. fedtschenkoi* during leaf development, and *M. crystallinum* in response to abiotic stress. These TFs belong to the NAC, MYB, Homeobox (HB) and Nuclear factor Y (NFY) families (Amin *et al.*, 2019), which were also identified here as potentially associated with the drought-stress associated CCM transition in *P. oleracea*. In addition, *EFM*, *HB7, MYB82*, *WRKY44*, *NAC22*, *NFYC9*, *NFYC4* and *NFYA7* belong to TF families that have commonly been reported to be involved in drought responses in non-CAM species (Söderman *et al.*, 1996; Nakashima *et al.*, 2007; Li *et al.*, 2008; Tripathi *et al.*, 2014; Baldoni *et al.*, 2015). After monitoring the transcript profiling of the nine identified TFs during the time-course experiment in *P. oleracea* leaves, the up-regulation of *EFM*, *HB7, MYB82*, *NFYA7*, *TT8* and *WRKY44* coincided with the start of CAM after 6 days of drought stress. In contrast, *NAC22*, *NFYC9* and *NFYC4* transcript abundances were impacted at a time that had already been demonstrated to be characteristic for the down-regulation of the C_4 genes, after 12-15 days of drought treatment. When considering the different TFs selected here, NF-Ys are known to regulate photosynthesis responses and can be affected by CK and light (Kusnetsov *et al.*, 1999). Moreover, *NFYC9* overexpression in Arabidopsis mutants conferred hypersensitivity to ABA (Bi *et al.*, 2017), and here, *NFYC9* was responsive to ABA and CK treatments. Likewise, overexpression of *NFYA7* in Arabidopsis conferred resistance to drought stress (Leyva-González *et al.*, 2012), and this gene was reported to be associated with high temperature and salt stress (Maheshwari *et al.*, 2019). *TT8* is a basic helix-loop-helix (bHLH) transcription factor (Nesi *et al.*, 2000) that was also up-regulated during drought induction of weak CAM from C_3 in *T. triangulare* (Brilhaus *et al.*, 2016). In Arabidopsis, loss-of-function mutants for *TT8* were more sensitive to ABA (Rai *et al.*, 2016). *NAC22*, also referred to as *NAC21/22* or *NAC1*, was induced by ABA in grapevine leaves, and increased tolerance to osmotic, salt and cold stresses was induced in Arabidopsis plants

overexpressing this gene (Hénanff *et al.*, 2013). Lastly, *MYB82* can act as a negative regulator of drought-related responses in wheat (Mia *et al.*, 2019).

Among the TFs closely associated with CAM-associated *PPC-1E1c* (Fig. 8), *HB7* was up-regulated by ABA and drought in the C_3-CAM facultative species, *T. triangulare* (Brilhaus *et al.*, 2016; Maleckova *et al.*, 2019). *HB7* acts positively regulating the transcription of *PP2C* genes in C_3 *A. thaliana* (Valdés *et al.*, 2012). In *P. oleracea*, MYB-transcription factor-encoding gene *EFM* was up-regulated by drought (Fig. 7A), as also reported for *T. triangulare* (Brilhaus *et al.*, 2016), and down-regulated by CKs (Fig. 7C), as reported in Arabidopsis (Bhargava *et al.*, 2013). Finally, *TT8, NAC22*, and *MYB82* were more weakly correlated to *PPC-1E1c*, but all were up-regulated during long-term ABA treatment (Fig. 8). Therefore, these results represent an initial screening for regulatory events potentially involved in controlling the C_4 to CAM transition in *P. oleracea*. However, the complex signal transduction cascades controlling this poorly understood photosynthetic adaptation will undoubtedly hold many more interacting partners that remain to be elucidated. The generation of additional transcriptomic data during this photosynthetic transition, accompanied by comprehensive mining of the data for regulatory and TF transcripts based on larger databases, and the application of functional genomics approaches to test candidate gene function *in planta*, remain important future goals. Such approaches will undoubtedly lead to exciting new models of TF–phytohormone interaction networks controlling the facultative C_4-CAM system in *Portulaca*.

Conclusions

Overall, our study reveals that the endogenous circadian clock coordinates and optimizes the daily timing of both C_4 and CAM gene regulation in well-watered and drought-stressed leaves of *P. oleracea*, respectively. Among all CCM genes analyzed, *PPCK-1E* was found to be coupled most closely with changes measured in the temporal regulation of core circadian clock genes under free-running LL conditions. ABA was implicated as a major signal connecting plant water status with the regulation of core C_4 and CAM genes, with *NCED3* transcript accumulation responding quickly during drought and rewatering, and correlated with fast and fully reversible changes in the endogenous ABA level. In addition, based on the transcriptional responses of the CAM-marker gene *PPC-1E1c* and various TF transcripts to exogenous ABA and CK treatments, an antagonistic action of these two hormone classes was implicated in the regulation of CAM induction and repression in response to progressive drought and subsequent rewatering. Moreover, CK treatment recovered the levels of all six C_4-transcript markers analyzed in drought-stressed plants. Finally, *HB7*, *NFYA7*, *NFYC9*, and *ARR12* were identified

as the TFs mostly closely linked with CAM-associated *PPC-1E1c* transcript accumulation in response to drought stress and ABA and CK application, suggesting that these TFs were the most likely CAM effectors in the C_4-CAM system of *P. oleracea*. In addition, *TT8* was identified as a candidate TF that could act as an early messenger, since it responded to ABA and CK stimuli, and its induction preceded CAM-related transcript accumulation as drought developed. On the other hand, *NFYC4* and *ARR9* were tightly connected to the C_4 genes.

Since an efficient, stable *Agrobacterium*-mediated transformation protocol has been recently established for *P. oleracea* (Ferrari *et al.*, 2020*a*), future studies involving loss-of-function and overexpression mutants for the TFs listed here may provide further insights into the signaling networks controlling the coordinated, fast and completely reversible C_4-CAM transition in this species. *P. oleracea* holds enormous potential for furthering understanding of the intricate regulation and connectivity of both C_4 and CAM pathways within a single leaf, especially in the context of future endeavors aiming at biotechnological applications such as engineering crops for more resistant cultivars in a climate change context, and creating more sustainable agricultural systems with higher water use efficiency (Borland *et al.*, 2009; Covshoff and Hibberd, 2012; Yang *et al.*, 2015; Hartwell *et al.*, 2016; FAO 2020).

Author's contributions

LF and JH conceived the project and supervised the experiments; RCF and ABK conducted most of the experiments; SSF conducted the correlation network analysis; RCF, LF and JH wrote the article with contributions from other authors.

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

The following Supporting Information is available for this article:

Fig. S1 Day/night fluctuations in leaf acidity and clock- and CCM-related gene expression according to light treatments in *Portulaca oleracea.*
Fig. S2 Nocturnal acidification and CCM-related transcriptional changes upon 12 h and 24 h of rewatering.
Fig. S3 Impacts of the watering regime on the leaf endogenous abscisic acid (ABA) levels.
Fig. S4 Impacts of the watering regime on the transcript abundance of hormone-metabolism genes.
Fig. S5 Leaf nocturnal acid accumulation (ΔH^+) in response to hormonal treatments.
Fig. S6 Selected clusters generated via co-expression analysis for well-watered and drought-stressed plants.
Fig. S7 Transcriptional changes in transcription factors upon different water regimes.

Table S1 Primer sequences used for qPCR.
Table S2 Annotation and abundance of central circadian clock transcripts.
Table S3 Annotation and abundance of abscisic acid- and cytokinin-related transcripts.
Table S4 Annotation and abundance of transcription factor-encoding transcripts.
Table S5 Statistically significant modulations of transcription factors in response to drought.
Table S6 Filtered drought-modulated transcription factors after co-expression analysis
Table S7 Correlation matrix for CCM- and signaling-related genes.

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734

Figure legends

Fig. 1 Circadian clock-controlled expression of C_4- and CAM-related genes in *Portulaca oleracea*. (A) Schematic representation of the experimental design for the 12 h light / 12 h dark (LD) and constant light and temperature free-running (LL, 100 µmol m^{-2} s^{-1} at 22°C) time-course experiments. Well-watered or droughted plants were kept under LD or transferred to LL conditions, and mature leaves were sampled every three hours for 24 h, starting 48h into the LL treatment (Zeitgeber (ZT) 48 until ZT 72). (B) Transcript abundance of central circadian clock genes. (C) Transcript abundance of C_4- and CAM-related genes. Mean relative expression was normalized against the first-time point of either well-watered (left) or droughted (right) leaf samples under the LD regime. The shaded areas indicate the dark and subjective dark periods. Data are means (± SE) of at least three replicates. **P* < 0.05 compared with LD samples at each sampling time. AK, adenylate kinase; ALAAT, ALA aminotransferase; ALMT, aluminum-activated malate transporters; ASPAT, ASP aminotransferase; βCA, beta-carbonic anhydrase; CCA1; circadian clock associated 1; FKF1, flavin-binding kelch repeat F box 1; GI, gigantea; LHY, late elongated hypocotyl 1; NADME, NAD-malic enzyme; PPC, phospho*enol*pyruvate carboxylase; PPCK, phospho*enol*pyruvate carboxylase kinase; PPDK, pyruvate orthophosphate dikinase; PRR, pseudo-response regulator; RVE1, reveille 1; TOC1, timing of CAB expression; ZT, Zeitgeber time.

Fig. 2 Drought-induced CAM expression in *Portulaca oleracea* is accompanied by a progressive down-regulation in C_4-related transcript abundancies. (A) Schematic representation of the experimental design for the time-course analysis of CAM induction by drought. Leaves of 30-day old plants were sampled every three days under well-watered or drought conditions at dawn and dusk. (B) Soil volumetric water content (SVWC). (C) Osmotic potential. (D) Nocturnal titratable acid accumulation (ΔH^+). (E) Transcript abundance of C_4-related genes. (F) Transcript abundance of CAM-related *PPC* gene. In D, ΔH^+ indicates dawn-dusk differences and SE of the dawn-dusk difference = $\sqrt{((SE_{dawn})^2 + (SE_{dusk})^2)}$. In E and F, mean relative expression was normalized against well-watered leaves at the start of the treatment. All gene expression data are from samples harvested at dawn, except for *PPC-1E1c,* which was sampled at dusk. Data are means (± SE) of at least three replicates. **P* < 0.05 compared with well-watered samples at each sampling time. AK, adenylate kinase; ALAAT, ALA aminotransferase; ALMT, aluminum-activated malate transporters; ASPAT, ASP aminotransferase; βCA, beta-carbonic anhydrase; NADME, NAD-malic enzyme; PPC, phospho*enol*pyruvate carboxylase; PPDK, pyruvate orthophosphate dikinase.

Fig. 3 Rewatering promotes fast and synchronized up- and down-regulation of C_4- and CAM-related genes, respectively. (A) Schematic representation of the experimental design for the time-course analysis of CAM reversion by rewatering. Leaves of 30-day old plants were droughted for 21 days and sampled on day 22 after 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12 hours of rewatering. (B) Transcript abundance of *PPC* genes sampled after distinct rewatering periods either at day- or nighttime. (C) Transcript abundance of C_4-related genes sampled after nighttime rewatering. Mean relative expression was normalized against the droughted control at time 0. Data are means (± SE) of at least three replicates. Different letters indicate statistically significant differences between time points sampled after different rewatering periods ($P < 0.05$). AK, adenylate kinase; ALAAT, ALA aminotransferase; ALMT, aluminum-activated malate transporters; ASPAT, ASP aminotransferase; βCA, beta-carbonic anhydrase; NADME, NAD-malic enzyme; PPC, phospho*enol*pyruvate carboxylase; PPDK, pyruvate orthophosphate dikinase.

Fig. 4 Changes in hormonal metabolism and signaling during CAM induction and reversion in *Portulaca oleracea*. Treatment details as described in Figs. 2 (drought) and 3 (rewatering). (A) Endogenous abscisic acid (ABA) levels and transcript abundance of ABA- and cytokinin (CK)-related genes under progressive drought. In A, mean relative expression was normalized against well-watered leaves at the start of the treatment. *$P < 0.05$ compared with well-watered samples. (B) Endogenous ABA levels and transcript abundance of ABA and CK-related genes under progressive rewatering. In B, mean relative expression was normalized against the droughted control at time 0, and different letters indicate statistically significant differences between time points sampled after different rewatering periods ($P < 0.05$). In all cases, data are means (± SE) of at least three replicates. All genes were sampled at dawn, except for *NCED3*, *ABF2* and *PP2CA,* which were sampled at dusk. ABF, aba-responsive element-binding factors; AHPT, phosphotransmitter; CYP707A, cytochrome P450 type enzymes; LOG, lonelyguy; ARR, response regulator; NCED, 9-cis-epoxycarotenoid dioxygenase; PP2CA, clade A PP2C phosphatases.

Fig. 5 Impacts of short- and long-term hormonal treatments on C_4- and CAM-related gene expression in *Portulaca oleracea*. (A-C) Experimental design schemes for treatments and sampling points, followed by heatmaps indicating $\log_2$ (fold-change) of C_4- and CAM-related transcript levels after hormonal treatment, and plots representing the most significant changes in transcript abundance. (A) Short-term abscisic acid (ABA) and cytokinin (CK) treatment. Well-watered or droughted plants were treated with 500 µM ABA or 6-Benzylaminopurine (BA) for 12

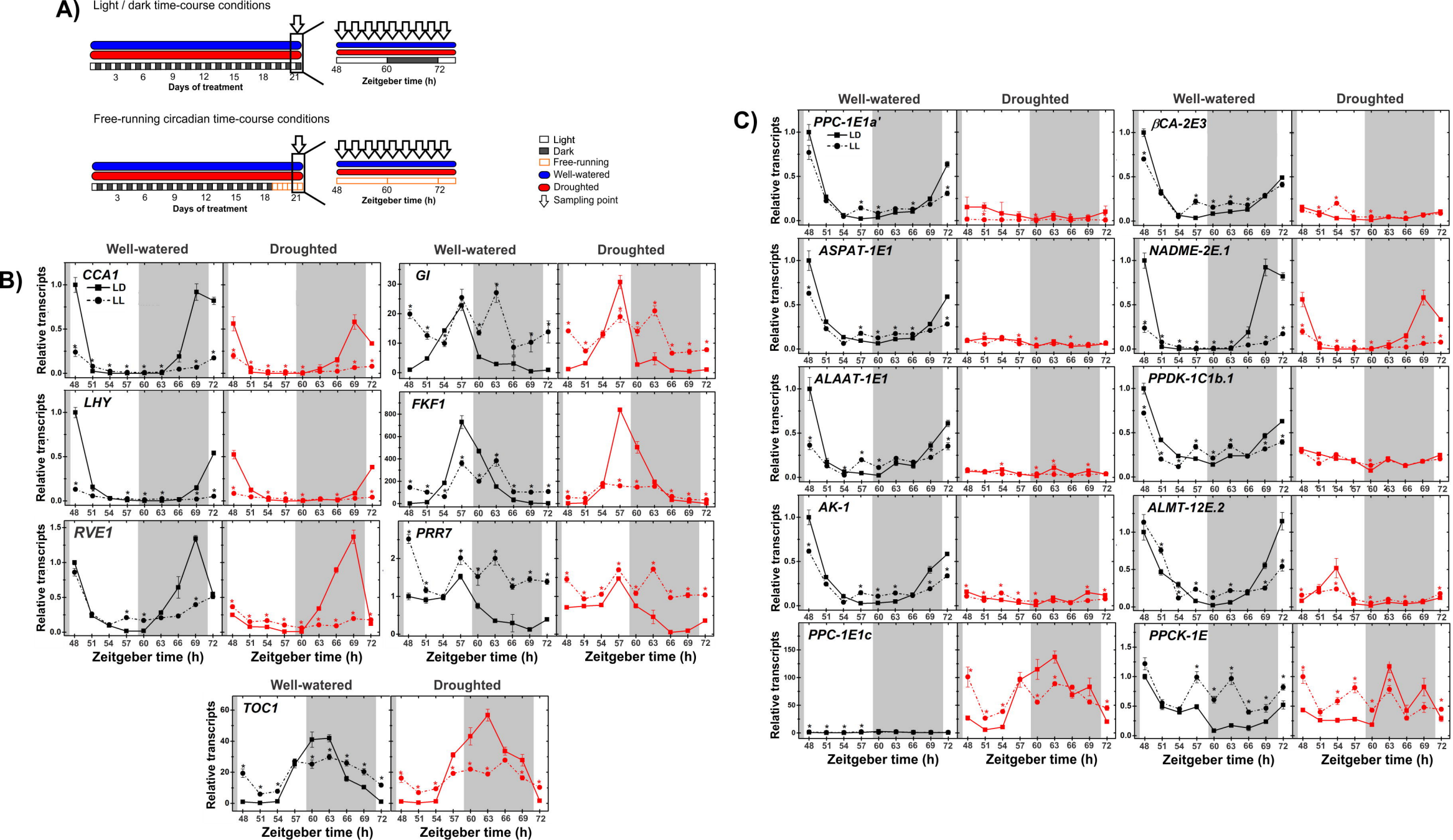
h (short-term). (B) Long-term ABA treatment. Well-watered plants were treated with 0, 25, 100 and 500 µM ABA for 4 consecutive days. (C) Long-term CK treatment. Droughted plants were treated with 0, 5, 10, 20 µM BA or *trans*-zeatin (Z) in four applications over 20 days. Mean relative expression was calculated using dawn samples for all C_4-markers genes and dusk samples for *PPC-1E1c* as a CAM-marker gene, and normalized against water-sprayed controls. In heatmaps, statistically significant differences are compared to water-sprayed controls and are represented as colored squares ($P < 0.05$). In plots, different letters indicate statistically significant differences between treatments ($P < 0.05$). Data are means (± SE) of at least three replicates. ALAAT, ALA aminotransferase; ALMT, aluminum-activated malate transporters; ASPAT, ASP aminotransferase; βCA, beta-carbonic anhydrase; NADME, NAD-malic enzyme; PPC, phosphoe*nol*pyruvate carboxylase; PPDK, pyruvate orthophosphate dikinase.

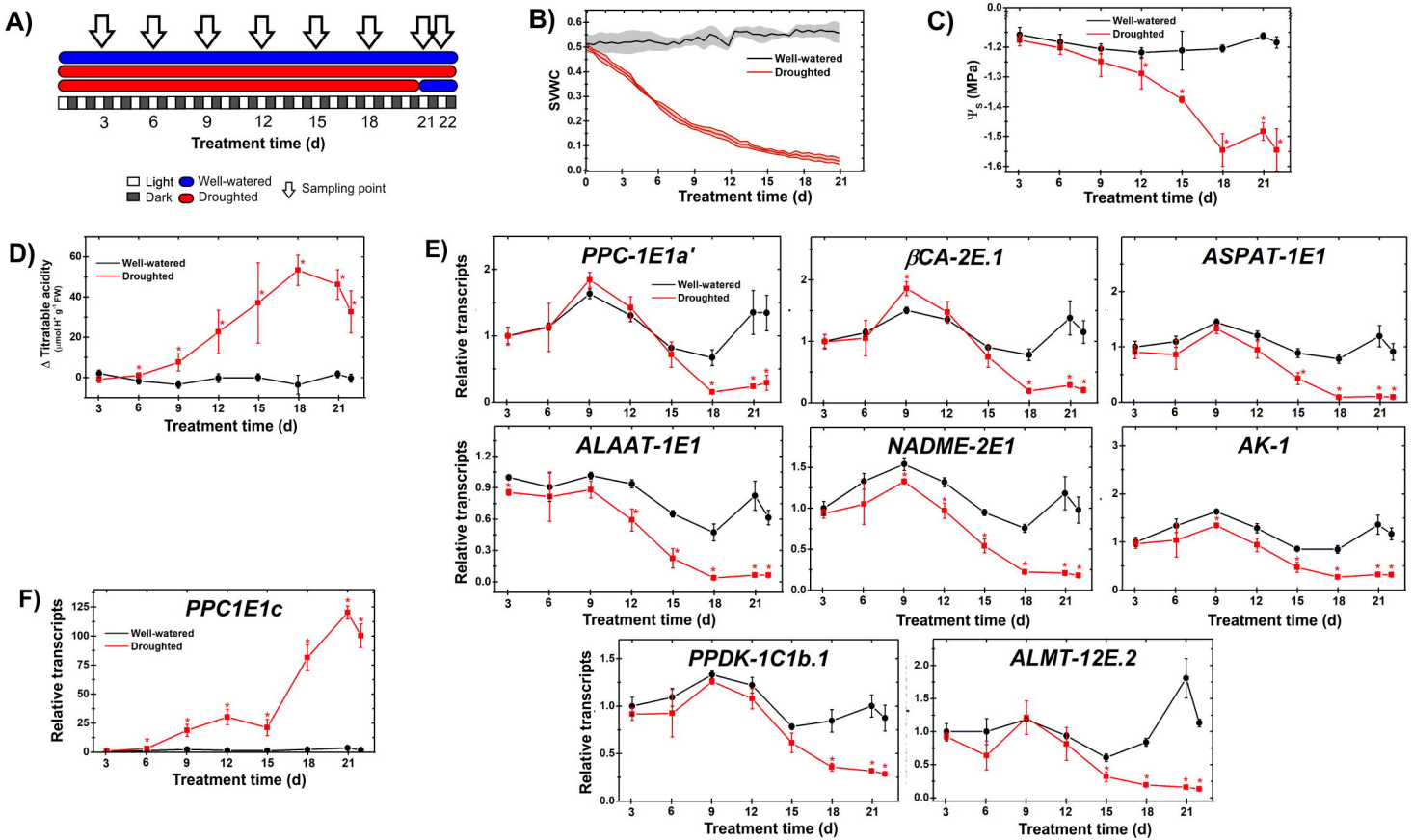
Fig. 6 Hormonal cross-interactions in well-watered and droughted *Portulaca oleracea* plants. Treatment details as described in Fig. 5. (A) Impacts of long-term abscisic acid (ABA) treatment on the transcript abundance of ABA and cytokinin (CK)-related genes in well-watered plants. (B) Impacts of long-term 6-benzylaminopurine (BA) or *trans*-zeatin (Z) treatments on the transcript abundance of ABA- and CK-related genes associated in droughted plants. Different letters indicate statistically significant differences between treatments ($P < 0.05$), and mean relative expression was normalized against the control. All genes were analyzed in samples harvested at dawn, except for *NCED3*, *ABF2* and *PP2CA,* which were sampled at dusk. ABF, aba-responsive element-binding factors; AHPT, phosphotransmitter; CYP707A, cytochrome P450 type enzymes; LOG, lonelyguy; ARR, response regulator; NCED, 9-cis-epoxycarotenoid dioxygenase; PP2CA, clade A PP2C phosphatases.

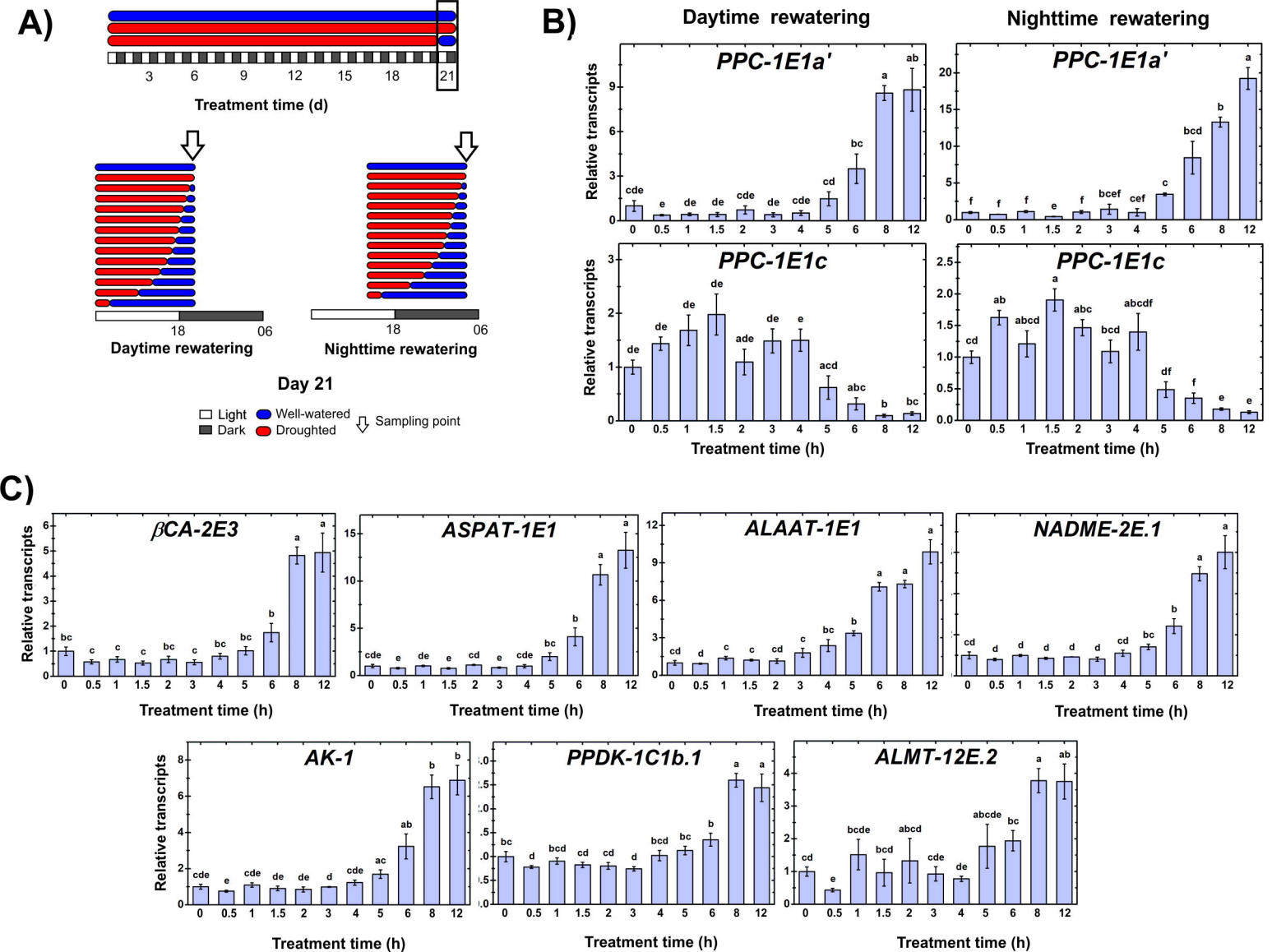
Fig. 7 Fluctuation in mRNA levels encoding candidate transcription factors (TFs) during CAM induction and reversion in *P. oleracea*. Treatment details as described in Figs. 2 (drought) and 3 (rewatering). (A-B) Transcript abundance of TF-encoding genes under progressive drought (A) and rewatering (B). In A, $*P < 0.05$ compared with well-watered samples. In B, different letters indicate statistically significant differences between time points sampled after different rewatering periods ($P < 0.05$). (C) Heatmaps indicate $\log_2$ (fold-change) of TF-encoding genes following hormonal treatment. In the heatmaps, statistically significant differences compared with water-sprayed controls are represented as colored squares ($P < 0.05$). (D-E) Plots represent TF-encoding transcript levels under long-term ABA and CK treatments, respectively, and different letters indicate statistically significant differences between treatments ($P < 0.05$). Mean relative expression was normalized against water-sprayed controls. Data are means (±

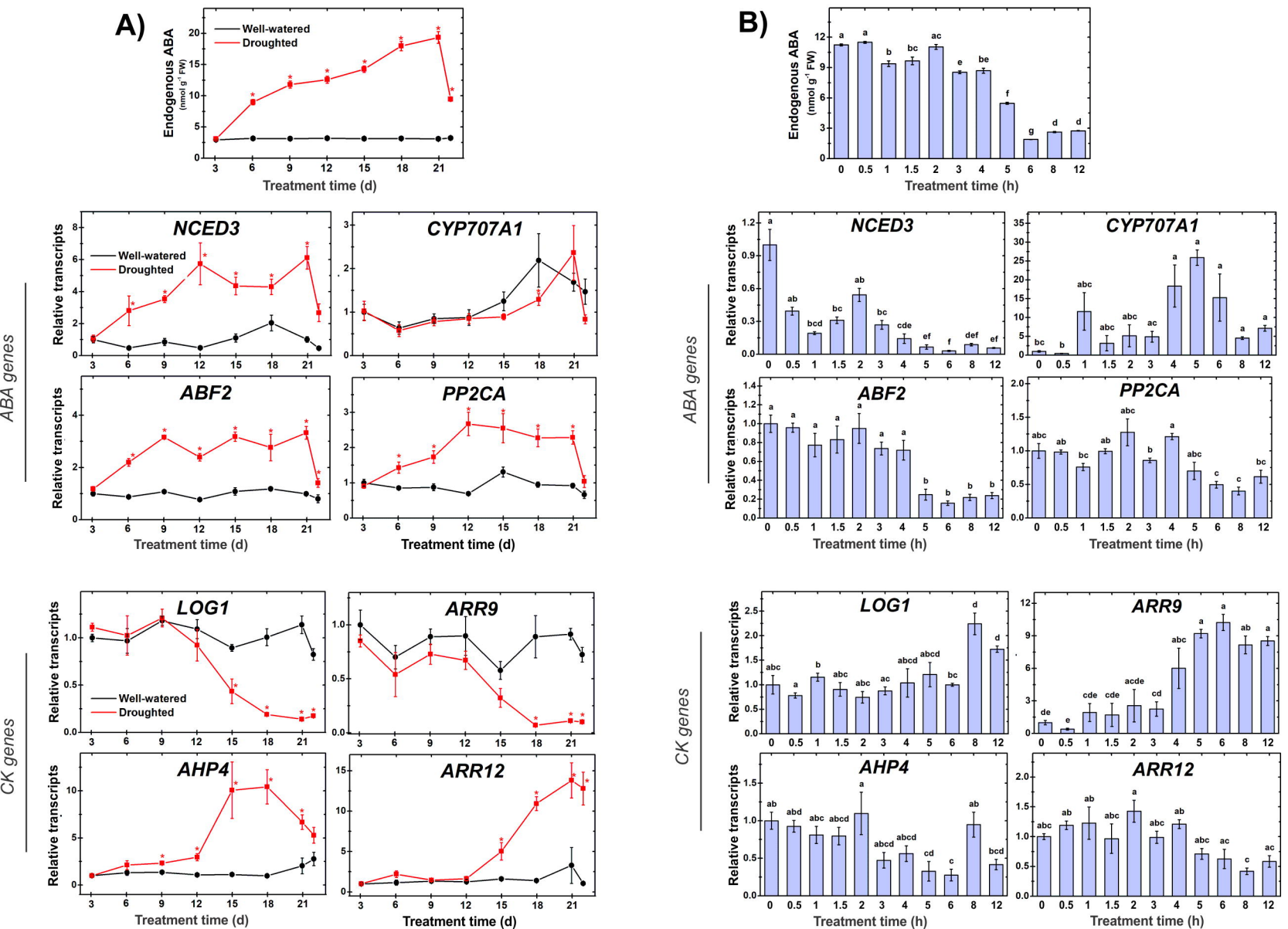
SE) of at least three replicates. All genes were analyzed in samples harvested at dawn, except for *EFM, WRKY44, HB7, MYB82*, and *TT8,* which were analyzed in afternoon/night samples. EFM, flowering MYB protein; HB7, homeobox; MYB, MYB domain protein; NAC, NAC domain-containing protein; NFYC and NFYA, nuclear factor Y, subunits C and A; TT, transparent testa; WRKY, WRKY transcription factor family protein.

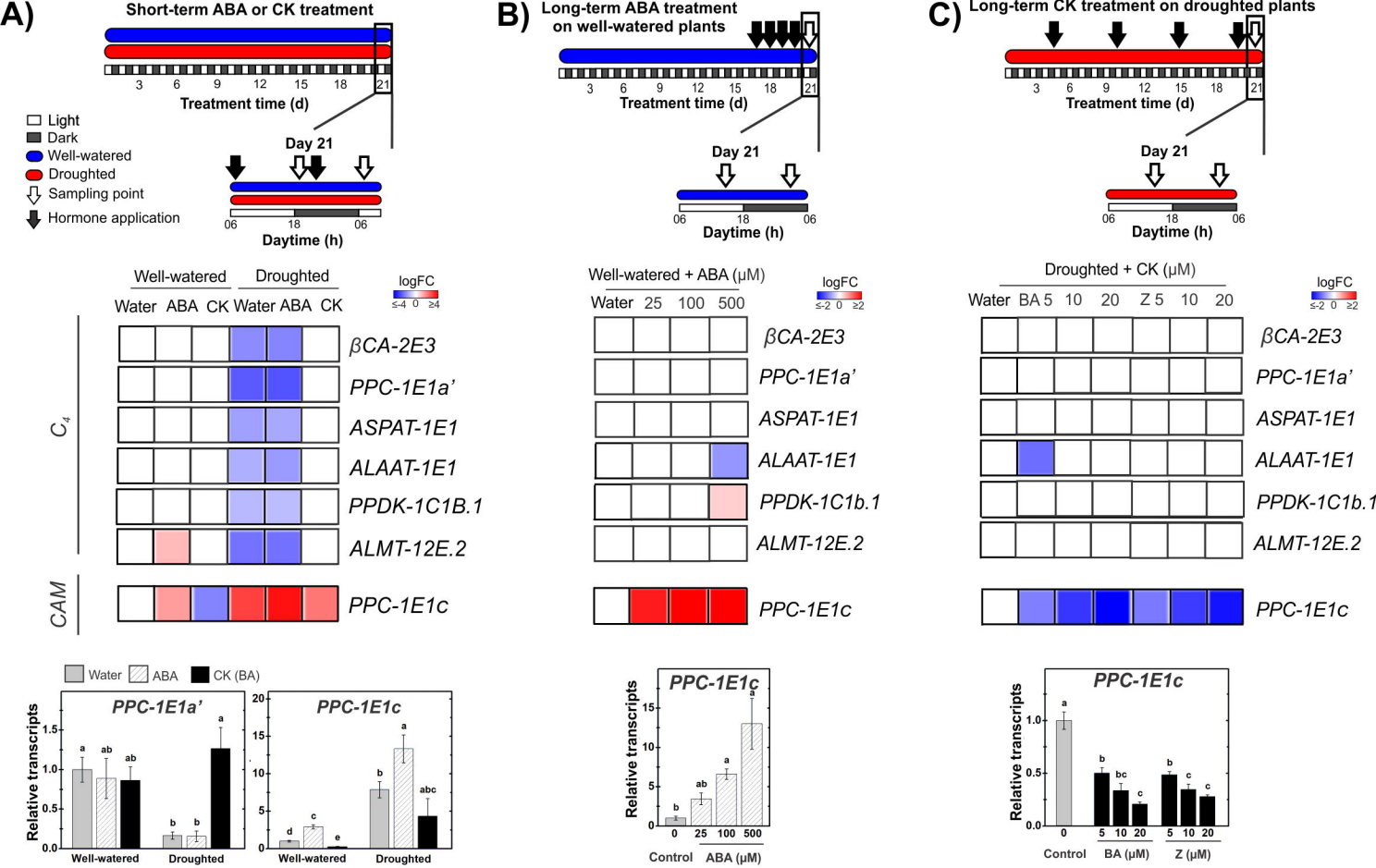
Fig. 8 Regulatory events controlling C_4 and CAM expression in *P. oleracea*. Diagram illustrating the level of connection between transcription factors (TFs) and the CAM- or C_4-related *PPC* genes according to correlation indexes (correlation indexes listed in Table S7). Gene abbreviations are described in the text.

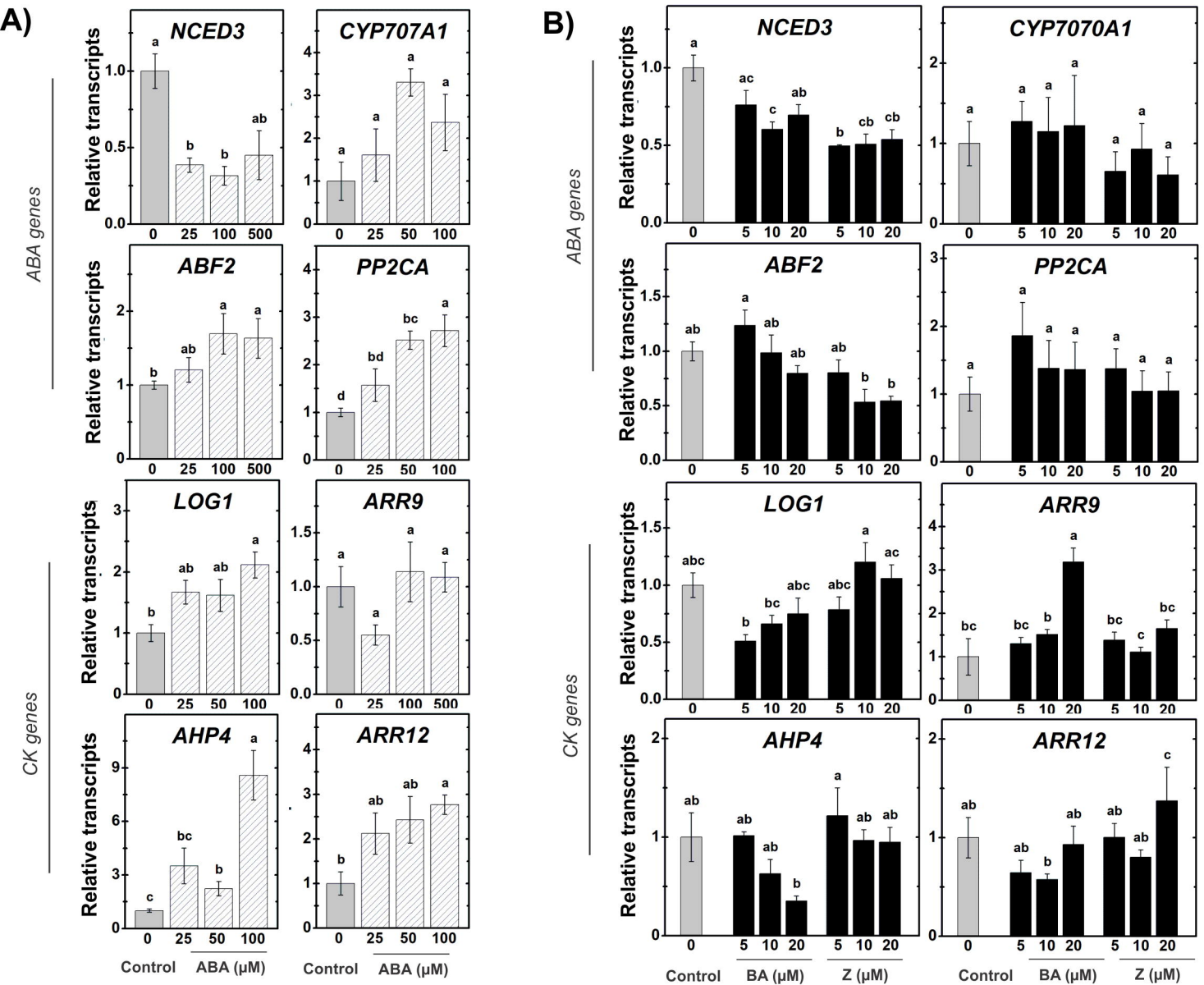

A)
Light / dark time-course conditions
Days of treatment
Zeitgeber time (h)
Free-running circadian time-course conditions
Light
Dark
Free-running
Well-watered
Droughted
Sampling point
B)
Well-watered
Droughted
CCA1
LD
LL
GI
LHY
FKF1
RVE1
PRR7
TOC1
Relative transcripts
Zeitgeber time (h)
C)
PPC-1E1a'
βCA-2E3
ASPAT-1E1
NADME-2E.1
ALAAT-1E1
PPDK-1C1b.1
AK-1
ALMT-12E.2
PPC-1E1c
PPCK-1E

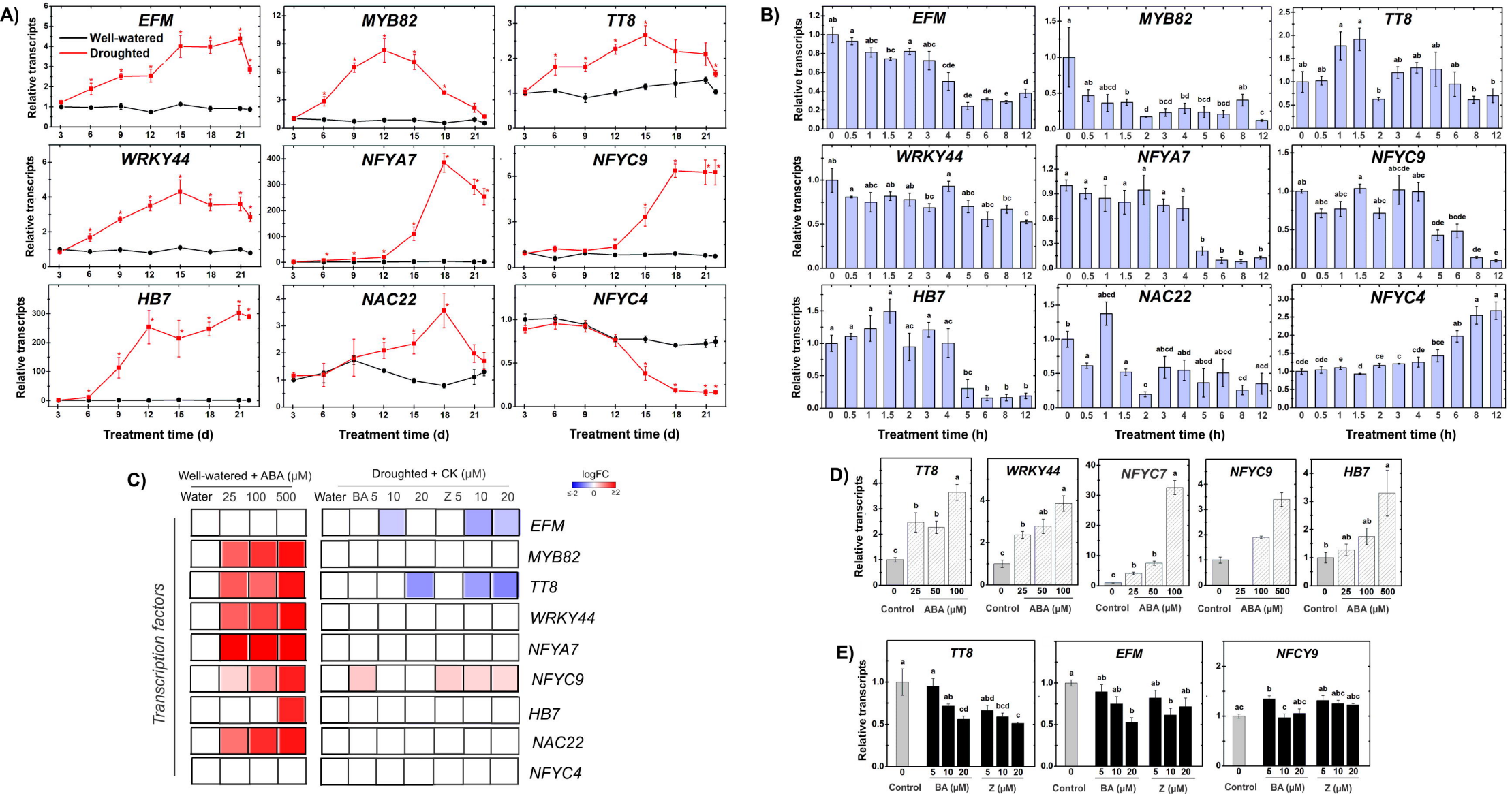

A)
3 6 9 12 15 18 21 22
Treatment time (d)
Light
Dark
Well-watered
Droughted
Sampling point
B)
SVWC
Well-watered
Droughted
Treatment time (d)
C)
Well-watered
Droughted
Ψs (MPa)
Treatment time (d)
D)
Well-watered
Droughted
Δ Titratable acidity (μmol H+ g-1 FW)
Treatment time (d)
E)
PPC-1E1a'
βCA-2E.1
ASPAT-1E1
ALAAT-1E1
NADME-2E1
AK-1
PPDK-1C1b.1
ALMT-12E.2
Well-watered
Droughted
Relative transcripts
Treatment time (d)
F)
PPC1E1c
Well-watered
Droughted
Relative transcripts
Treatment time (d)

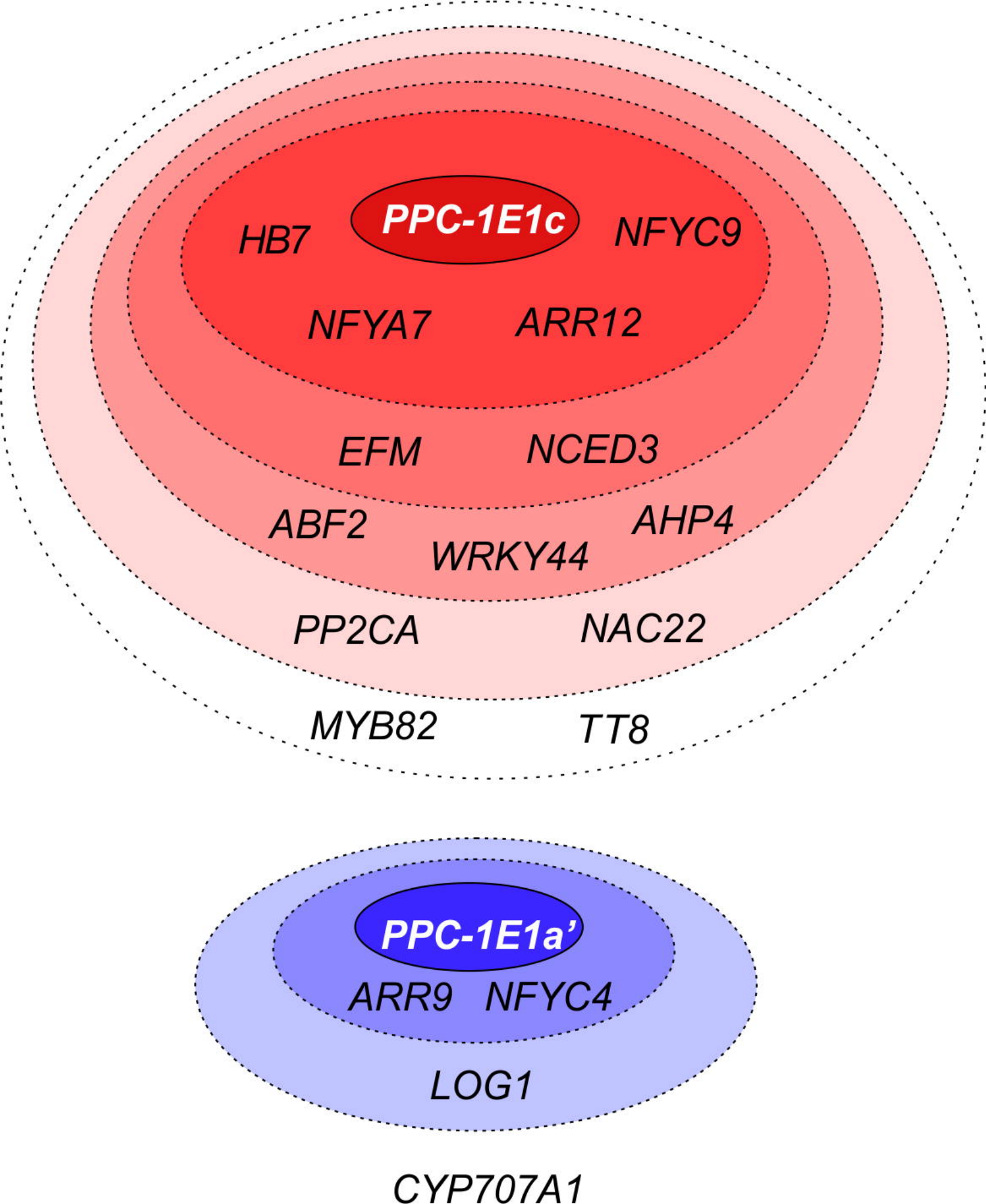

A)
3 6 9 12 15 18 21
Treatment time (d)
18 06
Daytime rewatering
18 06
Nighttime rewatering
Day 21
Light
Dark
Well-watered
Droughted
Sampling point
B)
Daytime rewatering
Nighttime rewatering
PPC-1E1a'
PPC-1E1a'
PPC-1E1c
PPC-1E1c
Relative transcripts
Treatment time (h)
C)
βCA-2E3
ASPAT-1E1
ALAAT-1E1
NADME-2E.1
AK-1
PPDK-1C1b.1
ALMT-12E.2
Relative transcripts
Treatment time (h)


A)
B)
Well-watered
Droughted
Endogenous ABA (nmol g-1 FW)
Treatment time (d)
Treatment time (h)
ABA genes
CK genes
Relative transcripts
NCED3
CYP707A1
ABF2
PP2CA
LOG1
ARR9
AHP4
ARR12


A)
Short-term ABA or CK treatment
3 6 9 12 15 18 21
Treatment time (d)
Day 21
06 18 06
Daytime (h)
Light
Dark
Well-watered
Droughted
Sampling point
Hormone application
B)
Long-term ABA treatment on well-watered plants
3 6 9 12 15 18 21
Treatment time (d)
Day 21
06 18 06
Daytime (h)
C)
Long-term CK treatment on droughted plants
3 6 9 12 15 18 21
Treatment time (d)
Day 21
06 18 06
Daytime (h)
Well-watered
Droughted
Water ABA CK Water ABA CK
logFC
≤-4 0 ≥4
C4
CAM
βCA-2E3
PPC-1E1a'
ASPAT-1E1
ALAAT-1E1
PPDK-1C1B.1
ALMT-12E.2
PPC-1E1c
Well-watered + ABA (μM)
Water 25 100 500
logFC
≤-2 0 ≥2
βCA-2E3
PPC-1E1a'
ASPAT-1E1
ALAAT-1E1
PPDK-1C1b.1
ALMT-12E.2
PPC-1E1c
Droughted + CK (μM)
Water BA 5 10 20 Z 5 10 20
logFC
≤-2 0 ≥2
βCA-2E3
PPC-1E1a'
ASPAT-1E1
ALAAT-1E1
PPDK-1C1b.1
ALMT-12E.2
PPC-1E1c
Water
ABA
CK (BA)
PPC-1E1a'
PPC-1E1c
Relative transcripts
Well-watered
Droughted
PPC-1E1c
Relative transcripts
Control
ABA (μM)
PPC-1E1c
Relative transcripts
Control
BA (μM)
Z (μM)


A)
B)
ABA genes
CK genes
NCED3
CYP707A1
CYP7070A1
ABF2
PP2CA
LOG1
ARR9
AHP4
ARR12
Relative transcripts
Control
ABA (μM)
BA (μM)
Z (μM)


A)
Well-watered
Droughted
EFM
MYB82
TT8
WRKY44
NFYA7
NFYC9
HB7
NAC22
NFYC4
Relative transcripts
Treatment time (d)
B)
Relative transcripts
Treatment time (h)
C)
Well-watered + ABA (μM)
Droughted + CK (μM)
Water 25 100 500
Water BA 5 10 20 Z 5 10 20
logFC
≤-2 0 ≥2
Transcription factors
EFM
MYB82
TT8
WRKY44
NFYA7
NFYC9
HB7
NAC22
NFYC4
D)
TT8
WRKY44
NFYC7
NFYC9
HB7
Relative transcripts
Control ABA (μM)
E)
TT8
EFM
NFCY9
Relative transcripts
Control BA (μM) Z (μM)


PPC-1E1c
HB7
NFYC9
NFYA7
ARR12
EFM
NCED3
ABF2
AHP4
WRKY44
PP2CA
NAC22
MYB82
TT8
PPC-1E1a'
ARR9
NFYC4
LOG1
CYP707A1