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UNIVERSITY OF CALIFORNIA SAN DIEGO

Organ-specific mechanisms regulate circadian clock function across the plant shoot system

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biology

by

Nicholas Deason

Committee in charge:

Professor Jose L. Pruneda-Paz, Chair
Professor Christopher Benner
Professor Julie A. Law
Professor Martin F. Yanofsky

2024

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University of California San Diego

2024

DEDICATION

This dissertation is dedicated to Jackie, who left home more than seven years ago to begin a new life with me in San Diego as I pursued my PhD. Along the way she became my wife and gave birth to our beautiful son. I was blessed to have her constant love and support as I performed all the work contained herein.

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- 3. Deason_Table_S3_rhythmicTranscripts.xlsx**
- 4. Deason_Table_S4_enrichedGOterms.xlsx**
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Chapter 2, in part is currently being prepared for submission for publication of the material. Deason, Nicholas; Li, Zheng; Chen, Peiyun; Vegesna, Neeraja; Law, Julie A.; Doherty, Colleen; Pruneda-Paz, Jose L. The dissertation author was the primary researcher and author of this material.

Chapter 3 contains unpublished material coauthored with Li, Zheng; Chen, Peiyun; Vegesna, Neeraja; Law, Julie A.; Doherty, Colleen, and Pruneda-Paz, Jose L. The dissertation author was the primary author of this chapter.

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PUBLICATIONS

1. B. St Laurent, N. Harding, N. Deason, K. Oy, C. Sok Loeun, M. Sary, R. Sunly, S. Nhep, E. Drury, K. Rockett, S. Sovannaroeth, S. Goncalves, D. Kwiatkowski, A. Miles, Population genomics reveal distinct and diverging populations of *An. minimus* in Cambodia. *Communications Biology* **5**, 1308 (2022). doi: 10.1038/s42003-022-04259-y.
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FIELD OF STUDY

Major Field: Biological Science
Studies in Cell and Developmental Biology
Professor Jose L. Pruneda-Paz

ABSTRACT OF THE DISSERTATION

Organ-specific mechanisms regulate circadian clock function across the plant shoot system

by

Nicholas Deason

Doctor of Philosophy in Biology

University of California San Diego, 2024

Professor Jose L. Pruneda-Paz, Chair

The circadian clock is an internal timing mechanism that allows organisms to anticipate and respond to predictable changes in their environment throughout the day and across the year. Circadian processes are particularly important for plants because they are sessile organisms that must adjust their physiology to accommodate the changing world around them. This requires precise coordination of rhythmic gene expression across cells, tissues, and organs of the plant.

However, more research is needed to understand the complexity of spatial clock regulation in plants. Here, we present a survey of the circadian transcriptomes of two major organs of the *Arabidopsis* shoot system: the shoot apex and the leaves (Chapter 2). Our comparative analysis revealed that there are unique rhythmic gene sets in each organ, and that rhythmic genes tend to reach their peak expression earlier in the shoot apex. The notion of organ-specific phase regulation was bolstered by the identification of a shoot apex-enriched transcription factor that regulates the timing of expression of the core clock component CCA1. Ultimately, we formulated a model of the circadian clock architecture in which organ-specific mechanisms modulate the phase of rhythmic genes through upstream regulation of the core clock, while the amplitude of rhythmic expression is controlled independently in each organ downstream of the core clock. Our model was further supported by the results of an organ-level transcriptome survey of the shoot system following a single leaf bacterial infection (Chapter 3). We found that the phase of clock components and of rhythmic gene expression more broadly was altered by the distal infection. This, combined with the observation that infection alters the makeup of organ-specific rhythmic gene sets, reinforces our assertion that phase is controlled upstream of the clock, and amplitude is controlled downstream of the clock, in an organ-specific manner. Organ-specific control of rhythmic gene expression may allow for precision tuning of clock-regulated processes in response to intra- and inter-organ signals, integrating microenvironmental information with internal rhythms in order to balance the needs of growth and reproduction with responses to stressors like pathogen attacks.

Chapter 1 INTRODUCTION TO THE DISSERTATION

Each day, life on Earth is subjected to dramatic, but predictable, environmental fluctuations resulting from the rotation of the planet on its axis. Throughout evolutionary history, organisms have repeatedly evolved a strategy to anticipate and respond to these fluctuations via an internal timekeeping mechanism called the circadian clock (reviewed by Bell-Pederson *et al.* (1)). Questions of how the circadian clock is built and maintained are the focus of the field of chronobiology, which has its roots in an 18th century work by Jean-Jacques d'Ortous de Mairan. De Mairan observed that the daily rhythm of leaf movement in the sensitive plant *Mimosa pudica* was maintained even when the plants were moved to constant darkness (2), foreshadowing the eventual discovery of an internal timing mechanism. However, it is only in recent decades that scientists have begun to uncover the molecular-genetic details of these mechanisms and how they coordinate physiology accordingly. Numerous studies have shown that a functioning circadian clock can enhance fitness and survival in lifeforms as diverse as cyanobacteria (3-5), fungi (6, 7), plants (8, 9), and animals (10-13), and dysregulation of the clock has even been linked to human disease (14-16). Intriguingly, all known eukaryotic circadian clocks share a common fundamental architecture of time-phased, rhythmically expressed components that form a transcriptional-translational feedback loop (17). This suggests that evolution has converged on a solution common in function, though diverse in form, to a common problem (18). Therefore, discoveries in the mechanisms of timekeeping in one organism may provide useful insight to the field of chronobiology as a whole (1).

Plants were foundational model organisms for the discovery of circadian clocks, and critical breakthroughs are still being made in plant circadian biology today (reviewed in (19,

20)). The circadian clock is involved in nearly all aspects of plant physiology: from controlling growth and development (21) to photosynthesis and photoperiodic flowering (22, 23), with implications for agriculture, climate change, and beyond (24-26).

The current model of plant circadian clock function, based mostly on studies using *Arabidopsis thaliana* (Arabidopsis), consists of (1) input pathways that transduce environmental information to a core clock network, (2) the core clock, a transcriptional-translational feedback loop that coordinates endogenous rhythms, and (3) output pathways that result in physiological processes with approximately 24 hour rhythms (Figure 1.1A, adapted from (27)). The most important inputs to clock function are fluctuations in light and temperature as sensed by cryptochrome and phytochrome proteins, although uncovering the pathways through which these inputs are translated to directly regulate clock function is still an active area of research (28, 29). The outputs of the clock include the physiological processes previously mentioned, as well as others such as cell division and protein modification. In addition, information can flow bidirectionally from the clock leading to the circadian gating of sensitivity to inputs and magnitude of responses.

The molecular core clock network that underlies this functionality can be described generally as being composed of an inter-linked transcriptional-translational feedback loop built around a central repressilator (30), which is simplified and summarized in Figure 1.1B. Briefly, the transcription factors (TFs) LIGHT REGULATED WD 1 and 2 (LWD1/2) along with TCP DOMAIN PROTEINS 20 and 22 (TCP20/22) promote the morning-phased expression of CIRCADIAN CLOCK ASSOCIATED 1 and LATE ELONGATED HYPOCOTYL (LHY) (31). High CCA1/LHY levels lead to the indirect promotion of PSEUDORESPONSE REGULATORS (PRRs) 9 and 7, which reciprocally represses *CCA1* and *LHY* (32). Meanwhile, in the “central

loop” of this network, CCA1 and LHY repress the expression of their own transcript as well as that of *TIMING OF CAB EXPRESSION 1 (TOC1*, also known as *PRR1*) until CCA1/LHY levels decline enough that TOC1 levels subsequently begin to rise (33, 34). TOC1 further represses *CCA1/LHY* and TOC1 interaction with CHE enhances repression of *CCA1* in particular (35, 36). During the night, TOC1 is degraded by ZEITLUPE (ZTL) after ZTL dissociates from GIGANTEA (GI) (37, 38). Then, the multi-unit evening complex (EC, composed of EARLY FLOWERING 3 and 4 (ELF3/ELF4) and alternately LUX ARRHYTHMO (LUX) or BROTHER OF LUX ARRHYTHMO (BOA)) represses *PRRs*, which in conjunction de-repress *CCA1/LHY* as morning approaches again (39-42). Other identified components of the circadian clock network include COLD RESPONSIVE 27 and 28 (COR27/28), which inhibit expression of *TOC1* and *PRR5*, and members of the REVEILLE (RVE) and NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED GENES (LNK) protein families, which promote the expression of the EC, *PRRs*, and *GI* (43-47). Ultimately, rhythmic signals from the oscillating clock network are transduced throughout the transcriptome, controlling the daily cycle of gene expression, and indirectly, protein function and physiology.

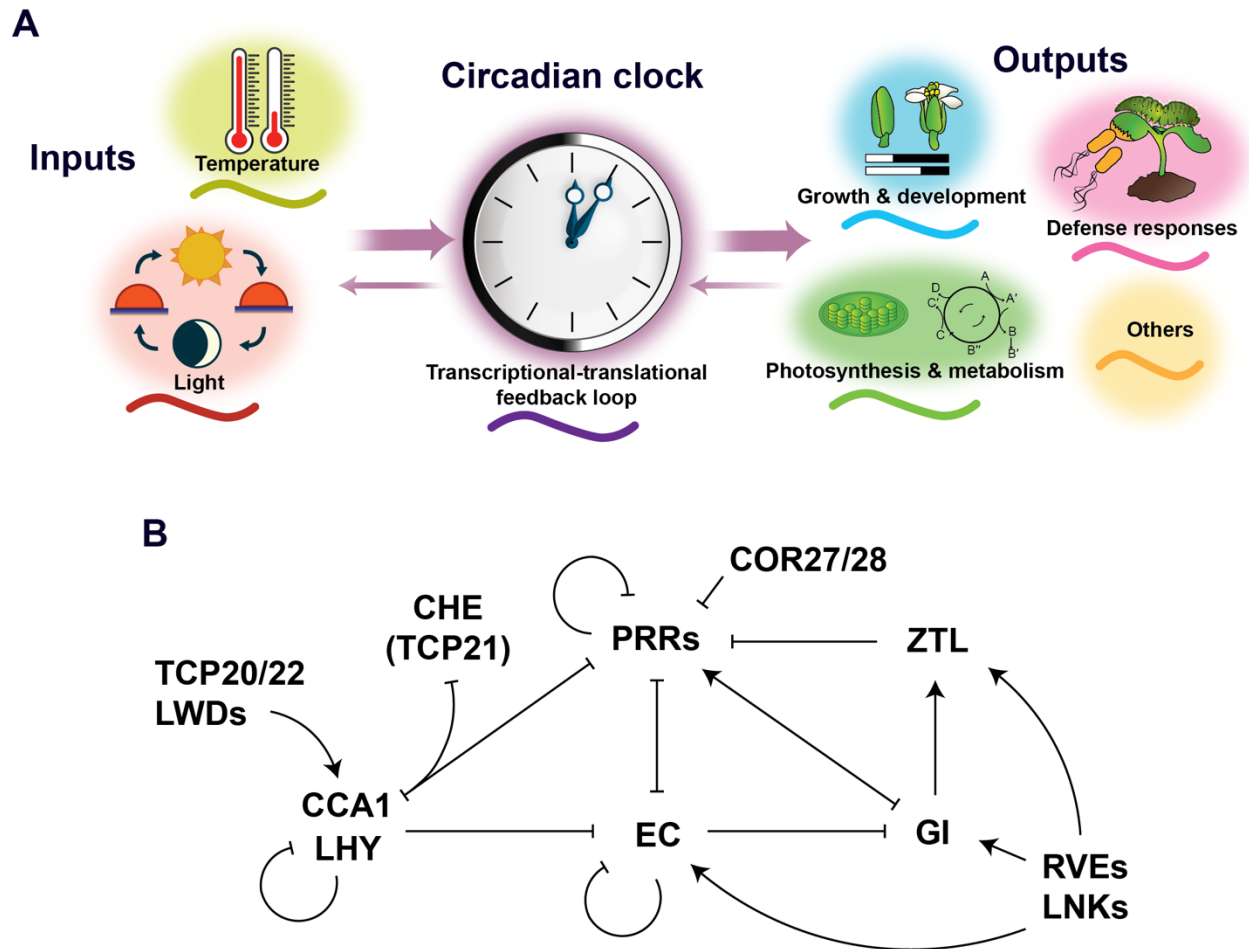


Figure 1.1. The circadian clock is a transcriptional-translational regulatory network that is entrained by the environment and controls rhythmic physiology.

(A) The plant circadian clock is entrained by rhythmic signals from the environment, namely the daily fluctuations in light and temperature. After entrainment, the circadian clock mechanism can drive rhythmic outputs even after entrainment cues are removed. Rhythmic outputs include growth and development (including flowering), metabolism, and defense responses, among many others. Bidirectional arrows to and from the clock indicate that information can flow both ways, leading to time-restricted sensitivity and response modulation (circadian gating). Figure adapted from (27). (B) Simplified core clock network, summarized from (25, 27, 30). Pointed arrows indicate positive input and blunt arrows indicate negative input.

The clock network regulates gene expression at the level of single plant cells, yet the organism must coordinate rhythmic gene expression across time and space for proper physiology and development (21). Discrepancies in the abundance of certain clock components have been

reported in different cell types (48), and it has been proposed that clocks in neighboring cells are loosely coupled together (49-51). In addition, some studies suggest that there is long-distance communication of circadian signals across tissues and organs of the plant (52, 53). However, more studies of spatial variation in clock entrainment and regulatory output are required in order to gain a fuller picture of the coordination of circadian rhythms across the organism.

Interestingly, coordination of circadian clock function across the plant is controlled not only by predictable daily fluctuations in the environment but is also influenced by more acute abiotic and biotic stressors. Studies have shown that *Arabidopsis* exhibits spatially-specific responses to extreme temperatures (54-59), osmotic and drought stress (54, 55, 59, 60), wounding and pathogen elicitors (55, 59), and more (61, 62). Furthermore, many of these responses intersect with the regulatory network of the circadian clock (55, 63-65). Of particular note, Li *et al.* (65) showed that a localized, single-leaf infection by *Pseudomonas syringae* pathovar *tomato* (*Pst*) DC3000 caused a lengthening of the circadian period and reduction of rhythmic amplitudes across the shoot system, even in distal uninfected tissues. Moreover, this localized infection caused major growth defects and delayed flowering, likely through disruption of the circadian clock in the shoot apex (66). This highlights the nuanced interplay between organ-specific stress responses and regulation of the circadian clock and underscores the need for further study of these complex phenomena.

To examine the architecture of circadian clock networks across organs, we turned to the plant shoot system, which provides an excellent model for studying spatial variation in circadian gene expression. Anatomical structures like the shoot apex and leaf can be close in proximity, but have highly specialized roles – for example, cell and organ differentiation in the shoot apex, and photosynthesis and gas exchange in the leaf. Each of these processes is tightly regulated by

the circadian clock, which itself appears to operate differentially across the shoot system. We hypothesized that spatial heterogeneity in clock function may be the result of organ-specific modulators of the core clock network, so we used a comparative transcriptomics approach to first characterize rhythmic gene expression in shoot apex and leaf and then guide our search for a spatially-restricted clock regulator. Using the model plant *Arabidopsis*, we unveiled the architecture and molecular components of the regulatory circuits underlying organ-specific regulation of the circadian clock, work which is discussed in Chapter 2.

In parallel, we performed a study of the shoot apex and leaf transcriptomes from plants that were subjected to a localized single-leaf infection of *Pst* DC3000. The collected organ samples were distal to the infection site and so were able to reveal the systemic and organ-specific effects of the localized pathogen exposure. We found that organs distal to the infection site were greatly affected by the localized treatment, with alterations in both gene expression levels and rhythmicity. These sets of genes revealed both known and novel associations with biological process ontologies and transcriptional regulatory motifs. From these results we conclude that a localized single-leaf bacterial infection has systemic and organ-specific effects on gene expression. These findings are presented in Chapter 3.

In summary, our work provides new insight on the complex organ-level regulation and coordination of the circadian clock and its transcriptional outputs across the shoot system in both basal and biotic stress conditions. Our findings set the foundation for understanding how circadian clocks are fine-tuned for timing organ-specific functions, including those that may allow plants to decide when and how to reproduce (Chapter 2) and respond to pathogens (Chapter 3). Ultimately, this understanding may lead to improvement of crop productivity through optimizing and exploiting the role of the plant circadian clock.

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Chapter 2 ORGAN-SPECIFIC MECHANISMS REGULATE CIRCADIAN CLOCK FUNCTION ACROSS THE PLANT SHOOT SYSTEM

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Abstract

The circadian clock is an internal timing mechanism common to most forms of life that drives ~24 hour rhythms in physiology and behavior, thus optimizing organismal fitness in a diurnally oscillating environment. The clock function is uniquely regulated across organs; however, our understanding of the molecular mechanisms underlying spatial specificity is incomplete. Here, we present a comparative analysis of clock-regulated transcriptomes from the shoot apex and leaves of *Arabidopsis* plants. We show that rhythmic genes are largely distinct between these two major organs of the plant shoot system and tend to reach their peak expression first in the shoot apex. Furthermore, our results reveal a core clock composition common to both organs and that organ-specific rhythmic gene expression arises from differences in clock input and output pathways. Building on this schema, we identified a shoot apex-specific regulator of the clock phase and found that organ-specific phase regulation provides previously unrecognized control of photoperiodic flowering.

Main text

Life on Earth has repeatedly evolved an internal timekeeping mechanism called the circadian clock that enhances fitness by synchronizing growth and physiology with daily fluctuations in the environment (1-3). In many organisms the clock runs with variable period, amplitude, and phase across organ structures, and it can even regulate the expression of spatially-specific subsets of rhythmic genes (4-7). However, the circadian clock function is generally cell-intrinsic and it is unclear if and how a conserved core clock machinery generates rhythmic specificity across diverse tissues and organs (5, 6, 8). To investigate the mechanisms behind spatial variation in circadian gene expression, we turned to the shoot system of the model plant *Arabidopsis thaliana* (Arabidopsis). In the shoot system, the clock operates differentially between organs in close proximity (7, 9-11) and therefore is likely modulated by local mechanisms. Furthermore, shoot organ structures have specialized functions intertwined with the circadian clock – for example, early cell and organ differentiation in the shoot apex, and photosynthesis and gas exchange in the leaves – which provides a powerful context to explore the biological relevance of locally regulated circadian rhythms. Therefore, we performed a comparative analysis of the rhythmic transcriptomes of the shoot apex and leaves of Arabidopsis plants. Our results indicated that organ-specific regulators of clock input and output pathways drive profound differences in rhythmic gene sets and their associated biological functions between organs. We used this model to guide a search for spatially restricted clock regulators and subsequently identified a previously unrecognized shoot apex-specific transcription factor that locally modulates the clock function and influences photoperiodic flowering. This work provides a basic architecture of the circadian system across plant organs, which may illuminate how spatial heterogeneity of rhythmic processes is achieved in other organisms.

Shoot apex and leaves have distinct circadian clock-regulated transcriptomes

To determine the extent of circadian clock transcriptional regulation in major organs of the plant shoot system, we performed time course mRNA sequencing (mRNA-seq) of shoot apices and leaves obtained from *Arabidopsis* seedlings grown under free-running conditions (Figure 1.1A). After verifying the quality of mRNA-seq data from independent replicate experiments (Supplemental Figure 1.1, A to D and Supplemental Table 2.1), we assessed the overlap in expressed gene sets between organs (Figure 1.1B and Supplemental Table 2.2). We found that most transcripts were expressed in both organs (13,536 out of 17,119, or 79%); however, among spatially-restricted transcripts ($n = 3,593$), more were present in the shoot apex ($n = 2,916$) than in the leaves ($n = 677$) (Figure 1.1B). We then identified which transcripts showed a circadian expression pattern and found that overall 29% ($n = 4,934$) were rhythmic in either organ (Supplemental Figure 1.2A and Supplemental Table 2.3), which is consistent with the proportion of rhythmic genes observed in previous whole plant transcriptomic studies (12, 13). However, the majority of these transcripts were rhythmic in only one organ ($n = 530$ in shoot apex only and $n = 3,596$ in leaves only, versus $n = 808$ in both), indicating that clock-regulated transcriptomes in the shoot apex and leaves were largely distinct (Figure 1.1C and Supplemental Table 2.3). This observation was not biased by the chosen thresholds for expression or rhythmicity, as varying these cutoff values had little effect on the relative sizes of rhythmic gene sets in one or both organs (Supplemental Figure 1.2, B to D). Interestingly, organ-specific rhythmicity was not a consequence of organ-specific expression, as the majority of transcripts that were rhythmic exclusively in the shoot apex or the leaves were actually expressed in both (3,781 out of 4,126, or 91.6%) (Figure 1.1D). In fact, most of these transcripts exhibited less than a twofold difference in abundance between the shoot apex and leaves (Figure 1.1E).

Likewise, organ-specific rhythmicity was not the result of an inherent difference in the robustness of oscillations between organs, as the amplitudes (half the peak to trough magnitude) of rhythmic gene waveforms showed similar distributions in the shoot apex and leaves (Figure 1.1F and Supplemental Figure 1.2E). Taken together, these results indicated that the distinct rhythmic transcriptomes of the shoot apex and leaves are not the result of organ-specific differences in mean expression levels or an inherent disparity in the overall robustness of clock-controlled oscillations in one organ. Rather, organ-specific transcript rhythmicity appears to be the result of damped oscillations in unique gene subsets depending on organ context (Figure 1.1G).

Next, to gain insight into the temporal coordination of the rhythmic transcriptome across the shoot system, we evaluated the phase (time of peak expression) of rhythmic gene waveforms. Genes that were rhythmic only in the shoot apex had an earlier phase on average than genes that were rhythmic only in the leaves (Figure 1.1H). To explore if organ- or gene-specific mechanisms regulate the temporal separation of rhythmic expression, we turned to the subset of genes rhythmic in both the shoot apex and leaves. Interestingly, we found that genes rhythmic in both organs also peaked earlier in the shoot apex (Figure 1.1I). In addition, a direct comparison of phases between organs for each of these genes (rhythmic in both organs) showed that the majority (614 out of 808, or 76%) had an earlier phase in the shoot apex (Figure 1.1J). These results showed a congruency in the phase offset between the shoot apex and leaves for each rhythmic gene, suggesting that organ-wide mechanisms likely regulate the timing of peak expression in each organ.

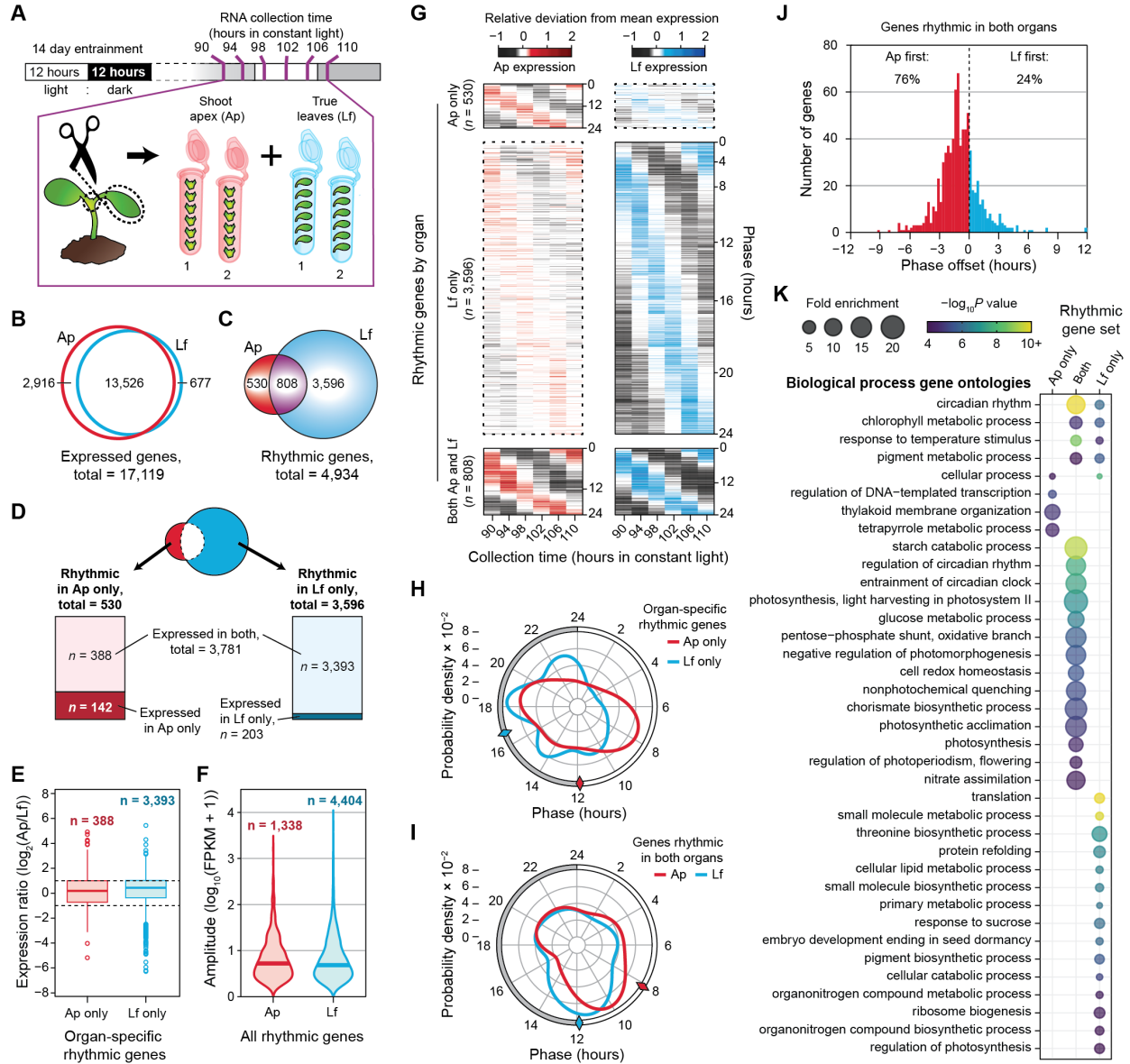
Finally, to identify physiological functions subject to organ-specific circadian regulation, we performed gene ontology (GO) enrichment analyses for biological processes associated with

genes that were rhythmic in one or both organs (Figure 1.1K, Supplemental Figure 1.3, and Supplemental Table 2.4). Broadly, genes rhythmic in the shoot apex were enriched for terms related to chloroplast development, while genes rhythmic in the leaves were associated with metabolism, protein synthesis and other biosynthetic pathways. Notably, genes rhythmic in both organs were enriched in circadian rhythm-related terms but were also linked to energy production and nutrient assimilation, perception of light and temperature, and photoperiodic flowering responses. These findings underscore the intricate regulation of circadian rhythms across the Arabidopsis shoot system, reflecting that some biological functions rely on organ-specific clock regulation, while others are rhythmically coordinated between organs.

Our analyses revealed that circadian gene expression is dependent on organ context. Most rhythmic genes oscillated exclusively in the shoot apex or the leaves despite being expressed at similar levels in both organs. While there were fewer rhythmic genes detected in the shoot apex than in the leaves, the amplitude distributions were similar between organs, suggesting that the robustness of clock-regulated oscillations was not a distinguishing factor between the two transcriptomes. Instead, we conclude that the features of circadian gene expression that characterize the two organs are the identities of specific sets of rhythmic genes (along with their associated biological processes), and the timing of their expression. These findings imply the existence of organ-specific mechanisms that independently control the amplitude and phase of rhythmic gene expression across the plant shoot system.

Figure 2.1. Shoot apex and leaves have distinct circadian transcriptomes.

(A) Schematic of the experimental conditions used for time course RNA collection from shoot apex (Ap) and leaves (Lf). Tubes 1 and 2 represent two biologically independent replicate experiments. (B and C) Numbers of genes categorized by their organ(s) of expression (B) and their organ(s) of rhythmic expression (C). (D) Numbers of organ-specific rhythmic genes categorized by their organ(s) of expression. (E) Ratios of mean gene expression (Ap/Lf) for genes expressed in both organs but rhythmic in only one. Boxplots show the interquartile range (IQR) (box) and median value (horizontal colored line) of each distribution with whiskers extending to $\pm 1.5 \times \text{IQR}$ (outliers outside whiskers are represented by individual symbols). Dashed lines bound the region with <2 -fold difference in expression between organs. (F) Amplitude distributions for rhythmic genes in each organ. Horizontal bars indicate the median. (G) Heatmap of relative transcript levels over the time courses for genes rhythmic in Ap, Lf, or both. Each row represents one gene (ordered by ascending phase in the rhythmic organ). (H and I) Density distributions of peak expression phases for genes rhythmic in only one (H) or both (I) organs. Colored diamonds indicate circular mean phase values. (J) Histogram of phase offsets ($\text{Phase}_{\text{Ap}} - \text{Phase}_{\text{Lf}}$) for each gene rhythmic in both organs (bin width = 15 min.). (K) Semantically representative biological process ontologies enriched in rhythmic gene sets. White and gray shaded boxes in (A), (H), and (I) denote subjective day and night periods, respectively.



A basic clock network common across the shoot system is phase-advanced in the shoot apex

We hypothesized that the observed differences in circadian gene expression between the shoot apex and leaves could be the result of distinct clockwork in each organ. To determine whether there were disparities in the composition of the circadian clock between organs, we examined the mean expression levels of 27 known clock components (14) in the shoot apex and leaf mRNA-seq datasets (Figure 1.2A and Supplemental Table 2.5). Remarkably, almost all of these clock genes (25 out of 27), including the core components *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, *LATE ELONGATED HYPOCOTYL (LHY)* and *TIMING OF CAB EXPRESSION 1 (TOC1)*, were expressed in both organs (only *PSEUDORESPONSE REGULATOR 9 -PRR9-* and *REVEILLE 6 -RVE6-* showed exclusive – but low – expression in leaves). Moreover, most of the clock components expressed in both the shoot apex and leaves (23 out of the 25 mentioned above) showed less than a twofold difference in transcript abundance between organs (only *NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED 3* and *4 -LNK3/4 -* expression in leaves was more than twofold higher than in the shoot apex) (Figure 1.2A). Thus, most known clock components were equivalently expressed in both the shoot apex and leaves reflecting a basic conserved structure of the clock mechanism across the shoot system.

Next, we evaluated organ-level differences in the circadian expression pattern of clock components. Of the 27 clock genes that we considered, 22 were previously shown to be expressed in a circadian manner in whole plant transcriptome surveys (12). We found the same 22 to be rhythmic in our dataset, with most of them (19 out of 22) expressed rhythmically in both organs (Supplemental Table 2.5). No detectable circadian expression was observed in either organ for the remaining five clock components that were previously found to be non-

rhythmically expressed in whole plants (12). Moreover, most rhythmic clock genes (17 out of 19) showed less than a twofold difference in amplitude between organs (Figure 1.2B). Three clock genes (*PRR9*, *TCP DOMAIN PROTEIN 22 -TCP22-*, and *CCA1 HIKING EXPEDITION -CHE/TCP21*) were rhythmic only in the leaves. However, *PRR9*, *TCP22* and *CHE* control the clock function via regulation of *CCA1* and *LHY* (among others) (15-17) which had equivalent expression levels and amplitudes between the shoot apex and leaves. Thus, organ-specific differences in *PRR9*, *CHE* and *TCP22* mRNA expression patterns may not play a significant role in the spatial regulation of clock gene expression. Remarkably, while almost all rhythmic clock genes – particularly those central to the core clock mechanism, such as *CCA1*, *LHY* and *TOC1* – exhibited similar expression amplitudes in both organs, there was a marked offset in their peak expression timing between the shoot apex and leaves. Of the 19 clock genes rhythmic in both organs, 17 had an earlier phase of expression in the shoot apex than in the leaves (Figure 1.2C). This pattern was a striking recapitulation of the earlier shoot apex peak expression phases that we found broadly in the organ-level circadian transcriptomes (shown in Figure 1.1, H to J), indicating that the phase offset between organs could be first established in the clock itself and then propagated to its target genes. To explore this idea, we performed motif enrichment analyses to search for signatures of transcriptional regulation in the promoter regions of genes rhythmic either in the shoot apex or in the leaves. We found that the promoters of both rhythmic gene subsets were highly enriched with two cis-elements, both of which are well-established circadian regulatory motifs: the evening element (EE) AAAATATCT, which is bound by the core clock components *CCA1*, *LHY*, and *RVE* family members (18-21), and the G-box motif CACGTG, which is linked to regulation by *TOC1* and related *PRRs* (22-24) (Figure 1.2D).

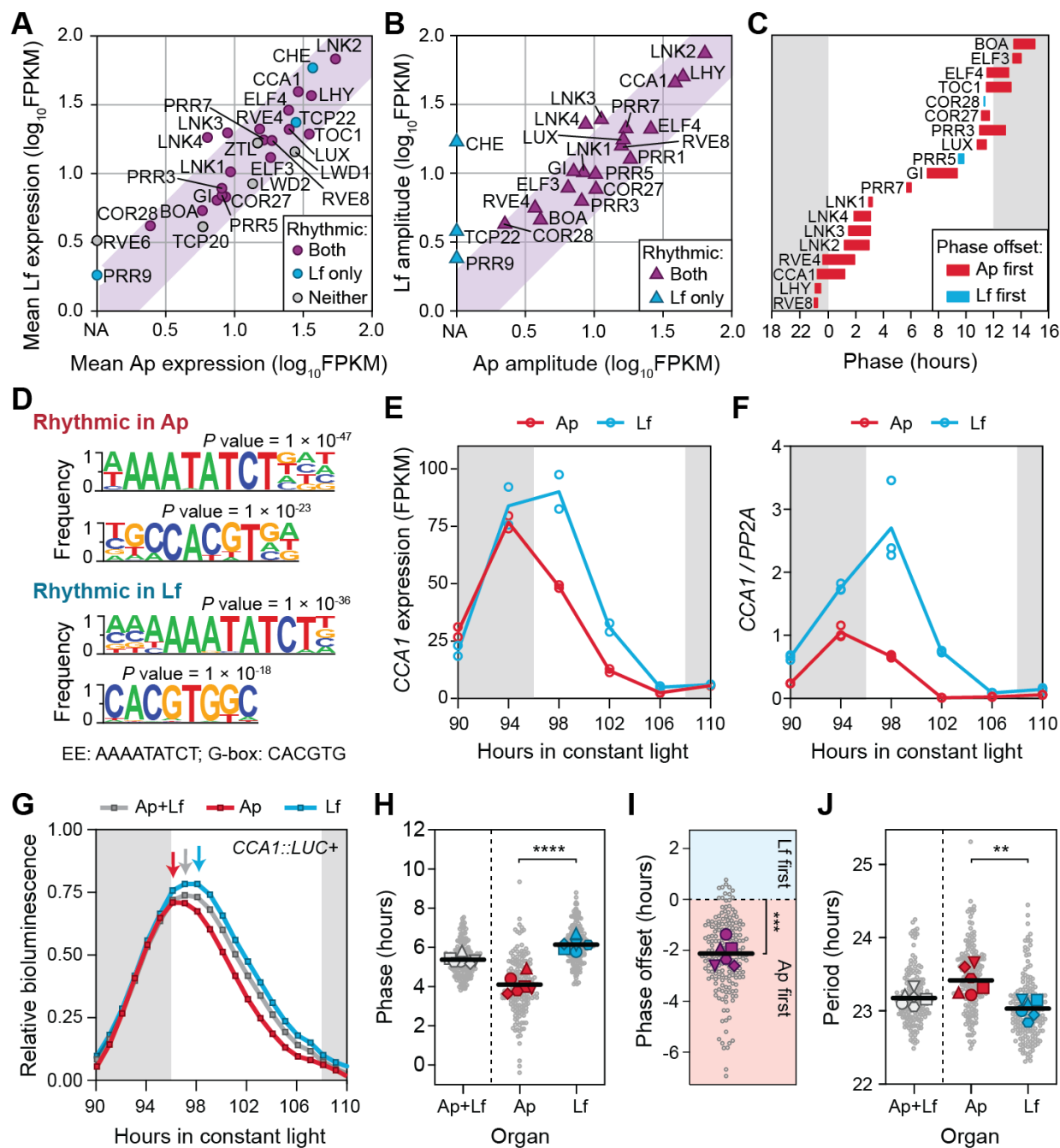
Together, these results indicate that a conserved clock structure drives circadian rhythms in the shoot system, although with an earlier phase in the shoot apex. The similar amplitudes of rhythmically expressed clock genes between the shoot apex and leaves contrasts with the finding that most clock-regulated genes are rhythmic in only one organ, suggesting that rhythmicity may be ultimately dictated by clock-independent mechanisms present in each organ. On the other hand, rhythmically expressed clock genes are consistently earlier phased in the shoot apex, resembling the overall phase distributions of clock-regulated genes (shown in Figure 1.1, H and I). This suggests that the timing of rhythmic gene expression may be dictated by the circadian clock itself. Thus, our results are consistent with a model in which organ-specific rhythmic gene sets are determined by regulatory mechanisms downstream of the core clock, whereas the timing of rhythmic gene expression is ultimately controlled by upstream mechanisms that set the phase of the core clock in each organ.

We used this model as a basis for further exploration of organ-specific circadian regulation, reasoning that it would be more promising to search for organ-specific regulators of the clock phase rather than components of a multifaceted downstream network of amplitude modulators. Therefore, we focused our subsequent analyses on the core clock component *CCA1* because it binds to the EE motif that was enriched among rhythmic gene promoters (shown in Figure 1.2D) and was also previously shown to be critical for setting the phase of the circadian clock in whole plants (25). First, we independently measured *CCA1* mRNA levels and validated that its expression peak was earlier in the shoot apex than the leaves (Figure 1.2, E and F), as previously indicated by our mRNA-seq data analyses. Then, we measured the *CCA1* promoter activity in an Arabidopsis luciferase reporter line (*CCA1::LUC+*) and found that *CCA1::LUC+* rhythms were similarly robust in both organs (Figure 1.2G and Supplemental Figure 1.4) and,

consistent with *CCA1* transcript expression pattern, peaked first in the shoot apex (Figure 1.2, H and I). Notably, the period of *CCA1::LUC*+ expression was slightly longer in the shoot apex compared to leaves (Figure 1.2J), ruling out the possibility that the earlier phase in the shoot apex was driven by period differences. In conclusion, we confirmed the phase offset between shoot apex and leaves using measures of transcriptional control of a central clock gene, *CCA1*, that is essential for proper timing of circadian rhythms. This pointed to *CCA1* as a potential regulatory target of unrecognized organ-specific phase regulators.

Figure 2.2. Circadian clock components are similarly expressed across the shoot system but are earlier-phased in the shoot apex.

(A and B) Correlation of mean expression (A) and amplitude (B) for core clock transcripts in the shoot apex (Ap) and leaf (Lf). Shaded color background indicates the region with <2-fold difference between organs and “NA” indicates that gene expression (A) or rhythmicity (B) was below threshold in the corresponding organ. (C) Phase offsets ($Phase_{Ap} - Phase_{Lf}$) for core clock genes rhythmic in both organs. Bars span the shortest circular distance between the phases in Ap and Lf, and are colored according to the organ of earlier phase. (D) Sequence logos for significantly enriched motifs in the promoter regions of rhythmic genes in each organ. Consensus sequences for the evening element (EE) and G-box motifs are shown below. (E and F) Time course of *CCA1* expression determined by mRNA-seq (E) and quantitative polymerase chain reactions (normalized to *PP2A*) (F). Open circles indicate replicate measurements and solid lines connect average values. (G) Bioluminescence analysis of *CCA1::LUC+* expression in intact plants considering Ap and Lf together (Ap + Lf) or separately (Ap, Lf). Data show average values ($n = 26$) from one out of six independent experiments (represented in H to J). Arrows indicate the peak expression within the plotted timeframe. (H to J) Phase (H), phase offset (I) and period (J) of *CCA1::LUC+* expression in intact plants as indicated in (G). Plots show mean values (black bars) of independent experimental averages (large colored symbols with shapes corresponding to each experiment, $n = 6$). Values for individual seedlings are shown by the gray symbols ($n = 181$). Statistical comparisons were performed with experimental averages ($n = 6$) using t-tests paired by experiment in (H) and (J) (** $P < 0.01$, **** $P < 0.0001$) or a one sample t-test comparing to 0 in (I) (*** $P < 0.001$). White and gray shaded background regions in (C), (E), (F) and (G) indicate subjective day and night periods, respectively.



TCP19 is a shoot apex-specific regulator of *CCA1*

It was previously shown that the timing of *CCA1* expression is key to setting the clock phase in whole plants (25). Given that *CCA1* expression peaked earlier in the shoot apex (Figure 1.2H), we posited that organ-specific input pathways regulating *CCA1* could provide transcriptional control of the phase of shoot apex and leaf clocks. To test this idea, we interrogated our transcriptomic dataset and identified 302 transcription factors (TFs) that were enriched either in the shoot apex ($n = 270$) or leaves ($n = 32$) (Figure 1.3A and Supplemental Table 2.6). Of these, we obtained yeast expression plasmids for 254 (26) and measured their ability to interact with the *CCA1* promoter using a yeast one-hybrid (Y1H) system (27). This analysis yielded three shoot apex-enriched TFs, none of which were previously recognized as clock regulators: TCP DOMAIN PROTEIN 19 (TCP19), AINTEGUMENTA-LIKE 5 (AIL5) and DNA-BINDING ONE ZINC FINGER 1.8 (DOF1.8) (Figure 1.3B and Supplemental Figure 1.5, A to C). Notably, the top candidate from this screen, TCP19, was the only organ-enriched member of the class-I TCP family (Supplemental Table 2.2), from which three members were previously shown to regulate *CCA1* expression (TCP20, CHE/TCP21 and TCP22) (15, 16). Further DNA binding experiments confirmed that TCP19 interacts with the class-I TCP-binding site (TBS-I) in the *CCA1* promoter (16) through its N-terminal region which contains the conserved TCP DNA-binding domain (Figure 1.3C). We therefore decided to test the hypothesis that TCP19 is a shoot apex-specific regulator of *CCA1*. For that, we first independently evaluated the shoot apex expression of *TCP19* using Arabidopsis lines that expressed the β -glucuronidase (GUS) reporter under the control of the *TCP19* promoter (*TCP19::GUS*). GUS histochemical

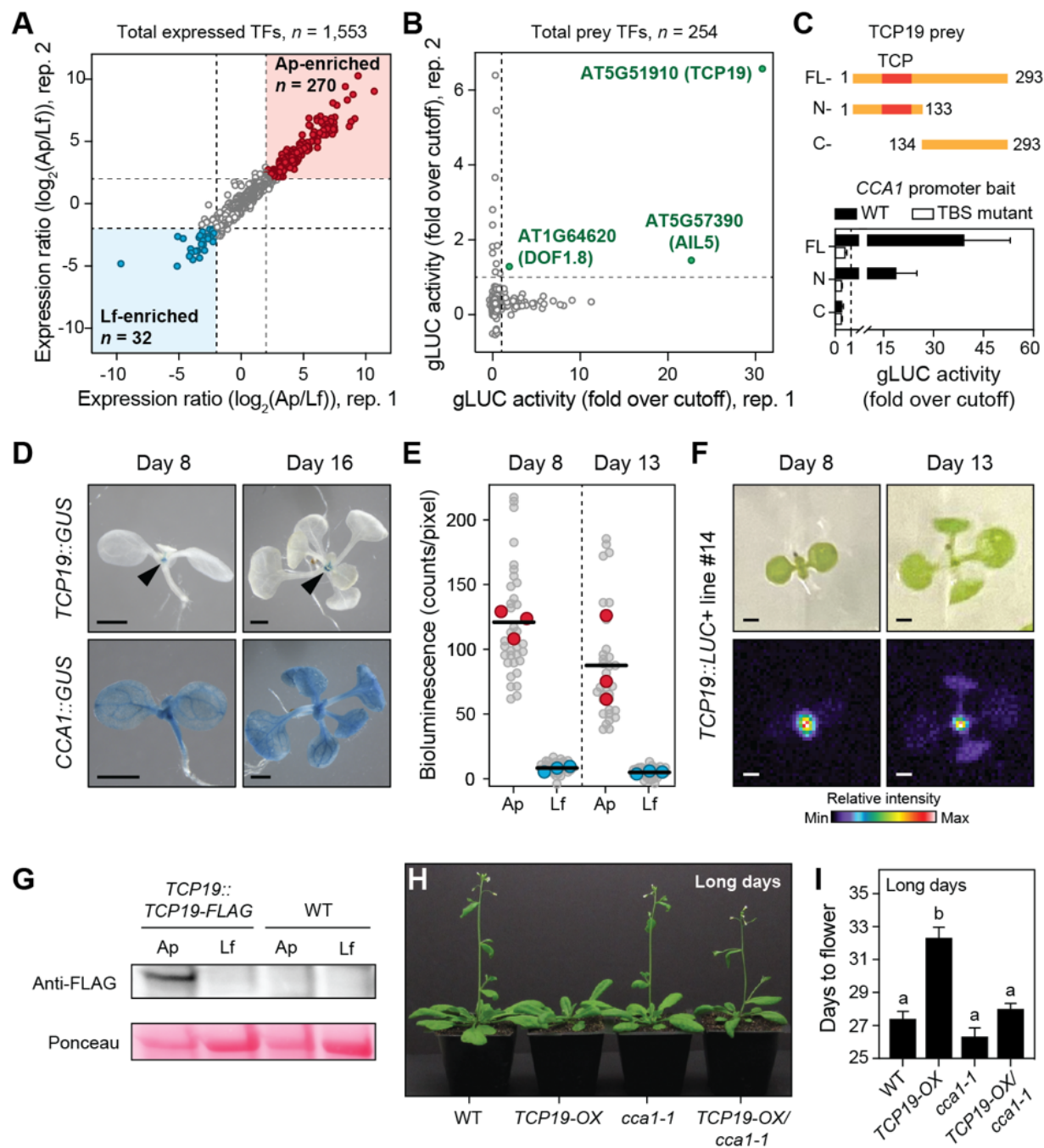
staining performed with these plants showed that the *TCP19* promoter activity was restricted to the shoot apex (Figure 1.3D and Supplemental Figure 1.6A). Importantly, *CCA1::GUS* lines processed in parallel showed that the *CCA1* promoter was active in the same shoot apical region as *TCP19*, as well as the plant shoot system more broadly (as previously shown (16)) (Figure 1.3D and Supplemental Figure 1.6B). In support of these findings, stable transgenic lines expressing the *LUC+* reporter under the control of the *TCP19* promoter (*TCP19::LUC+*) emitted bioluminescence primarily from the shoot apex region (Figure 1.3, E and F, and Supplemental Figure 1.7, A to D), while *CCA1::LUC+* plants luminesced broadly across the shoot system (Supplemental Figure 1.7, A to D). Moreover, time course imaging experiments showed that *TCP19::LUC+* activity from the shoot apex accounted for nearly all the bioluminescence detected from whole plants over several days, confirming the organ-specific expression of *TCP19* and ruling out daily changes in its spatial expression pattern (Supplemental Figure 1.8). Finally, we analyzed *TCP19* protein levels from plants expressing FLAG-tagged *TCP19* under the control of the *TCP19* promoter (*TCP19::TCP19-3xFLAG*) and found that *TCP19* protein followed the same shoot apex-specific expression pattern observed in *TCP19* promoter activity and transcript levels (Figure 1.3G).

Next, we reasoned that if *TCP19* regulates *CCA1*, then *TCP19* misexpression might phenocopy flowering time defects documented in plants carrying *CCA1* knockout or overexpression alleles (28, 29). Indeed, constitutive *TCP19* overexpression (*CaMV-35S::TCP19*; *TCP19-OX* line #19) (Supplemental Figure 1.9) resulted in a significant flowering time delay compared to wild-type controls in long days (16 hours light:8 hours dark) (Figure 1.3, H and I). Remarkably, the late flowering phenotype was reverted when *TCP19-OX* plants also carried the knockout *cca1-1* allele (Figure 1.3, H and I), indicating that *TCP19* regulates flowering time in a

CCA1-dependent manner. Altogether, these experiments confirmed our initial hypothesis that *CCA1* expression could be regulated at the organ-specific level by TFs that directly target the *CCA1* promoter. In particular, we singled out TCP19 as a shoot apex-specific regulator of *CCA1* and found that TCP19 regulates flowering time through *CCA1*, thus suggesting a broader implication of TCP19 in clock control.

Figure 2.3. TCP19 is a shoot apex-specific regulator of CCA1.

(A) Correlation of expression ratios (shoot apex (Ap) / leaf (Lf)) for transcription factors (TFs) in each replicate mRNA-sequencing datasets. Each symbol denotes one TF ($n = 1,553$). Colored background regions and symbols indicate expression enrichment >4-fold in both replicates. (B) Gaussia luciferase (gLUC) reporter activity from two independent yeast one-hybrid (Y1H) experiments testing the interaction of Ap- and Lf-enriched TFs ($n = 254$) with the *CCA1* promoter. Each symbol denotes one TF-bait combination, with green symbols and names indicating those above the interaction cutoff (shown by the dashed lines) in both experiments. (C) Interaction of full length (FL), N-terminal (N) or C-terminal (C) TCP19 protein fragments (orange bars with amino acid residue numbers, TCP domain in dark orange) with wild-type or class-I TCP binding site (TBS-I) mutant *CCA1* promoter regions (−363/−192) using a gLUC-based Y1H assay. Columns and error bars show the mean $\pm$ SEM of four independent experimental averages ($n = 3-6$ samples per experiment for each protein-DNA interaction tested) and the dashed line indicates the interaction cutoff. (D) *TCP19* and *CCA1* expression patterns determined by histochemical staining of *promoter:: β -glucuronidase (GUS)* reporter lines. Images are representative of three independent experiments ($n = 13-34$ individuals per experiment for each genotype-age combination). Where necessary, arrows point to stained regions. Scale bars, 1 mm. (E) Bioluminescence from Ap or Lf regions of 8- and 13-day old *TCP19::LUC*⁺ seedlings. Plots show mean values (black bars) of independent experimental averages (large colored symbols with shapes corresponding to each experiment, $n = 3$). Values for individual seedlings are shown by the gray symbols ($n = 34$). (F) Representative true color (top) and pseudo-colored bioluminescence intensity (bottom) images of *TCP19::LUC*⁺ seedlings analyzed in (E). Scale bars, 1 mm. (G) TCP19 protein expression in Ap and Lf from dissected *TCP19::TCP19-FLAG* and wild-type seedlings. Images are representative of blots from four independent experiments. (H) Photographs of 32-day old wild-type, *TCP19-OX* (line #19), *cca1-1* and *TCP19-OX* (line #19)/*cca1-1* plants grown under long days (16 hours light: 8 hours dark). Images are representative of six independent experiments. (I) Flowering time for wild-type, *TCP19-OX* (line #19), *cca1-1* and *TCP19-OX* (#19)/*cca1-1* plants grown under long day conditions (16 hours light: 8 hours dark). Columns and error bars indicate the mean $\pm$ SEM of 6 independent experimental averages ($n = 10-16$ individuals per genotype in each experiment). Statistical comparison was performed with experimental averages using a one-way ANOVA followed by Tukey's multiple comparisons test. Shared letters indicate non-significant differences ($P > 0.05$) while non-matching letters indicate significant differences ($P < 0.0001$) between group means.



TCP19 regulates the clock function and photoperiodic flowering responses

To investigate the potential role of TCP19 in clock regulation, we analyzed *CCA1::LUC*+ bioluminescence from plants carrying a *tcp19* loss-of-function allele (*tcp19-2*) (Figure 1.4A and Supplemental Figure 1.10, A and B). Wild-type and *tcp19-2* plants imaged in free-running conditions showed similarly robust *CCA1::LUC*+ rhythms (Supplemental Figure 1.10C). Remarkably, despite a minor period shortening (~15 min), the phase of *CCA1::LUC*+ expression was significantly delayed (~1 hour) only in the shoot apex of *tcp19-2* plants (Figure 1.4, B and C), resulting in a significantly reduced (~50%) phase offset between the shoot apex and leaves compared to wild-type controls (Figure 1.4D). Importantly, the phase of *CCA1::LUC*+ rhythms in the shoot apex of *tcp19-2* mutants was fully restored to wild-type by complementation of the mutation with a construct that expresses TCP19 under the control of its own promoter sequence (*TCP19::TCP19-FLAG*) (Supplemental Figure 1.11, A and B). These results were consistent with TCP19 expression being restricted to the shoot apex in wild-type plants and indicated that TCP19 regulates the timing of *CCA1* expression peak. To further confirm the role of TCP19 in clock regulation, we analyzed *CCA1::LUC*+ bioluminescence from the shoot apex and leaves of plants that ectopically overexpress *TCP19* under the control of the constitutively active 35S promoter (*TCP19-OX* lines #2 and #19, Supplemental Figure 1.9). Wild-type and *TCP19-OX* plants imaged in free-running conditions showed similarly robust *CCA1::LUC*+ rhythms (Supplemental Figure 1.12, A and B). However, we observed a phase advance of *CCA1::LUC*+ activity in leaves (Figure 1.4E and S12A), rather than both organs, suggesting that the function of TCP19 may be further controlled in the shoot apex. The magnitude of the leaf phase advance corresponded to the strength of *TCP19* overexpression (Figure 1.4E) and reduced the phase offset between organs accordingly (Figure 1.4F), indicating

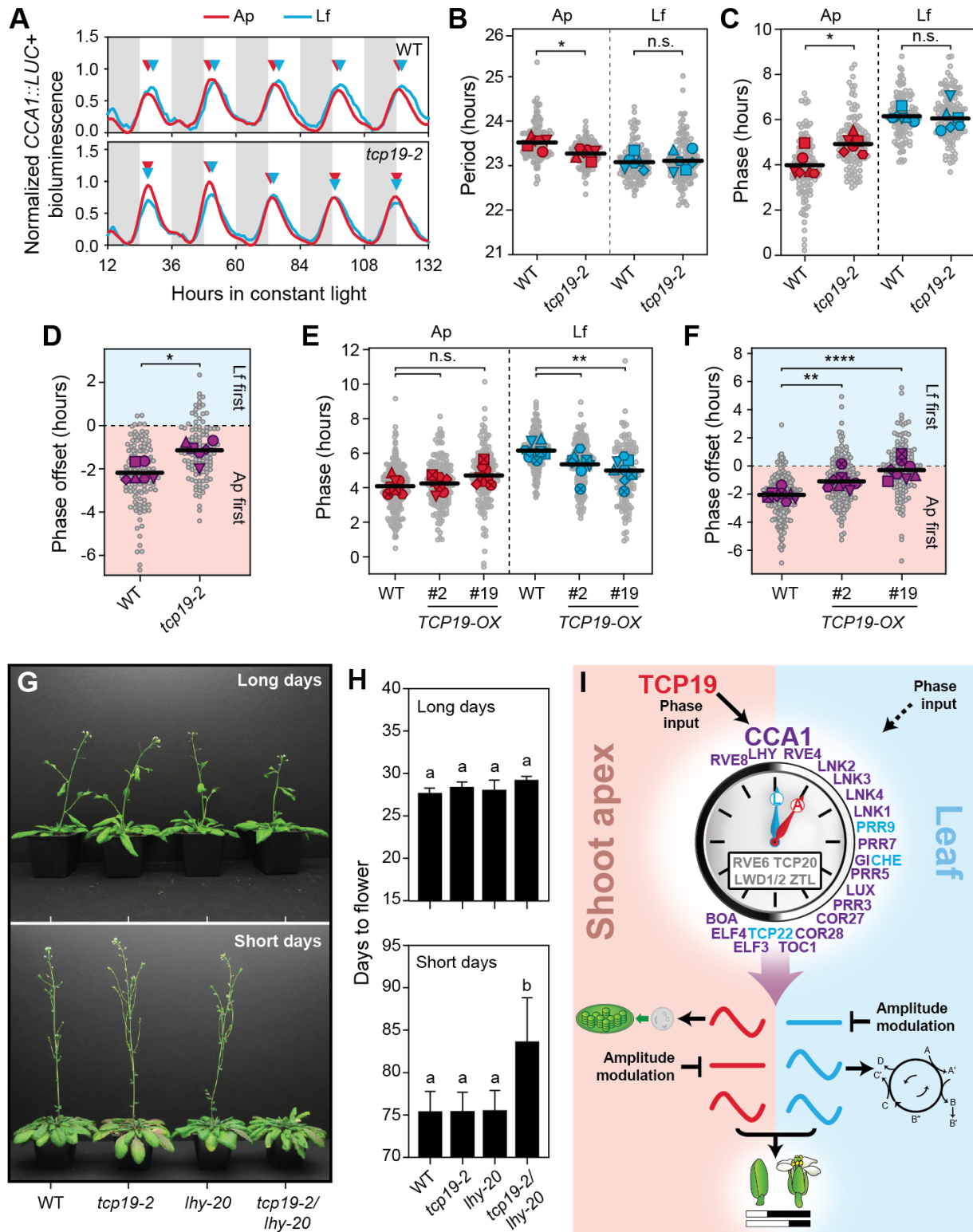
that phase determination is particularly sensitive to TCP19 levels. Additionally, the stronger overexpression line showed a significantly shorter period in both organs (Supplemental Figure 1.12C), which contrasts with the organ-specific regulation of phase and suggests that TCP19 controls period and phase by different mechanisms. Together, our findings reveal that the shoot apex-specific expression of TCP19 promotes an earlier expression peak of the key clock component *CCA1*, unveiling the broader function of TCP19 as an organ-specific regulator of the clock phase.

Next, we explored the role of the clock phase offset between shoot apex and leaves in downstream physiological processes. We anticipated that biological functions associated with genes rhythmic in both organs (*e.g.* photoperiodic flowering responses, as shown in Figure 1.1K) would be most sensitive to changes in the phase offset between organs. Notably, photoperiodic flowering has been shown to rely on proper phasing of clock-controlled gene expression (30) and we observed that *TCP19* regulates flowering in a *CCA1*-dependent manner (Figure 1.3I), so we explored whether *tcp19-2* plants display flowering time phenotypes when grown in long or short days. Although we found no significant differences between the mutant and wild type plants in either condition (Figure 1.4, G and H), previous studies suggested that class-I TCPs regulate only *CCA1* but not its close homolog *LHY*, and that the effect of single *tcp* mutants in clock regulation could be hindered by the functionally redundant role of LHY (16). Therefore, to determine if LHY masks flowering time phenotypes in *tcp19-2* mutant plants, we first investigated whether TCP19 can bind to the *LHY* promoter (using the same assay that revealed TCP19 binding to the *CCA1* promoter). As predicted, TCP19 did not interact with the *LHY* promoter region (Supplemental Figure 1.13, A and B), suggesting that flowering phenotypes in *tcp19-2* plants might be revealed in a sensitized *lhy* loss-of-function background (*lhy-20*) (16). In fact, we found

that *tcp19-2/lhy-20* double mutant plants showed a striking delay in flowering time compared to the respective single mutants and wild-type controls when grown in short day, but not long day conditions (Figure 1.4, G and H). These results reaffirm that TCP19 regulates the onset of flowering through *CCA1* and suggest that the clock phase offset between shoot apex and leaves, which is regulated in part by TCP19, provides previously unrecognized control of photoperiodic responses.

Figure 2.4. TCP19 is a shoot apex specific regulator of the clock function and photoperiodic flowering responses.

(A) Bioluminescence analysis of *CCA1::LUC* expression in shoot apex (Ap) and leaf (Lf) regions of intact WT and *tcp19-2* loss-of-function plants. Data show average values ($n = 14$) from one out of six independent experiments (represented in B, C, and D). Arrows indicate the peak expression within each cycle. (B) Period of *CCA1::LUC* expression in Ap (red) and Lf (blue) of intact wild-type (WT) and *tcp19-2* loss-of-function plants. (C and D) Phase (C) and phase offset (D) of *CCA1::LUC* expression. (E and F) Phase (E) and phase offset ($Phase_{Ap} - Phase_{Lf}$) (F) of *CCA1::LUC* expression in intact WT and *TCP19* overexpression (*TCP19-OX* lines #2 and #19) plants. (G) Photographs of 35- and 84-day old WT, *tcp19-2*, *lhy-20* and *tcp19-2/lhy-20* plants grown under long day (16 hours light:8 hours dark) or short day (8 hours light:16 hours dark) conditions, respectively. Images are representative of three (long day) or four (short day) independent experiments. (H) Flowering times for the experiments indicated in (G). Columns and error bars indicate the mean $\pm$ SEM ($n = 27-44$ individuals total for each genotype). Statistical comparisons were performed with experimental averages using a one-way ANOVA (blocked by experiment) followed by Tukey's multiple comparisons test. Shared letters indicate non-significant differences ($P > 0.05$) while non-matching letters indicate significant differences ($P < 0.05$) between group means within each growth condition. (I) Model for the proposed structure of the Arabidopsis clock across shoot system organs. Mechanisms upstream of the clock control phase (e.g., TCP19 regulation of *CCA1* in the shoot apex) and mechanisms downstream of the clock modulate the amplitude of rhythmic gene sets to control circadian processes in each organ. Plots show mean values (black bars) of independent experimental averages (large colored symbols with shapes corresponding to each experiment) in (B), (C), and (D) ($n = 6$), and (E) and (F) ($n = 8$). Values for individual seedlings are shown by the gray symbols in (B), (C), and (D) ($n = 118$), and (E) and (F) ($n = 139-237$). Statistical comparisons were performed with experimental means using one-way ANOVAs followed by t-tests paired by experiment in (B), (C), and (D) or Dunnett's multiple comparisons test in (E) and (F) (n.s., not significant; * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$).



Concluding remarks

The circadian clock is a mechanism conserved among most life forms that allows organisms to achieve optimal fitness and survival rates in the rhythmic environmental conditions present on our planet. Clock control dictates the timing of most critical organismal functions throughout the day to integrate the multiple signaling pathways that regulate physiology and behavior. Understanding the molecular mechanisms underlying the clock function is thus fundamental to illuminate a basic aspect of biological systems and is particularly relevant to plants due to their sessile nature. Several lines of evidence, including this work, support the notion that the clock function is controlled by a common mechanism conserved across most cells of an organism. However, the overall organismal clock function relies on local adaptations that lead to unique clock-controlled rhythms across tissues and organs. The extent of organ-specific clock regulation and, more importantly, the molecular mechanisms that allow a common clock mechanism to achieve spatial specificity were not well known in plants. In this work, we first characterized the clock-controlled transcriptomes in two major structures of the plant shoot system and found that although most clock components are similarly expressed between organs, rhythmic genes are unique to only one of them. Furthermore, we found that both clock components and rhythmic genes consistently peaked earlier in the shoot apex. Based on these findings, our work supports a model for the circadian system centered around a conserved organism-wide clock mechanism with organ-specific clock input and output pathways that determine the identity and timing of rhythmic gene expression in each structure (Figure 1.4I). In this model, organ-specific clock input control leads to the overall phase offset between rhythmic genes in the shoot apex and leaves, while organ-specific clock output control enables selective

amplitude regulation that ultimately determines whether a gene is rhythmically expressed. This molecular architecture was in part validated by identifying a shoot apex-specific clock regulator, TCP19, that locally controls the timing of expression of the key clock component *CCA1*. We postulate that organ-specific control of rhythmic gene expression allows a dynamic and selective modulation of clock-controlled processes in response to localized signals and that this is necessary for the proper regulation of highly integrated biological processes such as the onset of flowering. Thus, local clock control likely provides a critical homeostatic mechanism to maintain optimal organismal fitness in the context of multifaceted endogenous and environmental cues.

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Materials and Methods

Plant materials

Arabidopsis thaliana (Arabidopsis) seedlings used in this work were of the Columbia ecotype (Col-0). *CCA1::LUC+* reporter line (27), *cca1-1* (31, 32), *lhy-20* (SALK_031092) (33), *cca1-1* (*CCA1::LUC+*) (34) and *lhy-20* (*CCA1::LUC+*) (27) were previously described. In addition, we identified one *tcp19* loss-of-function mutant (*tcp19-2*) from two available T-DNA insertion lines: *tcp19-1* (SALK_017793, which was not a strong knockdown allele) and *tcp19-2* (SALK_024434).

To generate *TCP19* promoter activity reporter lines (*TCP19::LUC+* and *TCP19::GUS*), the region upstream of the *TCP19* translation start codon (-1/-1520) was amplified by PCR using the following primer pairs (lower case = plasmid overlapping region; upper case = genomic region) 5' taaagcttatcgatgTTACCACAAAACCCTAGAATAT 3' / 5' aggtcgactctagagACAAAGTCAGCGTCAACATTAG_3' (for *TCP19::LUC+*) or 5' agggactgaccaccTTACCACAAAACCCTAGAATAT 3' / 5' ctctagaggatccccACAAAGTCAGCGTCAACATTAG 3' (for *TCP19::GUS*). PCR fragments were cloned into *Bam*HI digested pZPomega-LUC+ (35) and *Sma*I digested pBI101 (36), respectively, using Gibson assembly (37) (New England Biolabs, Cat# E2621). The resulting plasmids (pZP-TCP19pro and pBI101-TCP19pro) were transformed into the Arabidopsis Col-0 background by Agrobacterium-mediated transformation (38). All experiments were performed using homozygous T3 or T4 seedlings.

To generate *TCP19* minigene transgenic lines (*TCP19::TCP19-FLAG*), a four fragment Gibson assembly approach utilizing a pGreenII-0179 backbone (39) was applied. The region upstream of the *TCP19* translation start codon (-1/-1520) was amplified by PCR from genomic DNA using the following primer pairs (lower case = plasmid overlapping region; upper case =

genomic region) 5' actagtggatcccccACAAAGTCAGCGTCAACATT 3' / 5' gtgattcgattccatTTACCACAAAACCCTAGAAT 3'. The CDS of *TCP19* (without STOP codon) was amplified by PCR from pENTR/D-TCP19 DNA, obtained from the pENTR/D-TF clone library available at the Arabidopsis Biological Resource Center (ABRC) (<http://abrc.osu.edu>) under stock #CD4-88 (24), using the following primer pairs 5'

ATGGAATCGAATCACGAAGG 3' / 5' AGTCTCATGACATGAAGACG 3'. A linker region was amplified by PCR from pMDC83-CHE DNA (27), and a partial triplicate FLAG extension sequence was added using the following primer pairs 5'

TCATGTCATGAGACTAAGGGTGGGCGCGCCGAC 3' / 5'

CATCATCATCCTTGTAATCCTTATCATCATCATCCTTGTAATCTTTTCTACCGGTACCG 3'. The remaining triplicate FLAG sequence with STOP codon and a sequence from the region downstream of the *TCP19* CDS (+1/+249 from STOP) was amplified by PCR from genomic DNA using the following primer pairs 5'

TGATGATGATAAGGATTACAAGGATGATGATGATAAGTAAGGAATTTTAACTCTTAG TTC 3' / 5' CGAATTCCTGCAGCCCTATTCCTTCTACTATTGTGTCG 3'. All four overlapping PCR fragments were assembled into *Sma*I digested pGreenII-0179 using Gibson assembly (37) (New England Biolabs, Cat# E2621). The resulting plasmid was transformed into the Arabidopsis *tcp19-2/CCAI::LUC+* background by Agrobacterium-mediated transformation (38). All experiments were performed using homozygous T3 or T4 seedlings.

To generate *TCP19* overexpression lines, TCP19 protein coding sequence was transferred from pENTR/D-TCP19, to the cauliflower mosaic virus (CaMV) 35S promoter-containing binary vector pH7WG2 (40) using LR Clonase II (Thermo Fisher Scientific, Cat# 11791020). Finally, pH7WG2-TCP19 was transformed into the Arabidopsis *CCAI::LUC+* background by

Agrobacterium-mediated transformation (38). We identified two lines (*TCP19-OX* #2 and #19) that overexpressed *TCP19* mRNA at different levels (as measured by qPCR). All experiments were performed using homozygous T3 or T4 seedlings.

Homozygous *tcp19-2* (*CCA1::LUC*+), *TCP19-OX* (#19)/*cca1-1* (*CCA1::LUC*+), and *tcp19-2/lhy-20* (*CCA1::LUC*+) lines were generated by genetic crossing with resistance marker and PCR genotyping confirmation.

Plant growth conditions

Arabidopsis plants were grown either in Murashige & Skoog (MS) media or soil. For MS-grown plants, seeds were plated in petri dishes containing 1x MS basal salts (Caisson Labs, Cat# MSP01) and 0.8% agar (Sigma, Cat# A1296) (MS-agar medium). For soil-grown plants, seeds were sown in 11" x 21" growth trays containing 32" x 2.5" square pots (8x4 divider) filled with autoclaved soil (Berger, BM2). Seeds in Petri dishes were stratified for 2-3 days at 4°C on top of the MS-agar medium. Soil-grown seeds were stratified for 2-3 days at 4°C in a tube containing a 0.15% agar solution and then pipetted onto soil in pots. Plants were grown at 22°C under the various daily light regimes below, depending on the experiment. Standard light-dark cycles: 12 hours light ($\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$)/12 hours dark; constant light (free-running condition): ($\sim 60 \mu\text{mol m}^{-2} \text{s}^{-1}$); short days: 8 hours light ($80\text{-}115 \mu\text{mol m}^{-2} \text{s}^{-1}$)/16 hours dark; long days: 16 hours light ($60\text{-}90 \mu\text{mol m}^{-2} \text{s}^{-1}$)/8 hours dark.

RNA isolation, mRNA-sequencing library construction, and sequencing

Seedlings were grown in soil with standard light-dark cycles for 14 days, and then transferred to constant light. Harvesting occurred at 6 timepoints after transition to constant light:

90, 94, 98, 102, 106, and 110 hours. For each sample collection, 6-10 seedlings were snap frozen in liquid nitrogen and then cryodissected to isolate the shoot apex and true leaves. Two independent replicate experiments (“replicate 1” and “replicate 2”) were performed, rendering a total of 24 samples. Total RNA was isolated from each sample using the RNeasy Plant Mini kit (Qiagen, Cat# 74904) and mRNAs were purified from 0.46µg of total RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Cat# E7490) before library preparation with the NEBNext UltraII RNA Library Prep Kit (New England Biolabs, Cat# E7770). Sera-Mag Magnetic SpeedBeads (Thermo Scientific, Cat# 65152105050250) were used for all size selection and clean-up steps and the resulting libraries were pooled and sequenced (single end 50 bp, SE50) on a HiSeq 2500 machine (Illumina).

mRNA-seq mapping and quantification of expression levels

mRNA-seq reads were mapped to the TAIR10 reference genome using STAR v2.5.0c (41) with the following options: “—outFilterMismatchNmax 2 —outFilterMultimapNmax 1”. Sorted .bam files were used to generate HOMER (42) Tag Directories using the makeTagDirectory script with the “-mis 2 -unique” option. UCSC format tracks were generated using the makeUCSCfile script with the “tair10 -fragLength given -style rnaseq” options. Raw read counts and normalized values (fragments per kilobase of transcript per million mapped reads, FPKM) for each sample were obtained using the HOMER analyzeRepeats.pl program with the “-noadj” or “-fpkm” options, respectively, and mapped to the TAIR10 genome annotation (43) with isoforms collapsed by “-condenseGenes”.

Principal component and clustering analysis

Unnormalized, rlog-transformed read counts with blind dispersion estimation were used to perform principal component analysis and calculate sample-to-sample distances with the DESeq2 R package (44).

Analysis of circadian gene expression

For optimal downstream statistical detection of rhythmic genes we followed previously described recommendations to first define the set of “expressed” genes (45). For that, we filtered the mRNA-seq dataset of normalized reads by applying an “expression” threshold that retained only those genes that had >3 FPKM in both replicates of at least one time point (note that this criterion was defined after testing a range 1 to 10 FPKM cut off values, as shown in Supplemental Figure 1.2B). To identify genes that cycled with a 24-hour period, we analyzed the filtered dataset of normalized reads using the Metacycle package for R (46). We used the integrated meta2d output values for downstream analysis and considered a gene to be “rhythmic” if it passed a false discovery rate threshold of Benjamini-Hochberg $Q < 0.05$.

Motif enrichment analysis

The findMotifsGenome.pl script from HOMER v4.11.1 (42) was used to identify enriched motifs in the cis-regulatory regions of rhythmic loci. The queried genomic promoter sequence for each locus was defined as 750 bp upstream plus 500 bp downstream of the 5’ end of the representative gene model annotation from Araport11 (47). The two target gene sets were all rhythmic loci in the shoot apex and all rhythmic loci in the leaves and the background set included all expressed loci passing the 3 FPKM “expression” threshold (see previous section). We considered motifs significant at a level of $P < 10^{-15}$.

Gene ontology (GO) analysis

To find biological process GO terms enriched in rhythmic gene sets compared to the genomic background, we used the “elim” method from the topGO R package deposited in Bioconductor (48) and reported those with raw $P < 0.001$ using Fisher’s exact test (Supplemental Table 2.4). Then, to summarize and remove redundant GO terms, we used the Revigo tool (49) with a semantic similarity cutoff value of $C = 0.5$ and visualized the results using the ggplot2 R package, following previously reported recommendations (50).

Quantitative real-time PCR analysis

To determine *TCP19* expression level in *tcp19-2* and *TCP19-OX* plants, seedlings were grown in MS-agar under standard light-dark cycles for 12 days. Pooled tissue samples were collected at Zeitgeber time (ZT) 2 hours by snap freezing in liquid nitrogen. Total RNA from these samples was isolated using the RNeasy plant mini kit (Qiagen, Cat# 74904) and on-column DNase (Roche, Cat# 4716728001) treatment. RNA from each sample (1 µg) was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad, Cat# 1708890). *CCA1* and *TCP19* transcript levels in each sample were determined by real time quantitative PCR (qPCR) (Bio-Rad CFX96 Real-time PCR detection system) using the Maxima Sybr green qPCR master mix (Thermo Fisher Scientific, Cat# K0221). For *CCA1* we used the following primers: 5’ CCGCAA CTTTCGCCTCAT 3’ / 5’ GCCAGATTCGGAGGTGAGTTC 3’, and the following PCR conditions: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 25 seconds, and 72°C for 25 seconds. For *TCP19* we used the following primers: 5’ CGTTTTGGCCTA TGGGTATGGGTACT 3’ / 5’ CTGAAGCGTTTGAGACATAACTAGACAC 3’, and the

following PCR conditions: 95°C for 10 minutes, 40 cycles of 95°C for 10 seconds, 55°C for 20 seconds, and 72°C for 20 seconds. In both cases transcript levels were normalized against *PP2A* which was quantified in parallel qPCR reactions using the following primers: 5' TAACGTGGCCAAAATGATGC 3' / 5' GTTCTCCACAACCGATTGGT 3'. Gene expression levels (normalized to *PP2A*) were calculated using the comparative Ct method (51).

Bioluminescence detection and data analysis

Arabidopsis seeds were germinated in MS-agar under standard light-dark conditions. At the beginning of day 7 (one day before the imaging period began), plants were sprayed with 5 mM D-luciferin potassium salt (in 0.01 % Triton X-100 solution). At the beginning of day 8, plants were transferred to constant light and bioluminescence was captured every hour for *CCA1::LUC*+ seedlings (2 min. acquisition time) or every 2.5 hours for *TCPI9::LUC*+ seedlings (25 min. acquisition time) for the next 6 days using a Pixis 1024 CCD camera positioned with a top-down transverse view of the seedlings (Princeton Instruments).

Bioluminescence images were processed using the MetaMorph image analysis software (Molecular Devices) to quantify bioluminescence counts captured from total aerial regions and shoot apex-only regions. Bioluminescence originating from leaves was calculated by subtracting apex region counts from the corresponding individual's total aerial region counts.

Bioluminescence counts for each experiment were then analyzed with a fast Fourier transform non-linear least squares (FFT-NLLS) algorithm using the web interface provided by BioDare2 (52). Output values used for plotting included period, phase (method-specific, relative to 0), and relative amplitude error (RAE). Phase offset between organs was calculated by subtracting each individual's shoot apex phase from its leaf phase, taking into account the circular nature of the

data. Time course plots were generated using “normalized bioluminescence” counts, calculated from amplitude and baseline detrended average bioluminescence traces using the interface provided by BioDare2 (52) and then normalized from min (set to 0) to max (set to 1) over the time course; or “background subtracted bioluminescence” counts, calculated as the difference between equivalent regions of *LUC+* reporter lines and no-reporter (*Col-0*) control plants.

To directly compare the bioluminescence intensities between shoot apex and leaf areas, we calculated the mean bioluminescent counts per pixel in shoot apex and leaf regions of 8- and 13-day old seedlings (images taken at ZT2 and ZT142, respectively).

GUS staining experiments

The β -glucuronidase (GUS) assay was performed as described previously with modifications (53). In summary, sterilized seeds were grown on MS-agar medium with standard light-dark cycles for 4, 8, or 16 days. Samples were harvested at ZT2-3 and placed into 90% acetone on ice and then incubated at room temperature for 20 min. After incubation, the acetone was removed and replaced with wash buffer (50 mM sodium phosphate buffer-pH 7.0, 0.2% Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide). Samples were washed with GUS wash buffer solution 3 times, then the buffer was replaced with GUS staining solution with (50 mM sodium phosphate buffer pH 7.0, 0.2% Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and 1 mM X-Gluc). Samples were vacuumed in 5 min. intervals for a total of 20 minutes and then incubated at 37°C protected from light for 6 hours (*CCA1::GUS*) or 20 hours (*TCPI9::GUS*). After incubation, the GUS staining solution was replaced with 70% ethanol to de-stain overnight. The 70% ethanol was replaced with 90% ethanol every day until completely de-stained. Independent homozygous transgenic lines ($n = 8$ -

10) were initially stained using 4 different concentrations of potassium ferrocyanide and potassium ferricyanide (1mM, 2mM, 5mM, and 10mM) to identify representative *CCA1::GUS* and *TCP19::GUS* lines. Images were captured using a magnifying scope (Nikon SMZ1270). Exposure of all images was raised one exposure value in Adobe Lightroom to aid in visibility.

SDS-PAGE and immunoblotting experiments

14-day old seedlings were harvested at ZT8 with apex and leaf tissue dissected and collected separately. Protein was isolated in buffer (50 mM Hepes KOH pH7.5; 100 mM NaCl; 5 mM EDTA; 10% glycerol; 5 mM DTT; 0.5% Nonidet P 40 substitute (Sigma); 0.5% deoxycholate pH8; 0.1% SDS; 5 mM benzamidine; 1X protease inhibitor cocktail (Roche); 1 mM PMSF; 50 μ M MG132; 2% PVPP) at 4°C. Protein was quantified using the DC protein assay (Bio-Rad, Cat# 5000111) and 40 μ g of protein was loaded into 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, Cat# 4561094) for each sample. Protein electrophoresis and transfer were performed with the Mini-PROTEAN Tetra cell system (Bio-Rad), followed by immunoblotting with 1:4,000 (3% milk in TBS-T) anti-FLAG (GenScript, Cat# A00187) and 1:100,000 (3% milk in TBS-T) anti-mouse peroxidase polymer (WestVision, Cat# WB-2000) antibodies. Protein was detected using the Super Signal West Femto chemiluminescence kit (Thermo Fisher Scientific, Cat# 34095) and the ChemiDoc XRS+ (Bio-Rad) imaging system. Total protein was reversibly detected by a 30 min. incubation with AdvanStain Ponceau (Advansta, Cat# R-03021-D50).

Yeast one-hybrid assays

Yeast one-hybrid (Y1H) assays were performed in high- (384-well format) or low-throughput (96-well format) using a Gaussia luciferase (gLUC) reporter as described previously (25). Yeast promoter bait strains (YM4271, Clontech) carrying the wild-type or mutated class-I TCP binding site (TBS-I) (-363/-162) *CCAI* promoter region were previously described (25). Six additional bait strains for other *CCAI* promoter regions were generated by first using the following PCR primer pairs: 5' caccATCAAAGGAGGAAGAAGAAGAAG 3' / 5' TGAGCTAAAAAAGACCAGAGAC 3' (-813/-642), 5' caccGTCTCTGGTCTTTTTTAGCTC 3' / 5' TCAACAGTTCAGCATTAAGCT 3' (-663/-492), 5' caccAGCTTAATGCTGAACTG 3' / 5' CAATCGAACGCGCATT 3' (-563/-342), 5' caccTTGACCTTTTAAACCTT 3' / 5' TATTCGAGGCTACAAA 3' (-213/-42), 5' caccTTTCTTTTGTAGCCTCG 3' / 5' GGATAAGAAAACAGTAA 3' (-63/+109) and 5' caccATTTCTTACTGTTTTCT 3' / 5' AAAAAACAATCAAGTCAAGTTA 3' (+88/+259). In addition, eight bait strains for the *LHY* promoter region were generated by first using the following PCR primer pairs: 5' caccGGTTAGTAACAAAACTGGTCT 3' / 5' ATGGAATTCAGCAAACATCATT 3' (-860/-603), 5' caccGTAAGCTGGAATCTAAAAAAT 3' / 5' AACCCGACAAGCTTGGACTGCT 3' (-660/-410), 5' caccAGATTTTTTATACAGACGCAAGT 3' / 5' AGTGTGGTGGTCCACAATTGCT 3' (-460/-210), 5' caccAGTTTGTACCAATAATCGTTTA 3' / 5' CAATCTCAGCCACAACAATTTT 3' (-260/+15), 5' caccTGTTCCAGCCTCAAATAAACTT 3' / 5' CGGAAATAATCAAATCGGAAGC 3' (-60/+190) and 5' caccTTTATTTTTAGAGATTTTTTTT 3' / 5' TAACTTTTAGAGCTAATAAGAT 3' (+140/+390), 5' caccTGAGAACTTATAGAAGCGATT 3' / 5' AAGATAAGAGTGATGAGCGAAA 3' (+340/+590) and 5' caccTGGACTTTGAAAAGATTTCTCT 3' / 5'

AACAGGACCGGTGCAGCTATTC 3' (+540/+779). Each promoter fragment was cloned into pENTR/D-TOPO according to the manufacturer's protocol (Thermo Fisher Scientific, Cat# K240020), and then transferred into the pY1-gLUC59-GW reporter vector (25) using LR clonase II according to the manufacturer's protocol (Thermo Fisher Scientific, Cat# Cat# 11791020). Finally, pY1-gLUC59-GW vectors carrying each *CCAI* promoter region were recombined into the *URA3* genomic locus of the YM4271 yeast strain as previously described (54).

For high-throughput Y1H assays, transcription factor (TF) yeast expression prey vectors, pDEST22-TF ($n = 254$, Supplemental Table 2.6), were obtained from the pDEST22-TF clone library available at the Arabidopsis Biological Resource Center (ABRC) (<http://abrc.osu.edu>) under stock #CD4-89 (24). Y1H assays were performed as previously described (25, 55). Briefly, pDEST22-TF plasmids ($n = 254$, Supplemental Table 2.6) and pEXP-AD empty vector controls ($n = 21$) (Thermo Fisher Scientific) were transformed into the YU yeast strain in a 96-well format (24, 56). Transformed cells, YU-TF effector strains (MAT α), were then indexed into a 384-well microplate and individually mated with each of the seven aforementioned wild-type *CCAI* promoter reporter strains (MAT α). Finally, the gLUC reporter activity was quantified in each well containing the resulting diploid cells.

For low throughput Y1H assays, TF yeast expression prey vectors, pDEST22-TCP19(N) and pDEST22-TCP19(C) were generated by first PCR amplifying the regions encoding for amino acids 1-133 (TCP19-N-terminus) and 134-293 (TCP19-C-terminus) of TCP19, using the following primer pairs 5' caccATGGAATCGAATCACGAAGGC 3' / 5' TTAGCTCGTCGGGATTTTAAAG 3' (for TCP19-N) or 5' caccATGTCTCCAGTGTTGAATGACGG 3' / 5' TTAAGTCTCATGACATGAAGACGGAG 3' (for TCP19-C). These DNA fragments were cloned into pENTR/D-TOPO and then

transferred to pDEST22 using LR clonase II according to the manufacturer's protocols (Thermo Fisher Scientific). Y1H assays were performed in a 96-well format as described before (25) using either: 1) the pDEST22-TCP19, pDEST22-TCP19-N and pDEST-TCP19-C prey plasmids and YM4271 promoter bait strains carrying either the wild-type or TBS-I mutant *CCA1* (-363/-192) promoter sequences, or 2) the pDEST22-TCP19 prey plasmid and YM4271 promoter bait strains carrying wild-type *LHY* promoter sequences.

For both high- and low-throughput Y1H assays, the gLUC reporter activity was calculated as Lum/OD₆₀₀ for each well, and then normalized to the binding cut-off value which was set as 2x of the average+3SD gLUC activity obtained for pEXP-AD control wells (Fold change over cut-off).

Flowering time measurements

Plants were grown in MS-agar under short- or long-day conditions (see “Plant growth conditions”) for 7 days, then transferred to soil containing pots (one seedling per pot) where they continued their growth in the original short- or long-day condition. Each growth tray was watered 7 days after (1L of water) and then every 3-4 days (1L of water) as needed. Each experiment included 2-3 growth trays grown in parallel, each containing all genotypes arranged randomly ($n = 16$ plants per genotype). Flowering time was recorded when inflorescence shoots reached a height of 1 cm and the mean flowering time was calculated for each genotype. Results were calculated by averaging the means of all biological replicates ($n = 3-4$), as indicated in figure legends). Representative pictures were taken from the average flowering plant for each genotype at the time that the latest genotype flowered.

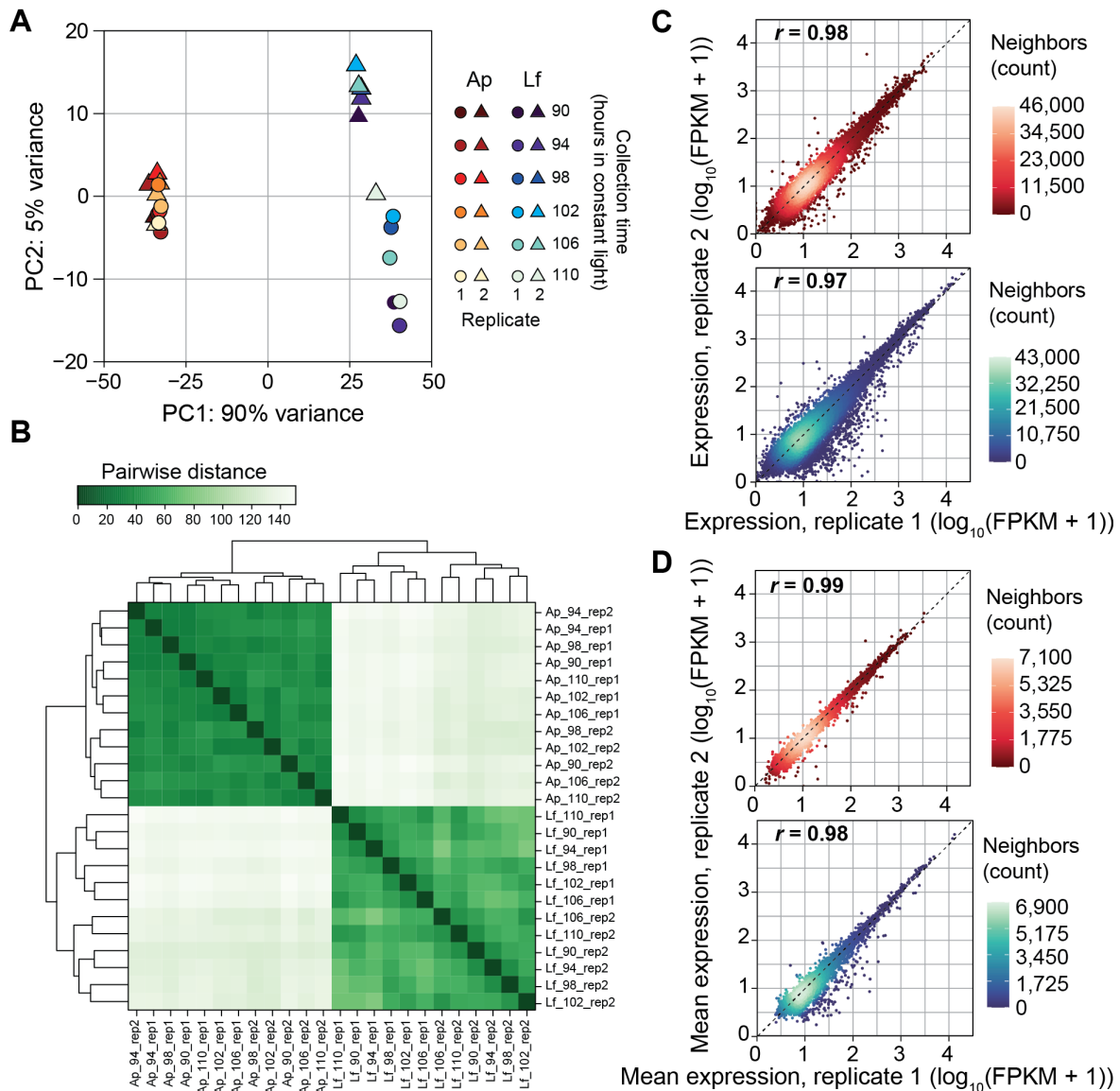
Statistical analyses and graphing

All statistical analyses were performed in GraphPad Prism 10 as indicated in figure legends. Graphing was either done using GraphPad Prism 10 (Figure 1.2, E to J, Figure 1.3, A, B, C, E, and I, Figure 1.4, A to F, and H, Supplemental Figure 1.4, Supplemental Figure 1.5C, Supplemental Figure 1.7, A and B, Supplemental Figure 1.8, Supplemental Figure 1.9, Supplemental Figure 1.10, B and C, Supplemental Figure 1.11, Supplemental Figure 1.12, and Supplemental Figure 1.13B) or R software (Version 2023.06.1+524) using packages including ggplot2 (57) (Figure 1.1, E to K, Figure 1.2, A to C, Supplemental Figure 1.1, A, C and D, Supplemental Figure 1.2, Supplemental Figure 1.3, and Supplemental Figure 1.5A), ggpointdensity (58) (Supplemental Figure 1.1, C and D), ggrepel (59) (Figure 1.2, A and B), RColorBrewer (60) (Supplemental Figure 1.1B), and pheatmap (61) (Supplemental Figure 1.1B). Adobe Illustrator was used to achieve uniformity of style.

Accession numbers

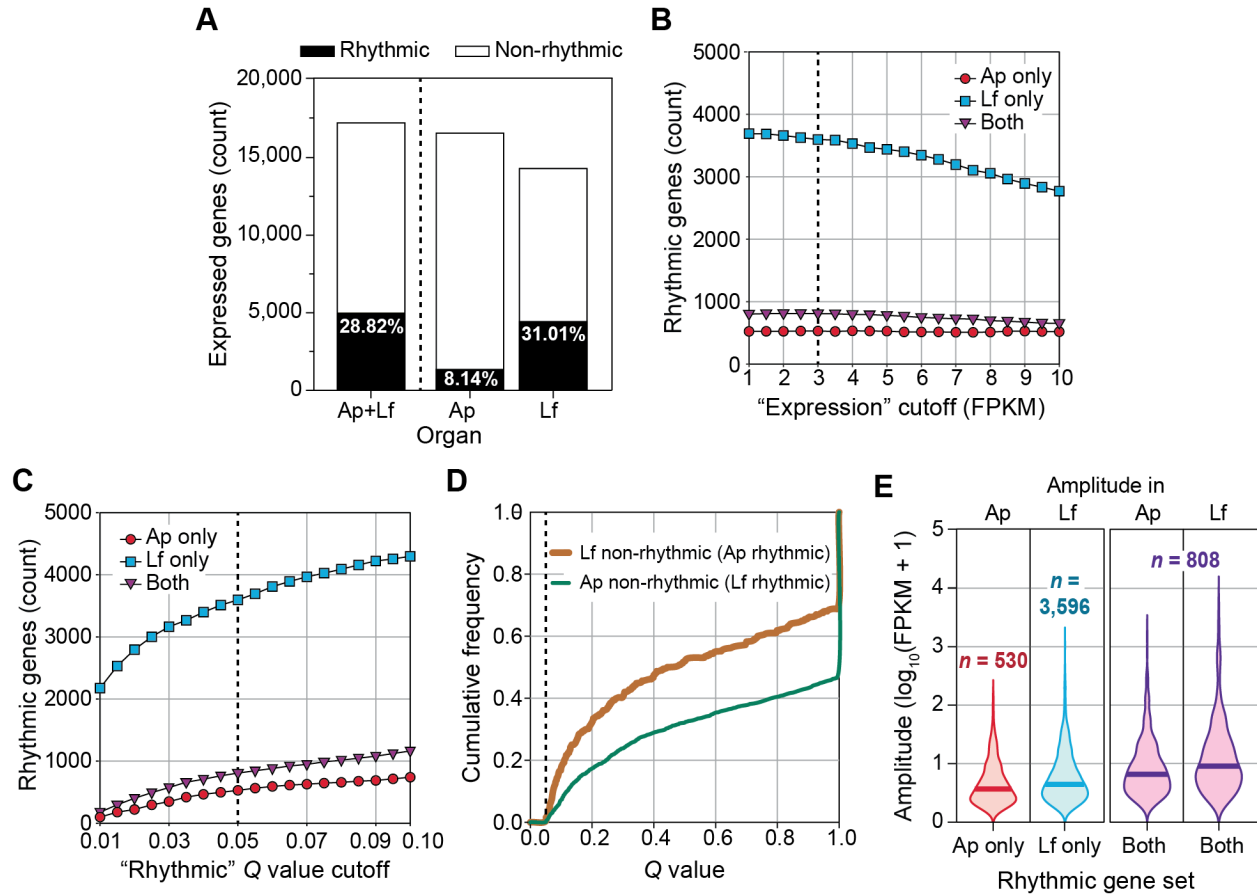
Gene models in this article can be found in The Arabidopsis Information Resource (TAIR) (<https://www.arabidopsis.org>) with the following accession numbers: *BOA*, AT5G59570; *CCAI*, AT2G46830; *CHE/TCP21*, AT5G08330; *COR27*, AT5G42900; *COR28*, AT4G33980; *ELF3*, AT2G25930; *ELF4*, AT2G40080; *GI*, AT1G22770; *LHY*, AT1G01060; *LNK1*, AT5G64170; *LNK2*, AT3G54500; *LNK3*, AT3G12320; *LNK4*, AT5G06980; *LUX*, AT3G46640; *LWD1*, AT1G12910; *LWD2*, AT3G26640; *PP2A*, AT1G13320; *PRR3*, AT5G60100; *PRR5*, AT5G24470; *PRR7*, AT5G02810; *PRR9*, AT2G46790; *RVE4*, AT5G02840; *RVE6*, AT5G52660; *RVE8*, AT3G09600; *TCP19* AT5G51910; *TCP20*, AT3G27010; *TCP22*, AT1G72010; *TOC1*, AT5G61380; *ZTL*, AT5G57360.

Supplementary Materials



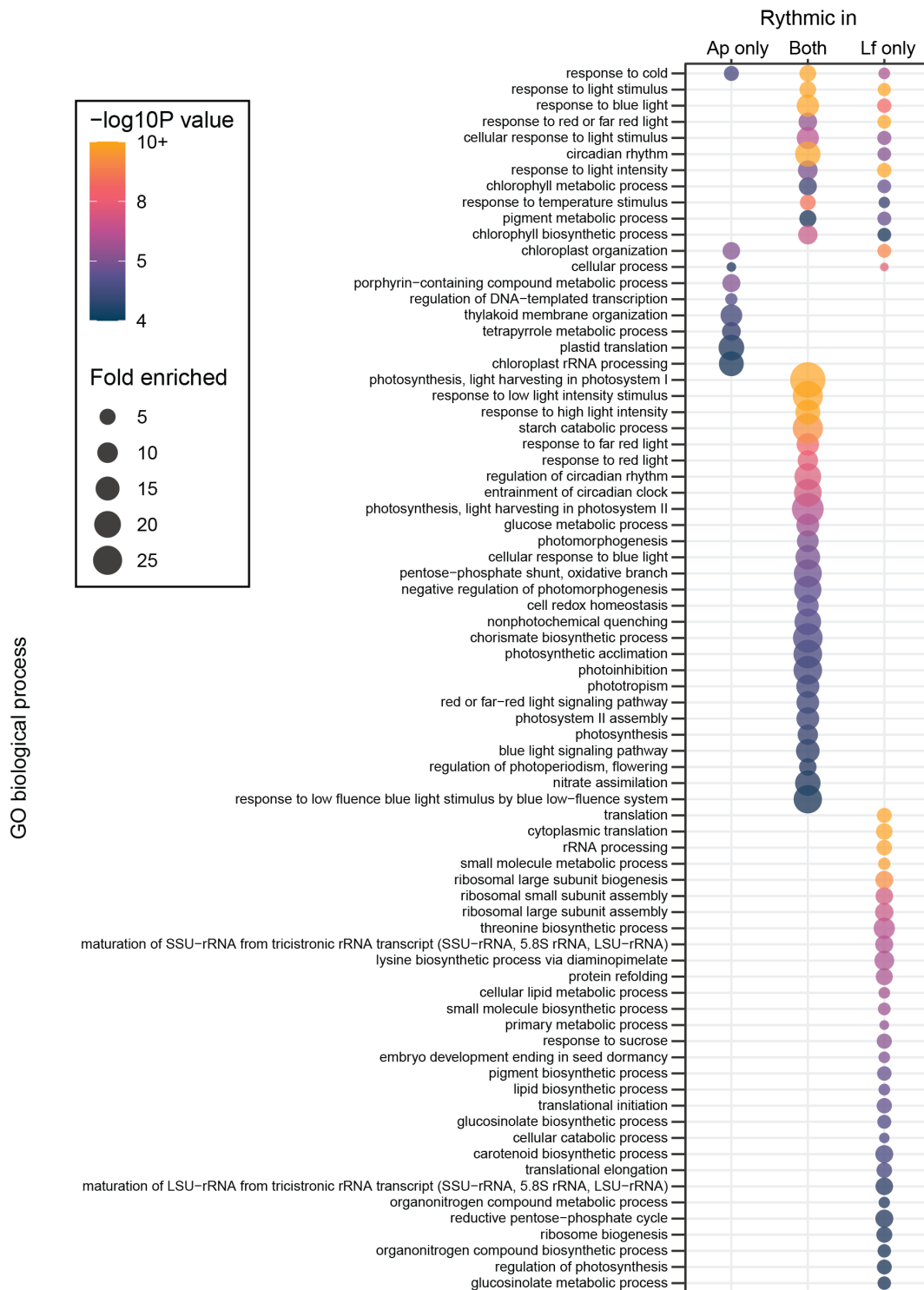
Supplemental Figure 2.1. Gene expression measurements from replicate mRNA-seq experiments cluster by organ and are highly correlated.

(A) Data groupings based on the two primary sources of variance (PC1 and PC2) in a principal component analysis of the gene expression profiles from the 24 mRNA-seq samples (two independent biological replicates for each of six timepoints from both the shoot apex (Ap) and leaf (Lf)). (B) Pairwise sample distances for gene expression data. The color gradient and hierarchical clustering dendrograms indicate similarity of samples based on their gene expression profiles. Sample naming convention is "Organ_collection time_biological replicate." (C and D) Correlation of expression values between replicates for each gene at each time point (C) or the mean of each gene across all time points (D). Dots are colored based on the density of neighboring dots (Ap, red tones; Lf, blue tones). Correlation is reported with Pearson's r . Dashed lines show $y = x$.



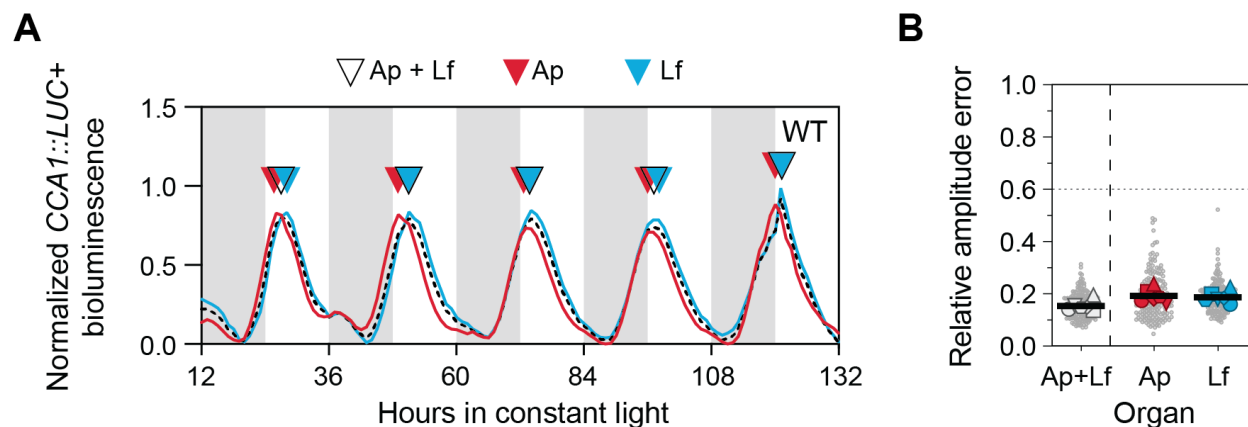
Supplemental Figure 2.2. Rhythmic gene sets maintain relative sizes across various cutoff thresholds and are similarly rhythmic in both organs.

(A) Percentage of rhythmic genes as a proportion of total genes expressed in each category ("Ap + Lf" is the union of genes in shoot apex (Ap) and leaf (Lf)). (B) Number of genes identified as "rhythmic" by passing the false discovery rate threshold of Benjamini-Hochberg Q value < 0.05 in Ap only, Lf only, or both organs as a function of the minimum "expression" cutoff applied to the mRNA-seq dataset. All downstream analysis in this study was performed using the selected cutoff of 3 FPKM (denoted by the dashed line). (C) Number of expressed genes (using the 3 FPKM cutoff) identified as "rhythmic" in Ap only, Lf only, or both organs as the minimum Q threshold varies from 0.01 to 0.1. The "rhythmic" threshold used for downstream analyses in this study was $Q < 0.05$ (denoted by the dashed line). (D) Cumulative frequency distributions for Q values of genes that were simultaneously found to be non-rhythmic in one organ but rhythmic in the other organ (considering a "rhythmic" cutoff of $Q < 0.05$ (dashed line)). (E) Amplitude distribution of rhythmic genes sets. Horizontal bars indicate median values.



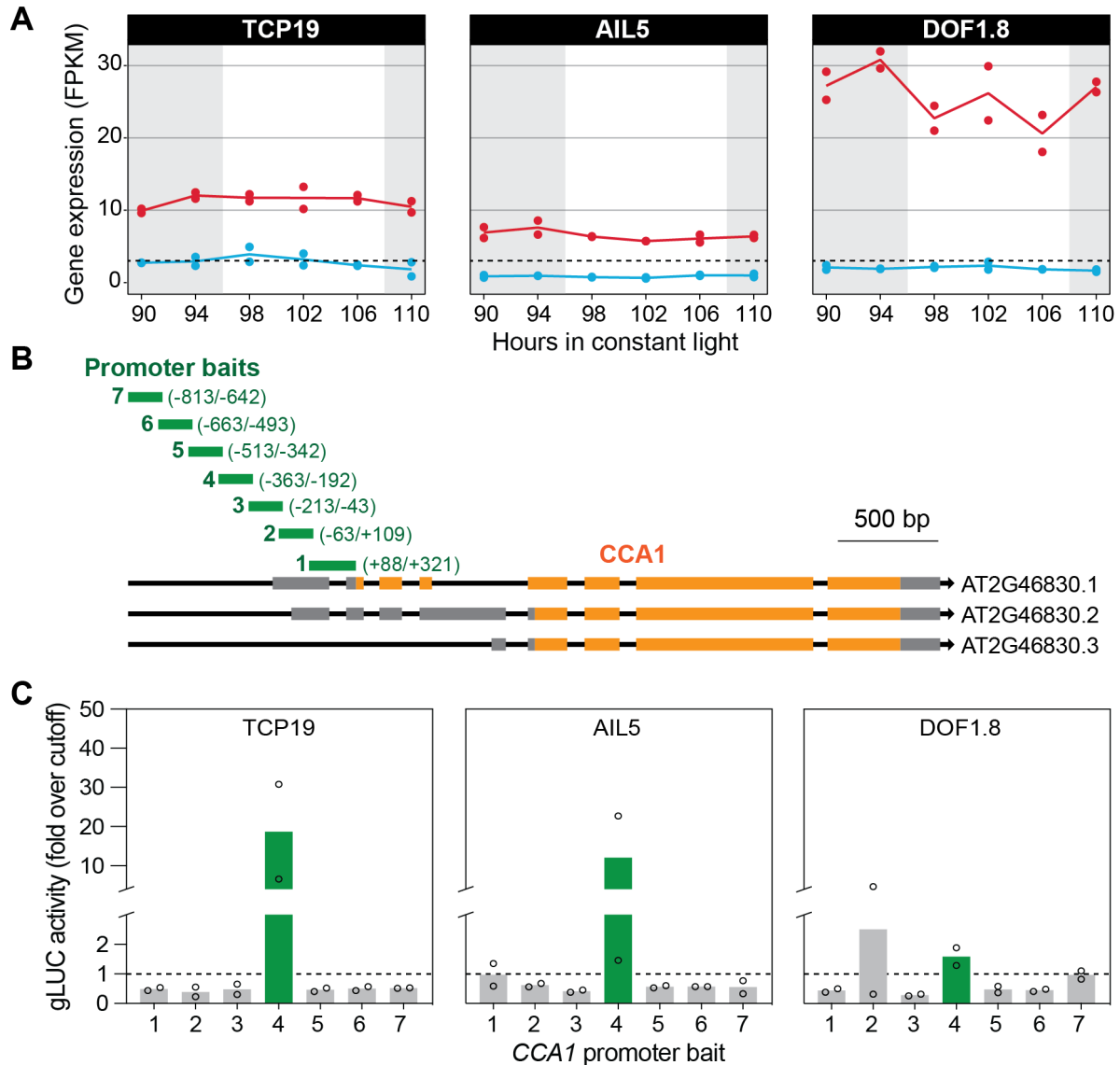
Supplemental Figure 2.3. Biological process ontologies enriched in rhythmic gene sets.

Full list of gene ontology terms found enriched ($P < 0.0001$) in genes rhythmic in the shoot apex (Ap only), leaves (Lf only) or both organs. Terms are ordered primarily by number of appearances across gene sets and secondarily by P value.



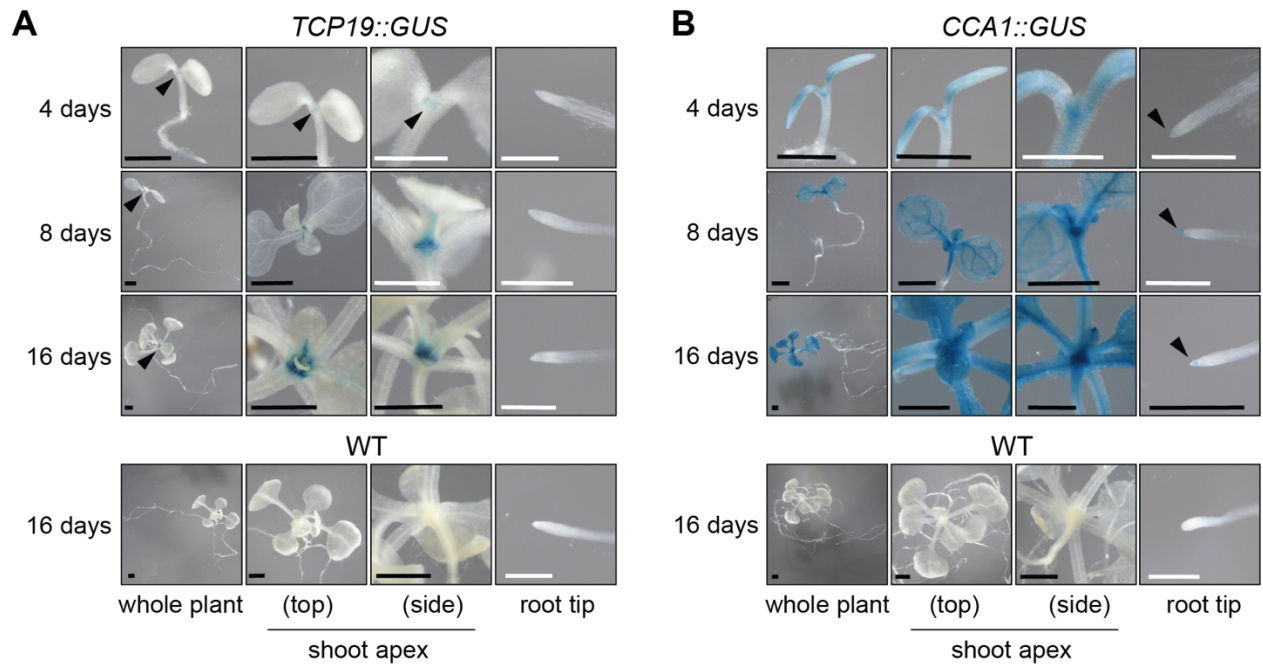
Supplemental Figure 2.4. Circadian rhythms of *CCA1* promoter activity are robust across the shoot system.

(A) Bioluminescence analysis of *CCA1::LUC+* expression in intact wild-type (WT) plants considering Ap and Lf together (Ap + Lf) or separately (Ap, Lf). Data show average values ($n = 26$) from one out of six independent experiments (represented in (B)). Arrows indicate the peak expression within each cycle. White and gray shaded background regions indicate subjective day and night periods, respectively. (B) Rhythmic robustness (measured as relative amplitude error, RAE) of *CCA1::LUC+* expression in Ap and/or Lf regions of WT plants. Plots show mean values (black bars) of independent experimental averages (large colored symbols with shapes corresponding to each experiment, $n = 6$) and individual values (small gray symbols, $n = 181$). Dotted line (RAE = 0.6) indicates the rhythmicity threshold value.



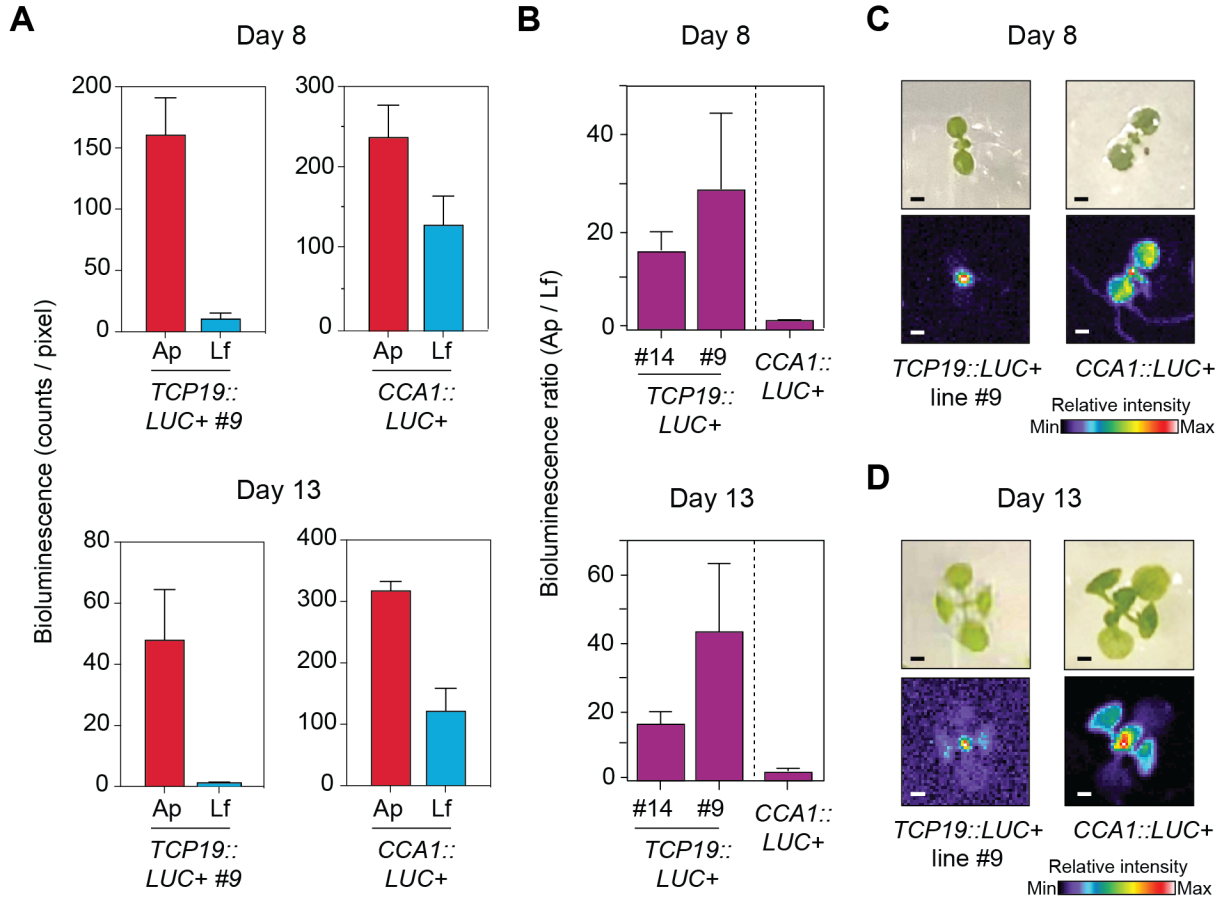
Supplemental Figure 2.5. Three shoot apex-enriched transcription factors interact with the *CCA1* promoter.

(A) Time course mRNA-seq expression in the shoot apex (red) and leaves (blue) for the top three TF hits identified from a yeast one-hybrid (Y1H) screen against the *CCA1* promoter. Dots indicate independent measurements and solid lines connect average values. Dashed lines denote the expression cutoff value (3 FPKM). White and gray shaded background regions indicate subjective day and night periods, respectively. (B) Diagram of Y1H promoter bait fragments (green boxes) in relation to *CCA1* gene models (untranslated regions, gray boxes; protein coding sequence, orange boxes; intronic and intergenic regions, black lines; arrows indicate direction of transcription). Coordinates are relative to the transcription start site of the AT2G46830.2 gene model. (C) Gaussia luciferase (gLUC) reporter activity from two independent Y1H experiments testing the interaction of the top three TF prey hits with the *CCA1* promoter bait regions indicated in (B). Bars indicate the mean value of independent experiments ($n = 2$) and are colored green when both replicates were above the interaction cutoff (dashed lines). Open circles plot values from each replicate experiment.



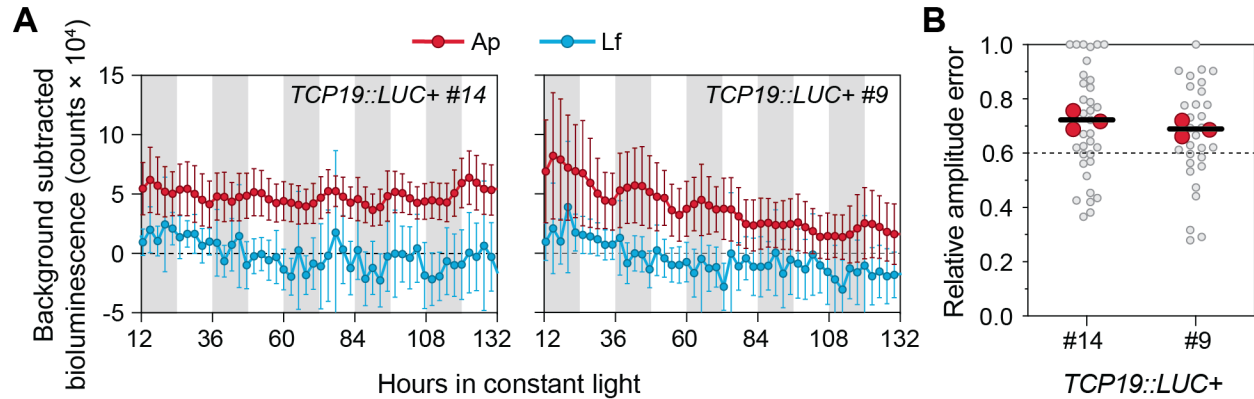
Supplemental Figure 2.6. TCP19 and CCA1 promoter activities co-localize in the shoot apex.

(**A** and **B**) *TCP19* (**A**) and *CCA1* (**B**) expression patterns determined by histochemical staining of *promoter::β-glucuronidase (GUS)* reporter lines. Images are representative of three independent experiments ($n = 13-34$ individuals per experiment for each genotype-age combination). Each row contains different views of the same individual and, where necessary, arrows point to stained regions. Scale bars, 0.5 mm (white) and 1 mm (black).



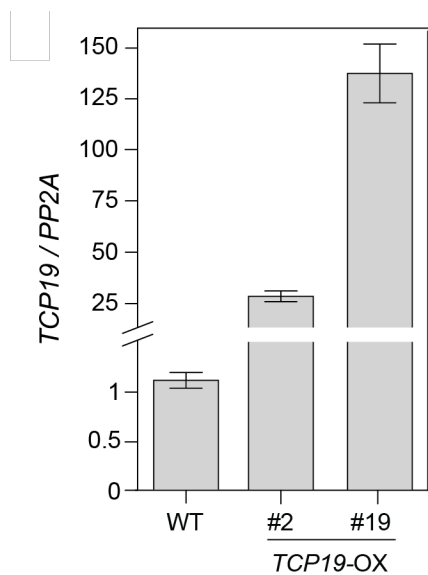
Supplemental Figure 2.7. TCP19 promoter activity is enriched in the shoot apex.

(A) Bioluminescence from shoot apex (Ap) or leaf (Lf) regions of 8- and 13-day old *TCP19::LUC+* and *CCA1::LUC+* seedlings. Columns and error bars show the mean + SEM of three independent experimental averages ($n = 34$ total individuals). (B) Ap/Lf bioluminescence ratios for *TCP19::LUC+* and *CCA1::LUC+* seedlings analyzed in (A) and Figure 1.3E. Data show mean + SEM. (C and D) Representative true color (top) and pseudo-colored bioluminescence intensity (bottom) images of 8-day old (C) and 13-day old (D) (ZT2 and ZT142, respectively) *TCP19::LUC+* and *CCA1::LUC+* seedlings analyzed in (A) and (B). Scale bars, 1 mm.



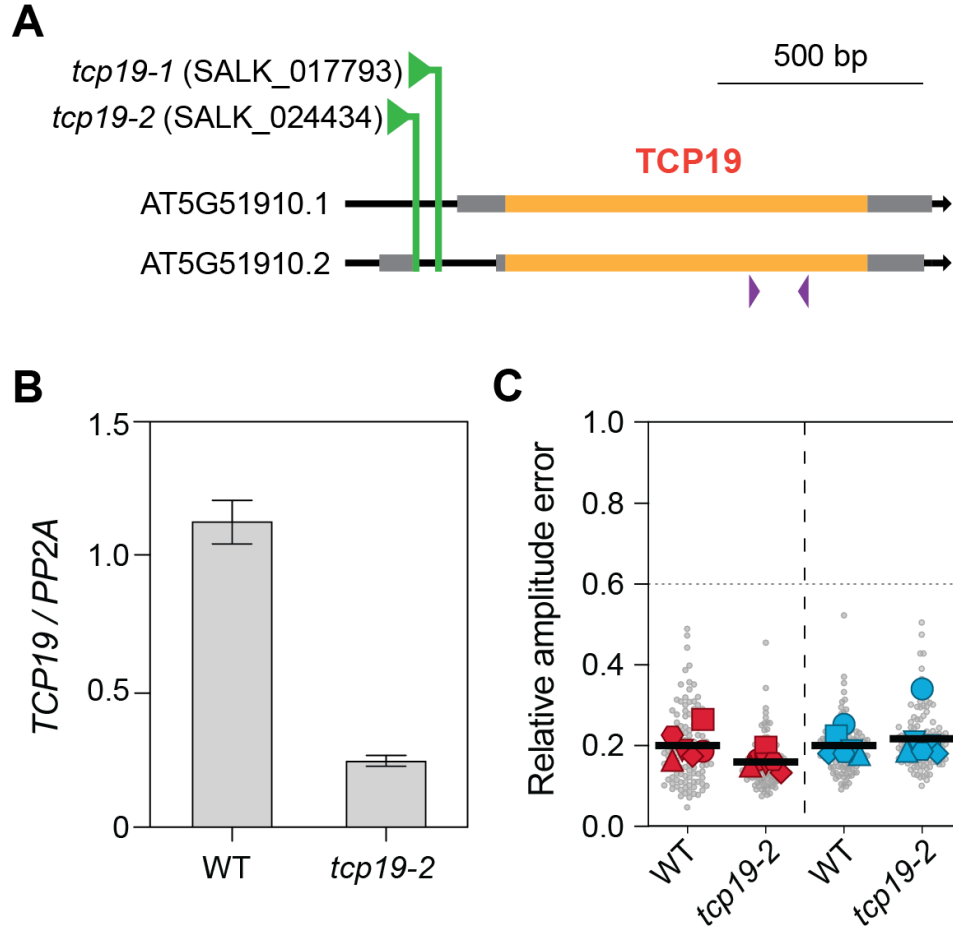
Supplemental Figure 2.8. Shoot apex-specific expression of *TCP19::LUC+* persists over time.

(A) Bioluminescence analysis of *TCP19::LUC+* expression in shoot apex (Ap) and leaf (Lf) regions of intact plants. Counts are background-subtracted to aid direct comparison of Ap and Lf regions. Data show average $\pm$ SD values ($n = 6-12$) from one out of three independent experiments (represented in (B)). White and gray shaded background regions indicate subjective day and night periods, respectively. (B) Rhythmic robustness (measured as relative amplitude error, RAE) of *TCP19::LUC+* expression in whole plants. Plot shows mean values (black bars) of independent experimental averages (large colored symbols, $n = 3$) and individual values (small gray symbols, $n = 30-37$). Dotted line (RAE = 0.6) indicates the rhythmicity threshold value.



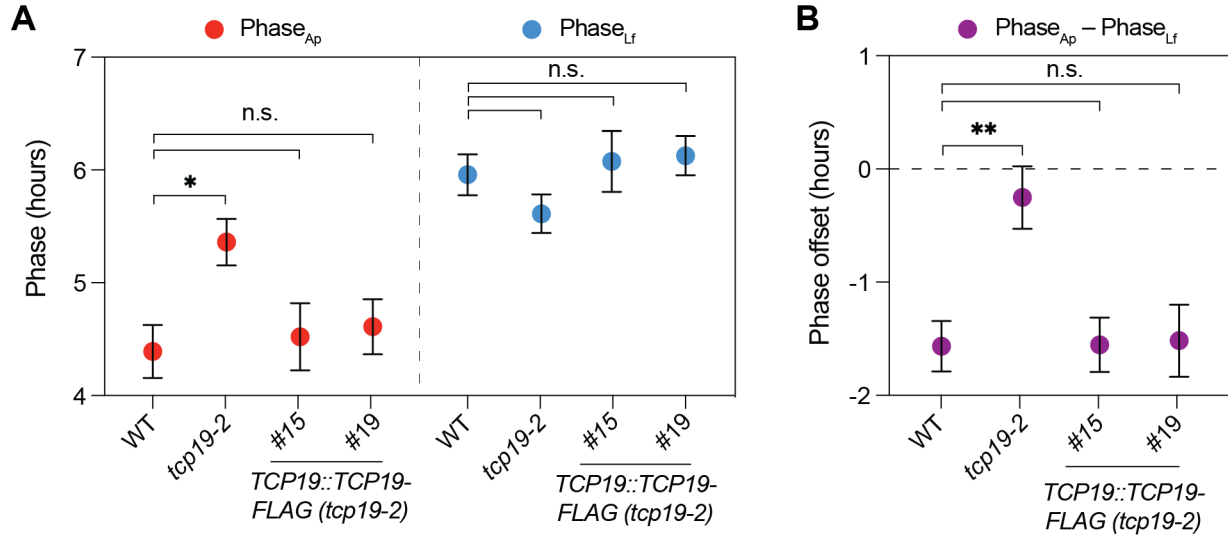
Supplemental Figure 2.9. TCP19-OX lines overexpress TCP19 transcript.

TCP19 expression in *TCP19-OX* lines determined by quantitative polymerase chain reactions. Data show mean $\pm$ SEM for three replicate experiments.



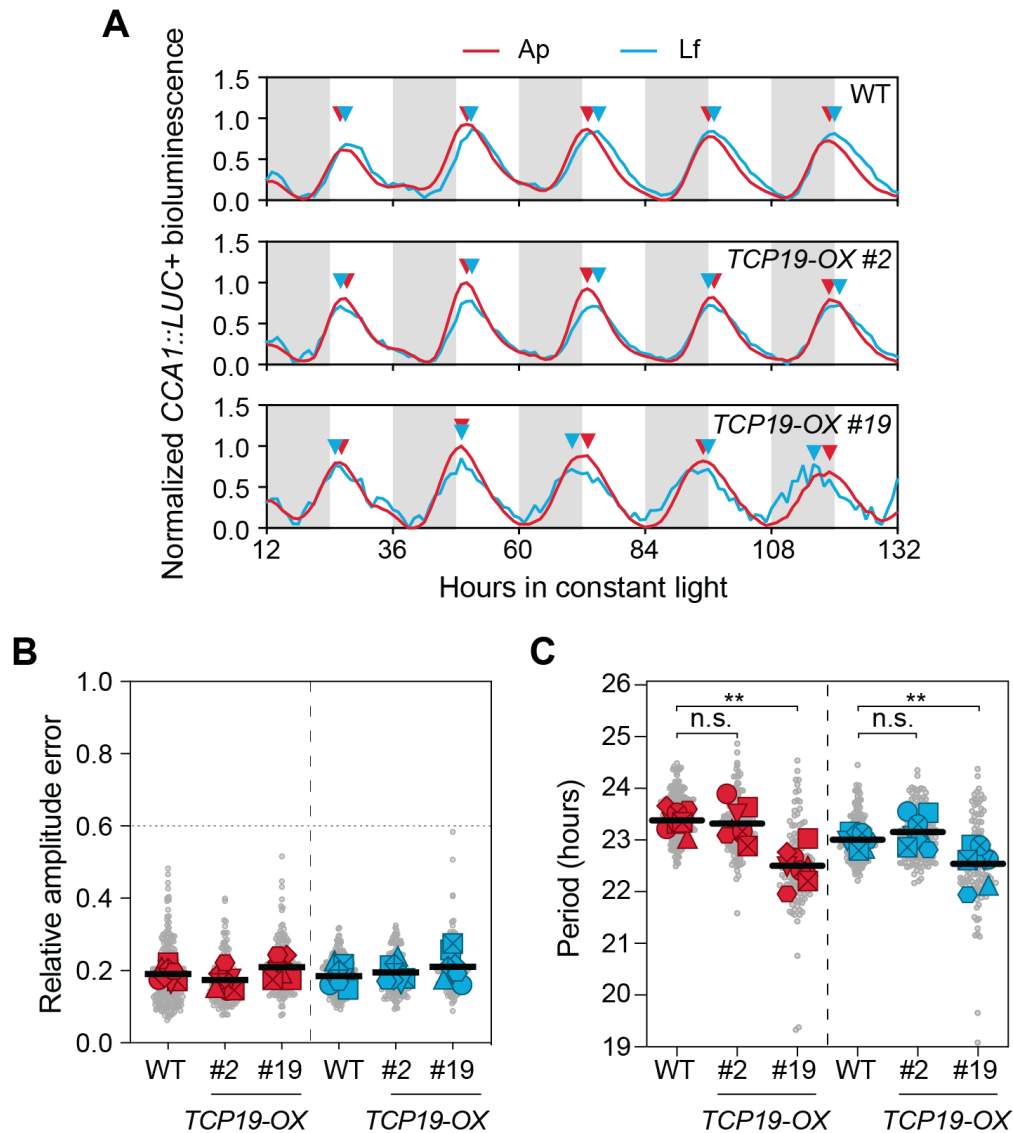
Supplemental Figure 2.10. The robustness of CCA1::LUC+ rhythms is not affected in plants carrying the *tcp19-2* loss-of-function mutation.

(A) Location of *tcp19-2* T-DNA insertion (green flag) in relation to *TCP19* gene models. Orange boxes, coding sequence; gray boxes, untranslated regions; black lines, intronic and intergenic regions; purple arrows, location of qPCR primers; black arrows indicate direction of transcription. (B) *TCP19* expression in wild-type (WT) and *tcp19-2* plants determined by quantitative polymerase chain reactions. Data show mean $\pm$ SEM for three replicate experiments. (C) Rhythmic robustness (measured as relative amplitude error, RAE) of *CCA1::LUC*+ expression in intact WT and *tcp19-2* plants. Data show mean values (black bars) of independent experimental averages (large colored symbols with shapes corresponding to each experiment, $n = 6$). Values for individual seedlings are shown by the gray symbols ($n = 118$). Dotted line (RAE = 0.6) indicates the rhythmicity threshold value.



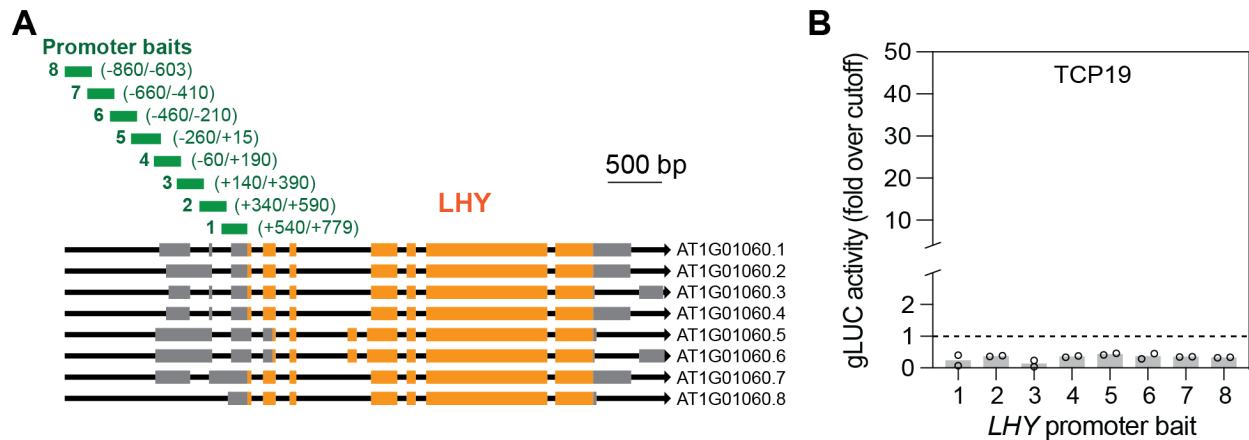
Supplemental Figure 2.11. The TCP19::TCP19-FLAG minigene construct rescues the phase offset phenotype of *tcp19-2* plants.

(A and B) Phase (A) and phase offset (B) of *CCA1::LUC* expression in wild type, *tcp19-2* and two independent lines carrying the *TCP19::TCP19-FLAG* construct in the *tcp19-2* background. Plots show mean $\pm$ SEM values ($n = 16-31$). Statistical comparisons were performed using one-way ANOVAs with Dunnet's multiple comparisons test to WT. (n.s., not significant; * $P < 0.05$; ** $P < 0.01$).



Supplemental Figure 2.12. Effects of TCP19 overexpression on *CCA1::LUC+* rhythms.

(A) Bioluminescence analysis of *CCA1::LUC+* expression in the shoot apex (Ap) and leaf (Lf) regions of intact wild-type (WT) and *TCP19* overexpression (*TCP19-OX* lines #2 and #19) plants. Data show average values ($n = 16$) from one out of eight independent experiments (represented in B and C). Arrows indicate peak measurement within each cycle. White and gray shaded background regions indicate subjective day and night periods, respectively. (B and C) Rhythmic robustness (measured as relative amplitude error, RAE) (B) and period (C) of *CCA1::LUC+* expression in WT and *TCP19-OX* plants. Data show mean values (black bars) of independent experimental averages (large colored symbols with shapes corresponding to each experiment, $n = 8$). Values for individual seedlings are shown by the gray symbols ($n = 139-237$). Dotted line (RAE = 0.6) indicates the rhythmicity threshold value (B). Statistical comparisons in (C) were performed with experimental means ($n = 8$) using one-way ANOVAs blocked by experiment, followed by Dunnett's multiple comparisons test between WT and *TCP19-OX* lines. (n.s., not significant; $**P < 0.01$).



Supplemental Figure 2.13. TCP19 does not bind to the LHY promoter.

(A) Diagram of yeast one-hybrid (Y1H) promoter bait fragments (green boxes) in relation to *LHY* gene models (untranslated regions, gray boxes; protein coding sequence, orange boxes; intronic and intergenic regions, black lines; arrows indicate direction of transcription). Coordinates are relative to the transcription start site of the AT1G01060.1 gene model. (B) Gaussia luciferase (gLUC) reporter activity from two independent Y1H experiments testing the interaction of TCP19 with the *LHY* promoter bait regions indicated in (A). Bars indicate the mean value of independent experiments ($n = 2$) and dashed lines the interaction cutoff. Open circles plot values from each replicate experiment.

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Chapter 3 LOCALIZED BACTERIAL INFECTION INDUCES ORGAN-SPECIFIC CHANGES IN THE CIRCADIAN TRANSCRIPTOME OF THE PLANT SHOOT SYSTEM

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Abstract

Many organisms synchronize organ-specific biological processes with daily environmental fluctuations via their internal circadian clock. Previously, we reported a circadian transcriptome survey of two major organs of the plant shoot system: the shoot apex and leaves. Here, we present a parallel transcriptomic analysis in the shoot apex and leaves of *Arabidopsis* plants that were subjected to a single-leaf bacterial infection. We performed mRNA sequencing on the uninfected shoot apices and leaves of treated seedlings to study the systemic effects of bacterial infection on the rhythmic transcriptome. We found that organs distal to the infection site were greatly affected by the localized treatment. Shoot apices had a large number of up-regulated genes in the infection condition, but fewer rhythmic genes than shoot apices from uninfected plants. In contrast, the leaves had fewer differentially expressed genes than the shoot apices, but also had more rhythmic genes than leaves from uninfected plants. Differentially expressed genes and genes gaining or losing rhythmicity after infection were associated with known infection-related biological processes ontologies and transcriptional regulatory motifs, in addition to processes and motifs that have not been previously linked to bacterial infection. Finally, we saw that genes rhythmic in the shoot apex had a striking phase delay in the infection condition, which abolished the shoot apex-first phase offset phenotype previously reported in uninfected plants. This was mirrored by a loss of the shoot apex-first phase offset in core clock genes. From these results we conclude that a localized single-leaf bacterial infection has systemic and organ-specific effects on gene expression, altering the identity of rhythmic genes and shifting the timing of their expression by changing the phase of upstream core clock genes.

Main text

The circadian clock is an endogenous timing mechanism that allows organisms to anticipate and respond to daily changes in their environment (1). In multicellular organisms, the clock function can vary across cells, tissues, and organs to allow for spatially-specific rhythmic activity (2-4). This is particularly relevant for sessile autotrophs like plants, which must respond to dramatic environmental fluctuations throughout the day and year, including abiotic and biotic stressors. Untangling the complex regulation and spatial coordination of the plant circadian clock is an active area of research (5-9). Previously, we used time course mRNA sequencing (mRNA-seq) of *Arabidopsis thaliana* (Arabidopsis) shoot apices and leaves to develop a model of the plant clock function in which organ-specific mechanisms modulate the phase of rhythmic genes through upstream regulation of the core clock, while the amplitude of rhythmicity is controlled independently in each organ downstream of the core clock (see Chapter 2). Here, we present the results of a parallel study of the shoot apex and leaf transcriptomes from plants that were subjected to a single-leaf infection of *Pseudomonas syringae* pathovar *tomato* (*Pst*) DC3000. We found that a single-leaf infection has profound effects on both the expression and rhythmicity of genes in distal uninfected organs of the plant shoot system. Furthermore, infection-associated changes in the timing of rhythmic gene expression were underpinned by changes in the phase of core clock gene expression. Overall, we show that systemic transcriptional changes result from a localized pathogen attack, suggesting that circadian regulation of gene expression is more complex and coordinated than previously understood.

Localized *Pst* DC3000 infection induces systemic and organ-specific differential gene expression

In Chapter 2 we reported a comparative time course transcriptomic analysis of *Arabidopsis* shoot apices and leaves dissected from seedlings grown in soil under free-running conditions after entrainment in light-dark cycles. Here, we present a study conducted in parallel, in which we collected shoot apices and leaves from seedlings that were treated with a single-leaf *Pst* DC3000 infection. The collected organs were distal to the treatment site and therefore uninfected. However, we hypothesized that mobile signals from the infection site would induce transcriptional changes across the entire shoot system. To test this hypothesis, we performed mRNA-seq analyses on distal uninfected shoot apices and leaves after a localized single-leaf *Pst* DC3000 infection (Figure 3.1).

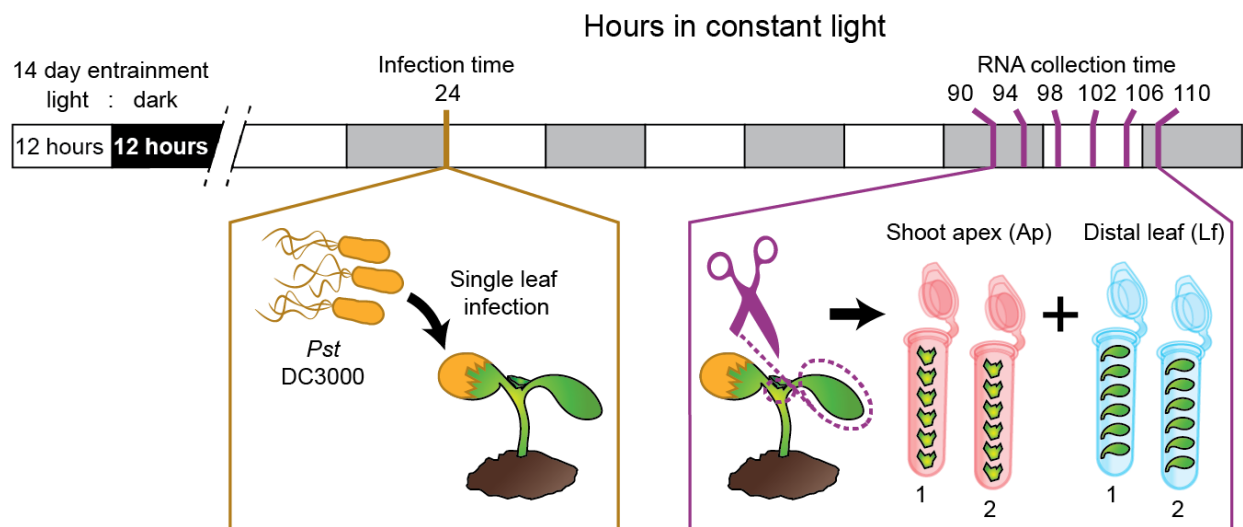


Figure 3.1. Experimental design of time course RNA collection from distal uninfected shoot apices and leaves after a single-leaf *Pseudomonas syringae* pathovar *tomato* (*Pst*) DC3000 infection.

Tubes 1 and 2 represent two biologically independent replicate experiments.

To verify the quality of sequencing data from replicate experiments and visualize the clustering of sequencing data, we performed a principal component (PC) analysis. This suggested that the shoot apex was more profoundly affected by the single-leaf infection, as

evidenced by the clear separation between infected and uninfected shoot apex samples along PC1, while leaf samples were more variable (Figure 3.2). Nevertheless, replicate samples from both shoot apices and leaves were in close proximity on at least one of the axes, indicating the good quality of independent replicate experiments.

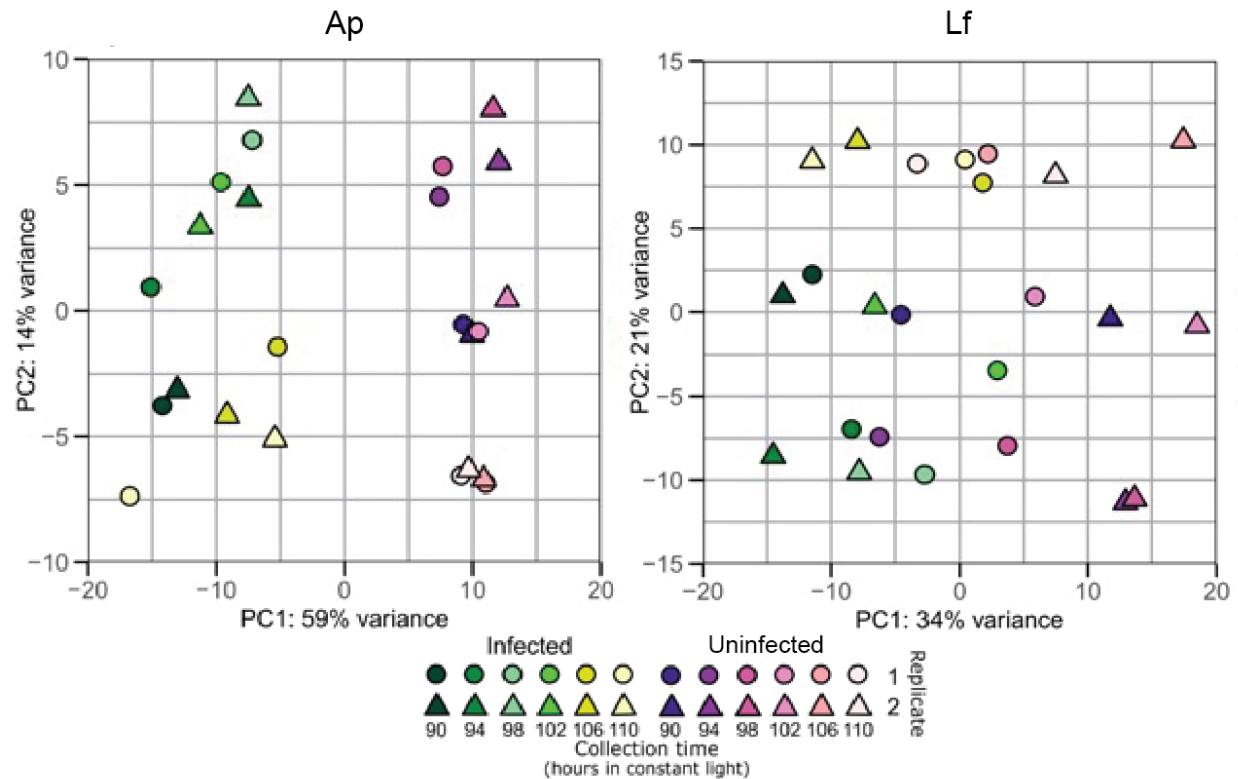


Figure 3.2. Principal component analysis of sequencing samples.

Data groupings based on the two primary sources of variance (PC1 and PC2) in the gene expression profiles from the 24 mRNA-seq samples (two independent biological replicates for each of six timepoints from both the shoot apex (Ap) and leaves (Lf)).

Next, we investigated whether infection influenced gene expression in distal uninfected organs of the shoot system, using DESeq2 (10) to identify differentially expressed genes (DEGs) between the organs of infected and uninfected seedlings. There were 787 upregulated DEGs and 52 downregulated DEGs in the shoot apex, whereas there were 222 upregulated DEGs and 53

downregulated DEGs in the leaves (Figure 3.3, A and B). These DEGs were largely organ-specific, as only 165 were differentially expressed in both the shoot apex and leaves (Figure 3.3C). This organ-specific differential gene expression was not a result of organ-specific expression generally, as most organ-specific DEGs were expressed in both organs; in other words, most organ-specific DEGs were expressed in both organs, but only exhibited significant differences in expression between infection conditions in one of the organs (Figure 3.3D). Altogether, our analyses showed that localized pathogen infection in just one leaf triggers systemic gene expression changes that are uniquely regulated in the shoot apex and leaves.

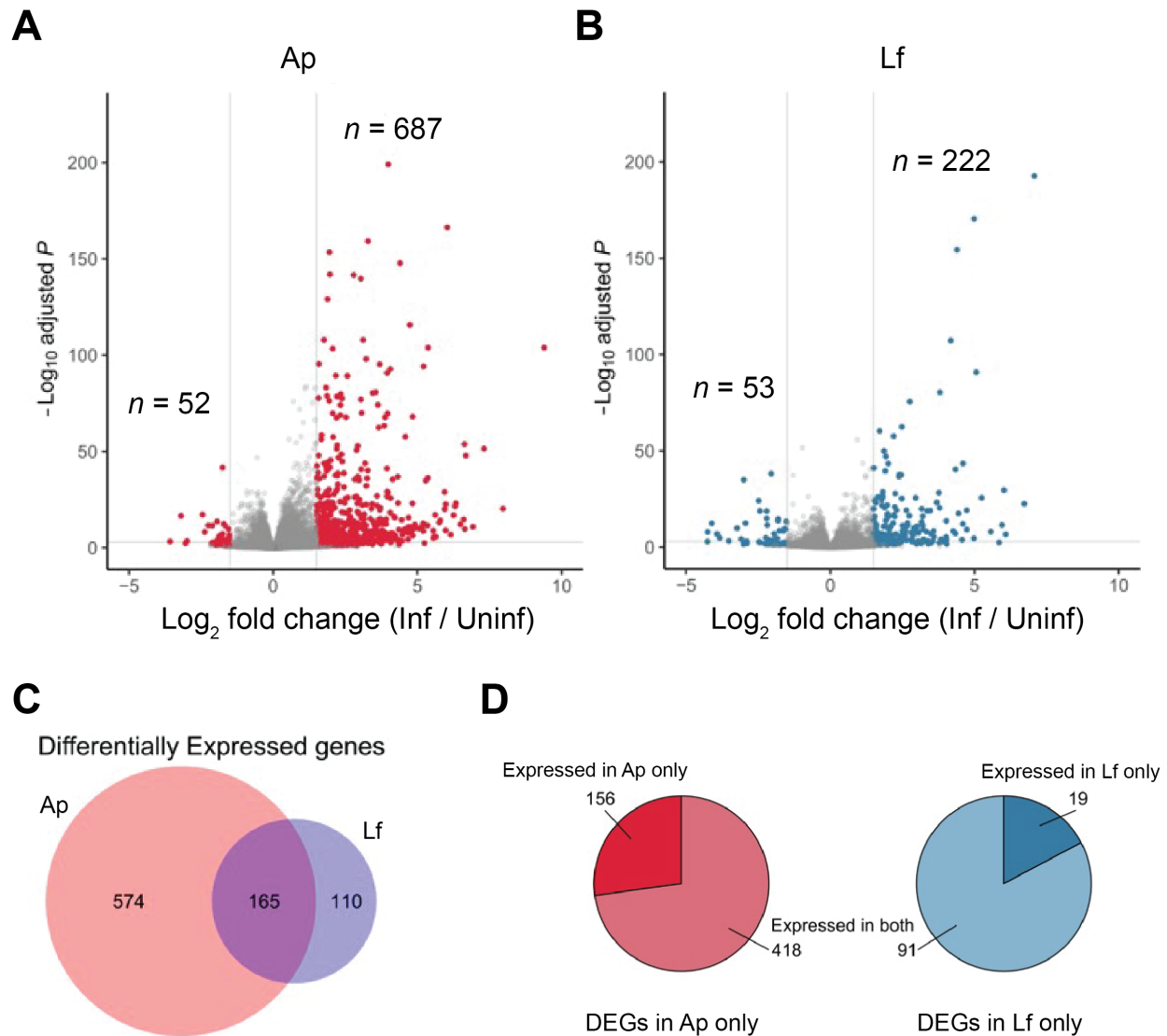


Figure 3.3. A localized bacterial infection differentially regulates transcriptional changes across the shoot system.

(A and B) Gene expression fold changes between organs (shoot apex (Ap) in (A) and leaves (Lf) in (B)) from infected (Inf) and uninfected (Uninf) seedlings plotted by the statistical significance of the difference. Significantly differentially expressed genes (colored red in (A) and blue in (B), with *ns* annotated) were defined by $P < 0.05$ and $|\log_2 \text{fold change}| > 1.5$. Only genes expressed in both organ-conditions were considered. **(C)** Numbers of differentially expressed genes categorized by their organ(s) of differential expression. **(D)** Numbers of organ-specific differentially expressed genes (DEGs) categorized by their organ(s) of expression.

To uncover biological functions important for systemic responses to localized bacterial infection, we performed a gene ontology (GO) enrichment analysis for biological processes associated with the identified sets of DEGs (Figure 3.4). We found that, like DEGs, enriched GO terms were largely organ-specific. While several terms were associated with defense against a bacterial attack (e.g. leaf senescence, responses to oxidative stress and salicylic acid, etc.) as expected from a *Pseudomonas* infection, we also unexpectedly found terms related to defense against necrotrophic pathogens such as insects and fungi. This implies a partially overlapping or generalized stress response to biotic attacks.

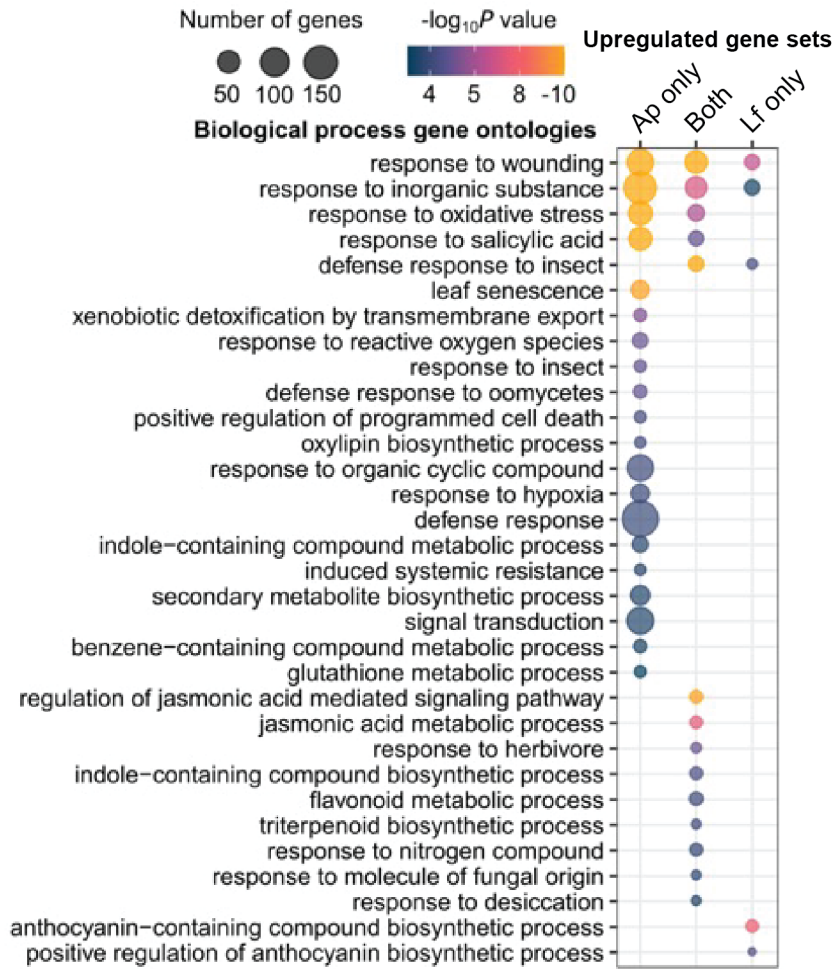


Figure 3.4. Biological process gene ontologies of single-leaf infection-upregulated genes across the shoot system are largely organ-specific.

Semantically representative biological process ontologies enriched in gene sets upregulated by infection.

Finally, to gain insight into how transcriptional responses to single-leaf infection might be wired, we performed motif enrichment analysis with the promoters of DEGs to search for signatures of transcription factor (TF) regulatory input. Sequences resembling WRKY binding sites and G-boxes (CACGTG) repeatedly appeared in several upregulated gene sets (Figure 3.5A), both of which have been linked with responses to pathogens (11, 12). A single sequence

was found to be enriched among all downregulated genes, which bears a loose resemblance to the GC or GCC box associated with zinc finger and ethylene-responsive element binding factors, perhaps indicating an avenue for negative input of these downregulated genes; however, more investigation into this enriched motif is need to validate its role in transcriptional control (Figure 3.5B).

A All upregulated DEGs ($n = 735$)

Rank	Motif	P-value	log P-value	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-33	-7.611e+01	31.84%	14.03%	294.9bp (371.7bp)	PIF7(bHLH)/col-PIF7-DAP-Seq(GSE60143)/Homer(0.890) More Information Similar Motifs Found
2		1e-18	-4.332e+01	39.18%	23.99%	333.6bp (389.1bp)	WRKY55/MA1305.1/Jaspar(0.915) More Information Similar Motifs Found
3		1e-13	-3.187e+01	3.40%	0.41%	291.6bp (381.9bp)	CMTA3/MA0970.1/Jaspar(0.715) More Information Similar Motifs Found
4		1e-13	-3.096e+01	3.95%	0.61%	376.0bp (413.9bp)	WRKY63/MA1092.1/Jaspar(0.633) More Information Similar Motifs Found
5		1e-13	-3.032e+01	26.80%	15.86%	344.8bp (392.7bp)	PIF3/MA0560.1/Jaspar(0.784) More Information Similar Motifs Found
6		1e-12	-2.872e+01	2.86%	0.31%	363.3bp (408.9bp)	GATA15(C2C2gata)/col-GATA15-DAP-Seq(GSE60143)/Homer(0.549) More Information Similar Motifs Found
7		1e-12	-2.868e+01	10.20%	3.93%	314.5bp (374.7bp)	HSF6(HSF)/col-HSF6-DAP-Seq(GSE60143)/Homer(0.666) More Information Similar Motifs Found
8		1e-12	-2.823e+01	4.63%	0.96%	380.9bp (369.7bp)	bHLH28(bHLH)/col-bHLH28-DAP-Seq(GSE60143)/Homer(0.655) More Information Similar Motifs Found
9		1e-12	-2.783e+01	1.63%	0.05%	407.4bp (293.7bp)	ERF018/MA1048.1/Jaspar(0.610) More Information Similar Motifs Found

All DEGs upregulated in Ap ($n = 672$)

1		1e-27	-6.239e+01	34.97%	17.21%	292.8bp (383.1bp)	ABF2/MA0941.1/Jaspar(0.802) More Information Similar Motifs Found
2		1e-14	-3.299e+01	15.48%	6.67%	305.6bp (392.0bp)	WRKY55/MA1305.1/Jaspar(0.895) More Information Similar Motifs Found
3		1e-13	-3.047e+01	10.71%	3.89%	318.6bp (379.7bp)	HSF6(HSF)/col-HSF6-DAP-Seq(GSE60143)/Homer(0.663) More Information Similar Motifs Found
4		1e-13	-3.023e+01	11.01%	4.09%	301.2bp (359.5bp)	WRKY50/MA1317.1/Jaspar(0.738) More Information Similar Motifs Found
5		1e-12	-2.886e+01	1.79%	0.05%	407.4bp (297.6bp)	ERF018/MA1048.1/Jaspar(0.610) More Information Similar Motifs Found
6		1e-12	-2.872e+01	16.52%	7.89%	337.7bp (414.4bp)	NAC025/MA0935.1/Jaspar(0.805) More Information Similar Motifs Found

DEGs upregulated in Ap only ($n = 518$)

Rank	Motif	P-value	log P-value	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-16	-3.757e+01	16.41%	5.89%	315.8bp (380.6bp)	WRKY55/MA1305.1/Jaspar(0.909) More Information Similar Motifs Found
2		1e-14	-3.387e+01	14.48%	5.11%	304.0bp (378.9bp)	HSF6(HSF)/col-HSF6-DAP-Seq(GSE60143)/Homer(0.705) More Information Similar Motifs Found
3		1e-12	-2.943e+01	11.97%	4.08%	350.1bp (364.4bp)	MP0010.1_Homeobox_class/Jaspar(0.649) More Information Similar Motifs Found
4		1e-12	-2.867e+01	5.02%	0.76%	343.7bp (383.2bp)	bHLH157(bHLH)/col-bHLH157-DAP-Seq(GSE60143)/Homer(0.748) More Information Similar Motifs Found

All DEGs upregulated in Lf ($n = 217$)

Rank	Motif	P-value	log P-value	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-23	-5.412e+01	46.08%	16.41%	278.8bp (374.4bp)	OJ1058_F05.8/MA1033.1/Jaspar(0.886) More Information Similar Motifs Found

DEGs upregulated in both Ap and Lf ($n = 154$)

Rank	Motif	P-value	log P-value	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-22	-5.110e+01	58.44%	21.62%	258.1bp (385.2bp)	PIF7(bHLH)/col-PIF7-DAP-Seq(GSE60143)/Homer(0.849) More Information Similar Motifs Found

B All downregulated DEGs ($n = 93$)

Rank	Motif	P-value	log P-value	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-12	-2.768e+01	12.90%	0.61%	253.9bp (336.9bp)	TF3A(C2H2)/col-TF3A-DAP-Seq(GSE60143)/Homer(0.705) More Information Similar Motifs Found

Figure 3.5. Enriched motifs in DEG promoters provide insight on wiring of regulatory response to localized bacterial infection.

(A and B) Sequence logos for significantly enriched motifs in the promoter regions of upregulated (A) and downregulated (B) DEGs.

Localized *Pst* DC3000 infection induces systemic and organ-specific changes in circadian gene expression

In order to ascertain the effects of localized bacterial infection on circadian transcription across the shoot system, we assessed the rhythmicity of gene expression over the time course mRNA-seq dataset. Rhythmic gene expression was highly organ-specific (Figure 3.6A), as we saw in our previous investigations on rhythmic gene expression across the shoot system in uninfected plants (Chapter 2). Importantly, organ-specific rhythmic genes were generally expressed in both organs (albeit rhythmic in only one) and had similar amplitude distributions between organs and infection conditions (Figure 3.6, B and C). This supports the idea that the effect of distal infection on rhythmic gene expression involves precise regulation of certain subsets of genes depending on organ context and does not cause a general organ-wide change in expression level or the robustness of oscillations.

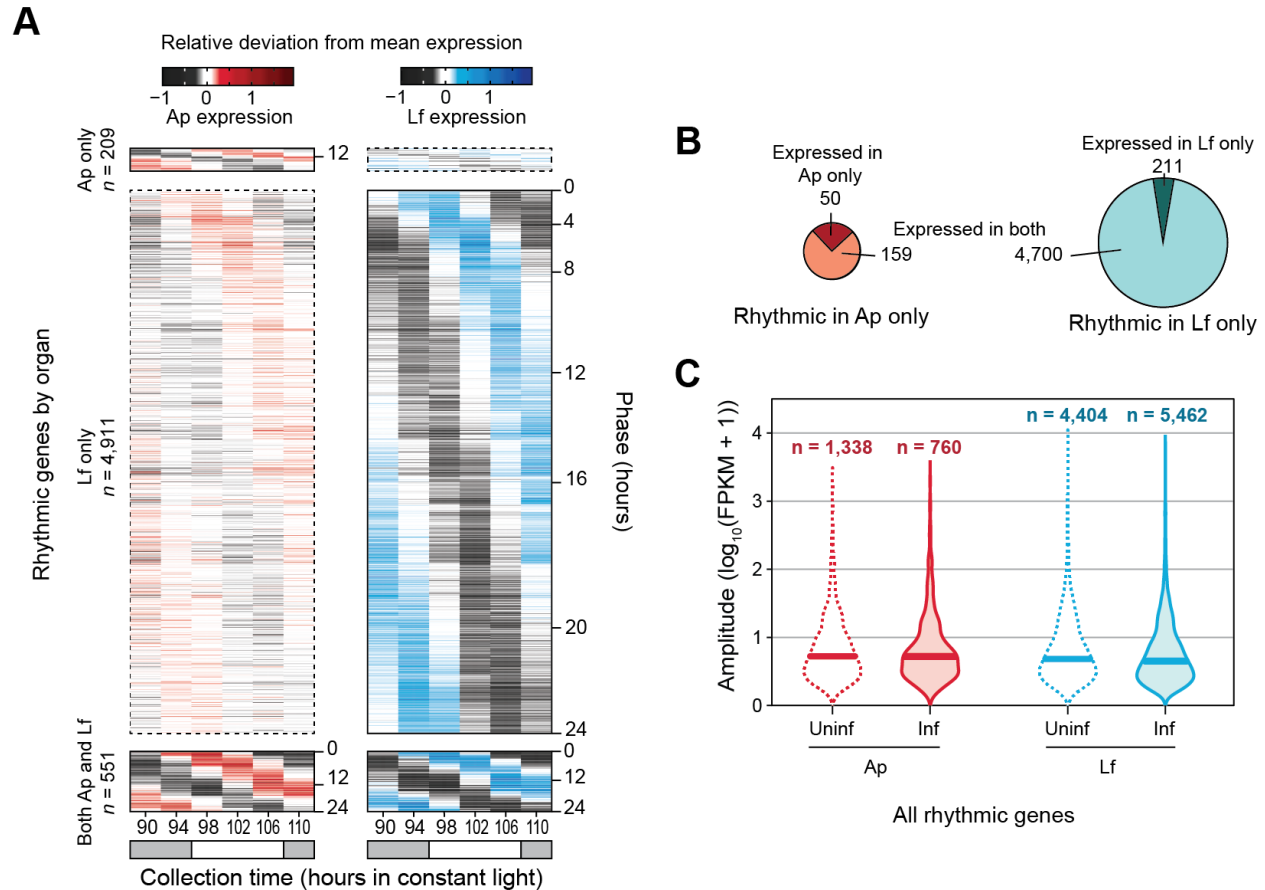


Figure 3.6. Expression patterns of rhythmic genes across the shoot system in plants subjected to single-leaf bacterial infection.

(A) Relative transcript levels over the time course of genes rhythmic in shoot apex (Ap) and leaves (Lf). Each row of the heatmap is one gene. Genes are ordered by ascending phase in the rhythmic organ. (B) Numbers of organ-specific rhythmic genes categorized by their organ(s) of expression. (C) Amplitude distributions for rhythmic genes in each organ in both the infected (Inf) and uninfected (Uninf; data reproduced from Chapter 2) conditions. Horizontal bars indicate the median.

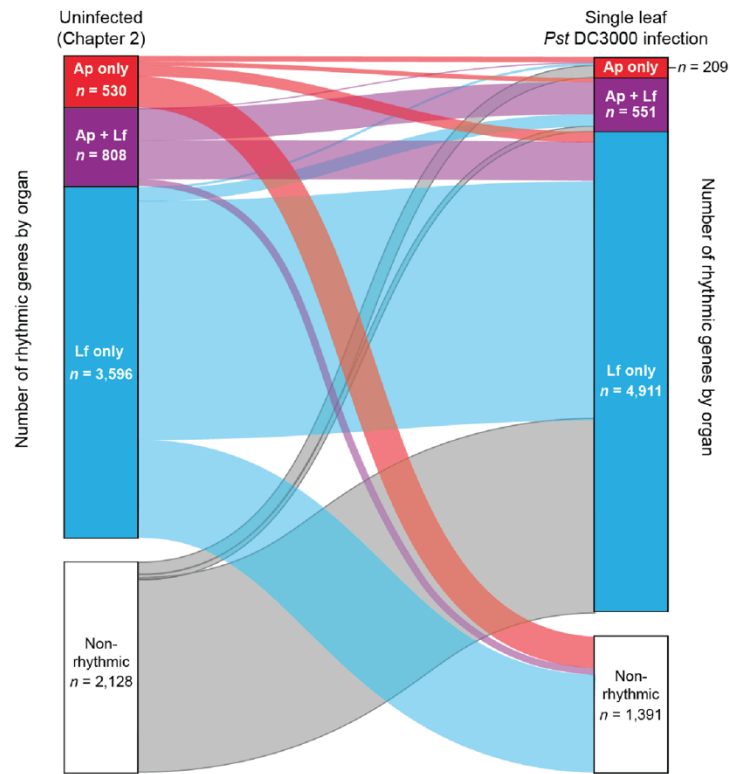
Next, with the aim of uncovering infection-induced changes in circadian gene expression, we assessed the overlap in rhythmic gene sets between organs from infected and uninfected plants (Figure 3.7A). We found that there were fewer rhythmic genes in the shoot apex after infection than in uninfected plants. The opposite was true for the numbers of rhythmic genes in leaves, with more genes being rhythmic in plants that were subjected to infection. Interestingly,

more than 2,000 genes that were not rhythmic in the uninfected plants became rhythmic in the infection condition, almost all of them specific to the leaves. This observation is robust at different levels of the statistical significance cutoff, as seen in the distributions of Benjamini-Hochberg Q values for genes that are rhythmic in only one condition (Figure 3.7, B and C). In addition, the genes that exhibited organ-specific rhythmicity only after infection were generally expressed in both organs but were only rhythmic in one, indicating that these gene sets were primarily defined by rhythmic regulation and not expression level (Figure 3.7D). Overall, these findings suggest that rhythmicity may be important for specific systemic responses to localized pathogen attacks.

Figure 3.7. A localized bacterial infection triggers organ-specific regulation of circadian gene expression across the shoot system.

(A) Numbers of rhythmic genes categorized by their organ(s) of expression. Bars on the left represent data from uninfected seedlings (reproduced from Chapter 2), while bars on the right show rhythmic genes in seedlings subjected to a single-leaf *Pst* DC3000 infection. Ribbons link identical genes between the two datasets and are colored by their organ(s) of rhythmic expression in the uninfected samples. (B and C) Distributions of Benjamini-Hochberg Q values for genes that gain (B) or lose (C) rhythmicity after infection. Boxes show median with interquartile range (IQR). Whiskers extend an additional 1.5 x IQR. Colored lines connect Q values (black circles) of the same gene in the two conditions. Dashed line marks the significance level cutoff used to define rhythmicity ($Q < 0.05$). (D) Numbers of genes that exhibit organ-specific rhythmicity only after infection categorized by their organ(s) of expression.

A

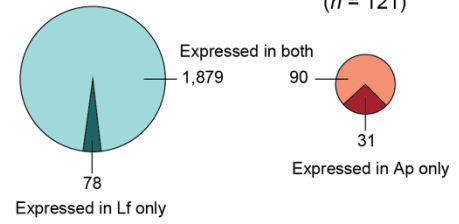


D

Genes that gain rhythmicity after infection

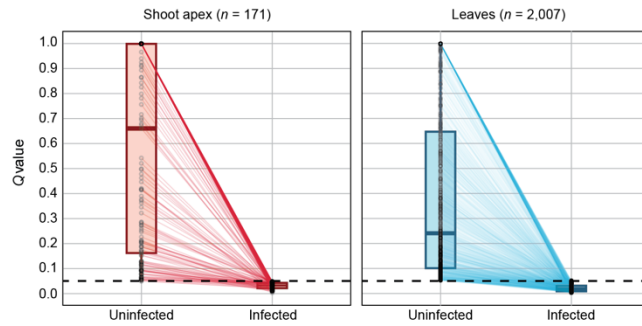
Rhythmic in Lf only
($n = 1,957$)

Rhythmic in Ap only
($n = 121$)



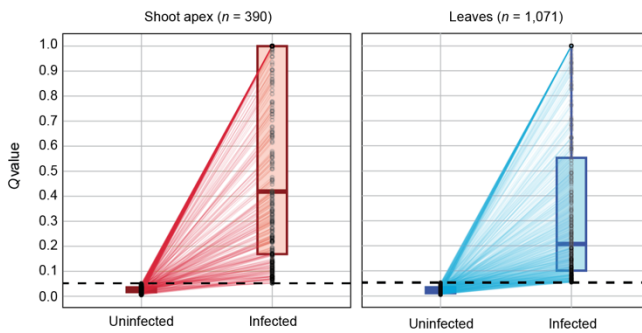
B

Genes that gain rhythmicity after infection



C

Genes that lose rhythmicity after infection



In Chapter 2 we saw that, in the uninfected context, genes that were rhythmic in both organs had a shoot apex-first offset in peak expression time. Therefore, we used the current dataset to investigate the phase of peak expression for genes that were rhythmic after a single-leaf *Pst* DC3000 infection. In comparison to the uninfected dataset, genes that were rhythmic in the infection context had a strong shift in their phases in the shoot apex, with a less dramatic effect on phases in the leaves (Figure 3.8A). Furthermore, when we calculated the offset in phase times between the shoot apex and leaves for each gene that was rhythmic in both organs after infection, we saw that the shoot apex-first phase offset observed in uninfected plants was abolished in infected individuals, with roughly equal numbers of genes peaking first in the leaves as in the shoot apex (Figure 3.8B). These findings indicate that a single-leaf *Pst* DC3000 infection disrupts the timing of rhythmic gene expression in distal uninfected tissues across the shoot system, especially in the shoot apex.

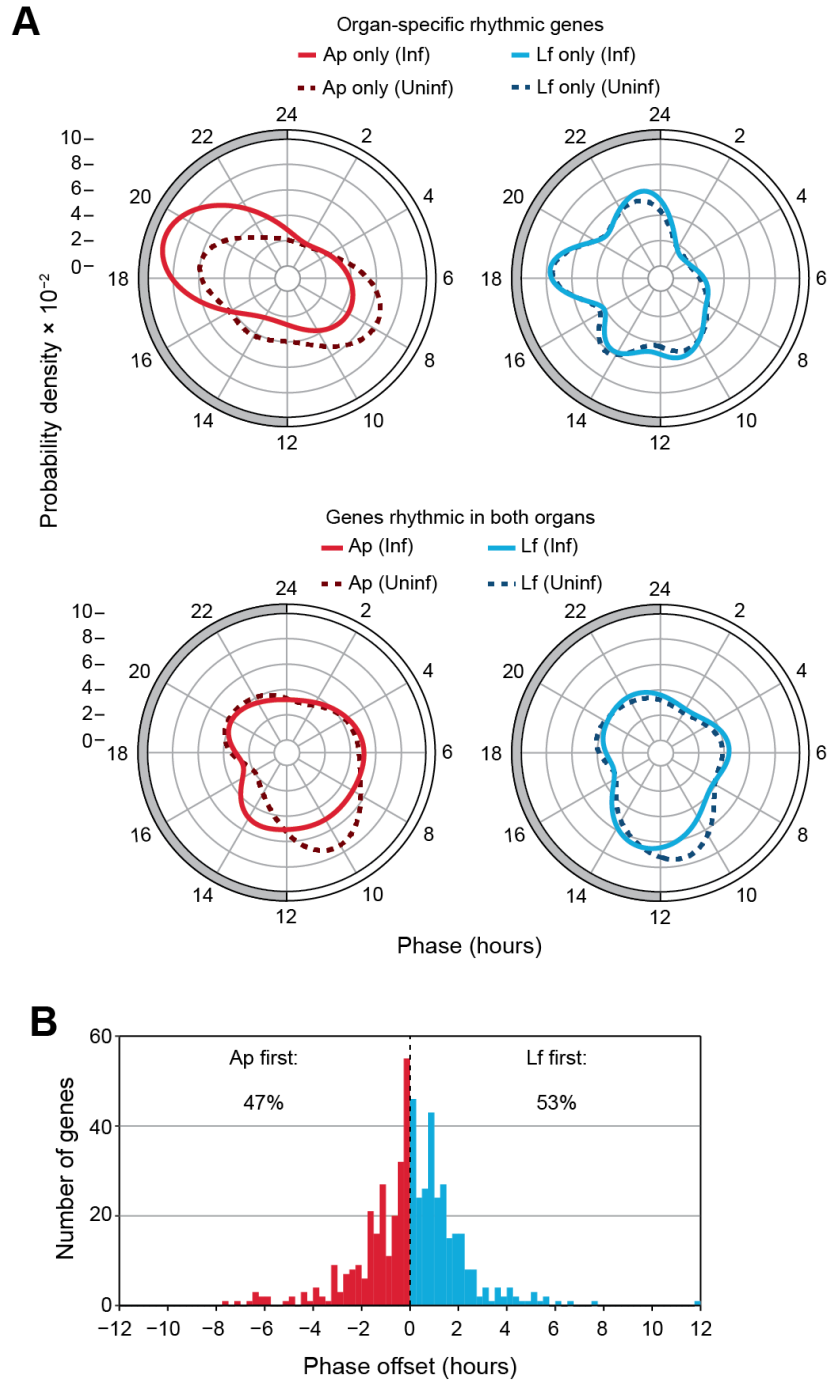


Figure 3.8. Single-leaf bacterial infection disrupts timing of rhythmic gene expression across the shoot system.

(A) Density distributions of peak expression phases for genes rhythmic in one organ (top) or both organs (bottom) for both infected (Inf) and uninfected (Uninf) plants. (B) Histogram of phase offsets ($Phase_{Ap} - Phase_{Lf}$) for each gene rhythmic in both organs of infected plants (bin width = 15 min.).

Based on the differences in rhythmic gene sets between the infected and uninfected plants, we hypothesized that the rhythmicity of certain processes may be shut down in the shoot apex upon a single-leaf infection, whereas others are ramped up in distal uninfected leaves. Therefore, we performed GO enrichment analysis for biological processes associated with different rhythmic gene sets (Figure 3.9). We found that terms associated with loss of rhythmicity in the shoot apex include “cell division” and “anatomical structure arrangement,” which may relate to the reduced growth and delayed flowering upon single-leaf infection previously reported (13). Furthermore, several terms associated with genes gaining rhythmicity in distal leaves after infection are related to metabolic and protein production processes, which may indicate rhythmic regulation of machinery needed for leaf-specific responses to distal infection.

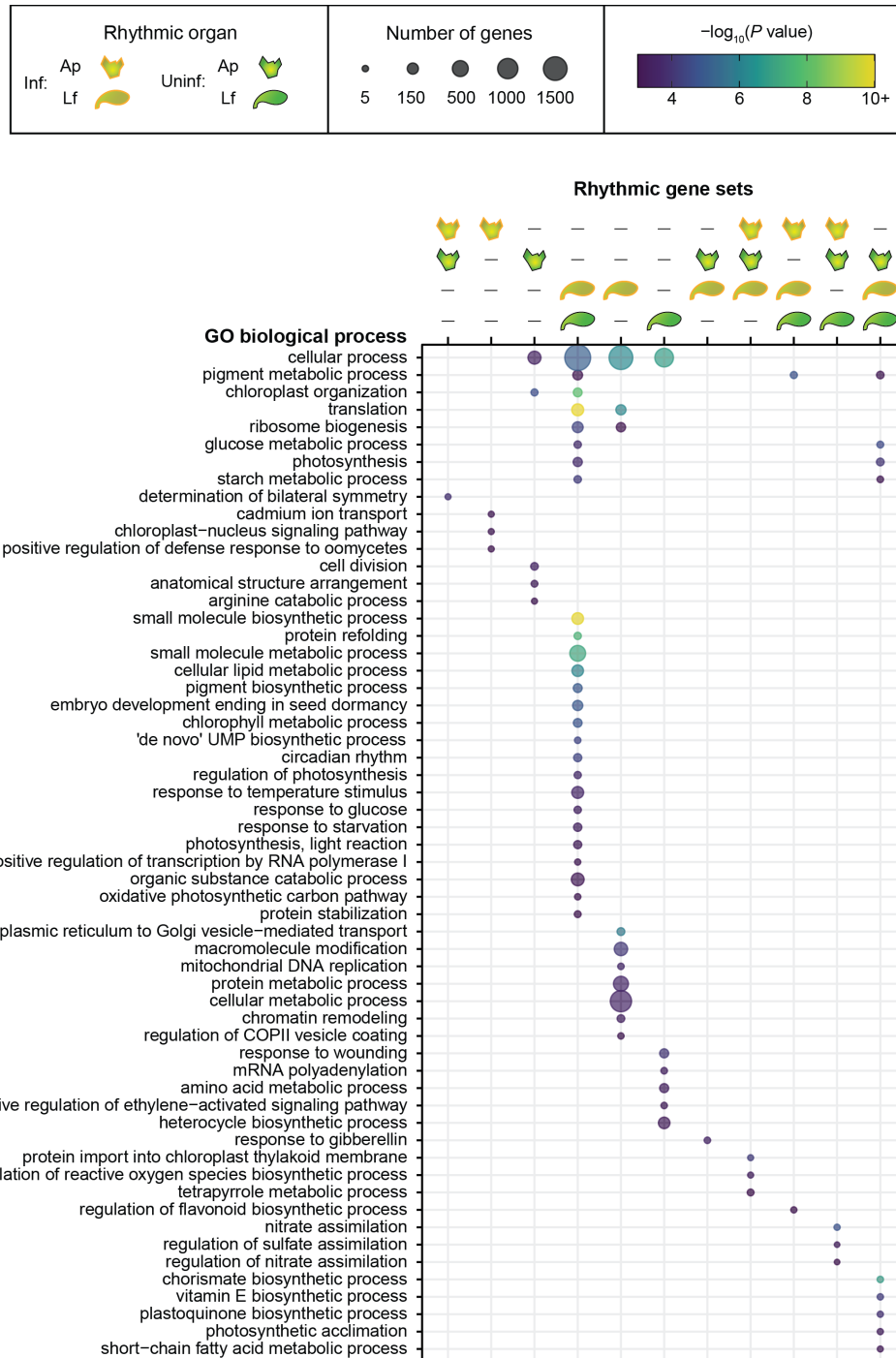


Figure 3.9. Biological process gene ontologies of gene sets based on their rhythmic status in the organs of uninfected (Uninf) and single-leaf-infected (Inf) seedlings.

Semantically representative biological process ontologies enriched in rhythmic gene sets. Rhythmic gene sets are represented graphically on the x-axis; a “—” in place of an organ indicates that the gene set was not rhythmic in that organ-condition. Possible rhythmic gene set combinations that are not depicted had no enriched biological process gene ontology terms at the $P < 0.001$ significance level.

Next, to gain insight on how rhythmic transcriptional responses to single-leaf infection might be wired, we performed motif enrichment analysis with the promoters of genes were rhythmic in only one condition (infected or uninfected) to search for signatures of TF input. Several motifs were associated with the gain or loss of rhythmicity due to single-leaf infection, but these were mainly driven by genes in the leaves (Figure 3.10, A and B). Notably, sequences that appeared multiple times across gene sets were associated with binding by MYB and homeobox (HB) TFs. MYBs have been well-documented to transmit rhythmic information through the circadian clock transcriptional network (*14-16*), while the association with HBs merits further investigation.

A Genes rhythmic only after infection

All ($n = 2,089$)

Rank	Motif	P-value	log P-value	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-12	-2.869e+01	4.12%	1.54%	294.2bp (326.2bp)	AT5g58900(MYBrelated)/colamp-AT5g58900-DAP-Seq(GSE60143)/Homer(0.657) More Information Similar Motifs Found
2		1e-12	-2.813e+01	0.91%	0.05%	323.4bp (239.6bp)	RAW1(2/AP2/EREBP)/Arabidopsis thaliana/AtHap0.654 More Information Similar Motifs Found
3		1e-12	-2.808e+01	5.27%	2.29%	304.5bp (290.8bp)	ATHB53/MA1215.1/Jaspar(0.674) More Information Similar Motifs Found

All Lf ($n = 1,971$)

Rank	Motif	P-value	log P-value	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-12	-2.763e+01	3.60%	1.23%	317.1bp (330.3bp)	AT2G40260(G2like)/colamp-AT2G40260-DAP-Seq(GSE60143)/Homer(0.610) More Information Similar Motifs Found

Only Lf ($n = 1,923$)

Rank	Motif	P-value	log P-value	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-14	-3.303e+01	3.28%	0.89%	329.5bp (349.6bp)	ATHB53(HB)/col-ATHB53-DAP-Seq(GSE60143)/Homer(0.654) More Information Similar Motifs Found
2		1e-12	-2.958e+01	2.45%	0.56%	338.6bp (387.1bp)	HAP3(CCAATHAP3)/col-HAP3-DAP-Seq(GSE60143)/Homer(0.745) More Information Similar Motifs Found

B Genes rhythmic only without infection

All ($n = 1,354$)

Rank	Motif	P-value	log P-value	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-14	-3.314e+01	1.26%	0.04%	218.5bp (449.7bp)	REF6(Z)/Arabidopsis-REF6-ChIP-Seq(GSE106942)/Homer(0.571) More Information Similar Motifs Found
2		1e-12	-2.992e+01	1.11%	0.03%	402.9bp (186.3bp)	POL13.1_MED-1/Jaspar(0.601) More Information Similar Motifs Found
3		1e-12	-2.969e+01	3.03%	0.64%	330.1bp (329.4bp)	SD0003.1_at_AC_acceptor/Jaspar(0.631) More Information Similar Motifs Found
4		1e-12	-2.919e+01	7.83%	3.41%	379.2bp (375.5bp)	NLP7(RWPRK)/col-NLP7-DAP-Seq(GSE60143)/Homer(0.566) More Information Similar Motifs Found

All Lf ($n = 1,041$)

Rank	Motif	P-value	log P-value	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-15	-3.487e+01	1.92%	0.10%	341.7bp (389.5bp)	AT5g47390(MYBrelated)/col-AT5g47390-DAP-Seq(GSE60143)/Homer(0.580) More Information Similar Motifs Found
2		1e-13	-3.027e+01	2.69%	0.36%	330.5bp (357.7bp)	AT5G18450/MA1226.1/Jaspar(0.666) More Information Similar Motifs Found
3		1e-12	-2.909e+01	1.15%	0.02%	133.3bp (62.1bp)	AT5G02460(C2C2dof)/col-AT5G02460-DAP-Seq(GSE60143)/Homer(0.675) More Information Similar Motifs Found
4		1e-12	-2.874e+01	1.25%	0.03%	403.3bp (376.3bp)	SPL9(SBP)/colamp-SPL9-DAP-Seq(GSE60143)/Homer(0.679) More Information Similar Motifs Found

Only Lf ($n = 972$)

Rank	Motif	P-value	log P-value	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-13	-3.176e+01	1.65%	0.06%	290.8bp (369.9bp)	AT5G02460(C2C2dof)/col-AT5G02460-DAP-Seq(GSE60143)/Homer(0.632) More Information Similar Motifs Found

Figure 3.10. Enriched motifs in the promoters of genes that gain or lose rhythmicity upon infection provide insight on wiring of rhythmic regulatory response to localized bacterial infection.

(A and B) Sequence logos for significantly enriched motifs in the promoter regions of genes rhythmic only after infection (A) and only in the absence of infection (B).

Based on the model of the organ-level shoot system circadian clock proposed in Chapter 2 (Figure. 2.4I), we hypothesized that the observed expression patterns of rhythmic genes in the shoot apex and leaves after distal infection would be driven by underlying changes in the expression patterns of known circadian clock component genes. In fact, we saw that nearly all of the known clock components that we investigated (25 out of 27) were expressed in both organs, and that most of the clock components expressed in both the shoot apex and leaves showed less than a twofold difference in transcript abundance between organs (Figure 3.11A). Likewise, most rhythmic clock components were expressed with similar amplitudes between organs (Figure 3.11B). This supports our notion that core clockwork is conserved across organs of the shoot apex and that rhythmic gene identity is controlled downstream of the clock in an organ-specific manner. Finally, we calculated the phase offset between organs for the clock components that were rhythmic in both organs and saw a striking reversal of the shoot-apex first pattern of expression that was previously seen in basal free-running conditions (see Figure 2.2C) for most clock components (Figure 3.11C). This suggests that the absence of the shoot apex-first tendency of rhythmic gene expression in infected plants (shown in Figure 3.8B) is underlain by a shift in the timing of expression for the core clock components. Additionally, we calculated the phase shifts of clock component expression as a result of the infection treatment and found that there was a strong phase delay in the shoot apex as a result of infection, but not in the leaves (Figure 3.11D), mirroring the result for rhythmic genes widely. These findings further support the idea that a localized single-leaf bacterial infection can have systemic and organ-specific effects on the timing of clock-controlled gene expression in a manner that is consistent with our proposed model of the organ-level shoot system circadian clock (formulated in Chapter 2).

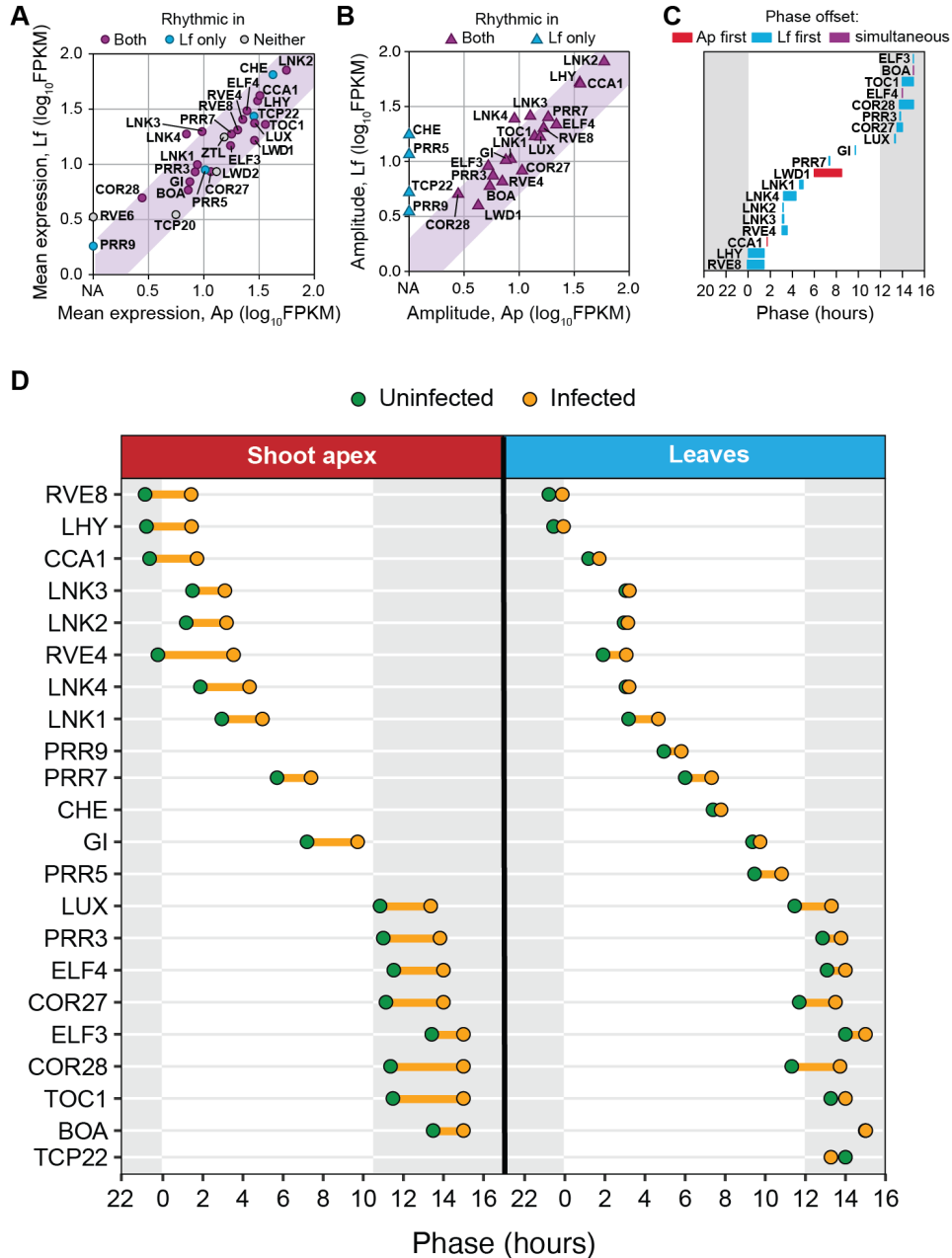


Figure 3.11. Gene expression characteristics of circadian clock components in distal organs after a single-leaf bacterial infection.

(A and B) Correlation of mean expression (A) and amplitude (B) values for core clock transcripts in the shoot apex (Ap) and leaf (Lf). Shaded background indicates the region with <2 -fold difference between organs, and "NA" indicates that gene expression (A) or rhythmicity (B) was below threshold in the corresponding organ. (C) Phase offsets (Phase_{Ap} - Phase_{Lf}) for core clock genes rhythmic in both organs. Bars span the shortest circular distance between the phases in Ap and Lf and are colored according to the organ of earlier phase. (D) Phase shifts as a result of infection for core clock genes. Yellow lines span the shortest circular distance between the phases in uninfected and infected conditions. Missing data means component was not rhythmic in one or both conditions.

Concluding remarks

Altogether, our work supports a model for the circadian network where a conserved shoot system-wide clock mechanism drives circadian rhythms in gene expression across different organs. However, the expression pattern of clock-controlled genes is ultimately dictated locally by organ-specific mechanisms that regulate clock input and output pathways. Based on this model, organ-specific clock input controls the phase of rhythmic genes in the shoot-apex and leaves, while organ-specific clock output control enables selective amplitude regulation that ultimately determines whether a gene is rhythmically expressed. This model of the clock architecture is supported by our findings from an mRNA-seq time course of shoot apices and leaves following a localized single-leaf infection by *Pst* DC3000. We observed that rhythmic gene sets were largely organ-specific, with rhythmicity being controlled independently from expression level and phase. Based on our model, we predicted that the core clock network itself should be expressed in common across the shoot system, but that the timing of the clock should be phase delayed in the shoot apex as a result of distal infection. Indeed, we saw that the expression level and amplitude of rhythmic core clock components was not affected by the infection, but that the timing of their peak expression was. This, coupled with the observation that infection alters the identity and phase of rhythmic outputs, reinforces the notion of organ-specific upstream phase control and downstream amplitude control of the rhythmic transcriptome. We postulate that organ-specific control of rhythmic gene expression allows dynamic and selective modulation of clock-controlled processes in response to local and distal signals, integrating information such as microenvironmental and stress fluctuations across the

shoot system. Such mechanisms likely provides a critical homeostatic mechanism to maintain optimal organismal fitness in the context of multifaceted endogenous and environmental cues.

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Investigation: ND, ZL, PC, NV, JPP

Visualization: ND, JPP, PC

Funding acquisition: JAL, CD, JPP, ND

Supervision: JAL, CD, JPP

Writing - original draft: ND

Writing - review & editing: ND, JPP

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (*Arabidopsis*) seedlings used in this work were of the Columbia ecotype (Col-0). *Arabidopsis* seeds were sown in 11” x 21” growth trays containing 32” x 2.5”

square pots (8 x 4 divider) filled with autoclaved soil (Berger, BM2) after 2-3 days stratification at 4°C in a tube containing a 0.15% agar solution. Plants were grown at 22°C under the daily light regimes below: Standard light-dark cycles: 12 hours light ($\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$)/12 hours dark; constant light (free-running condition): ($\sim 60 \mu\text{mol m}^{-2} \text{s}^{-1}$). Single-leaf infections were performed at the beginning of the second day (24 hours in constant light).

Pseudomonas syringae culture and single leaf infection protocol

Pseudomonas syringae pathovar tomato DC3000 (*Pst* DC3000) (17) liquid cultures in King's B medium (2% Proteose peptone No.3, 1% Glycerol, 8.6mM K₂HPO₄ and 6mM MgSO₄) were grown in the dark at 28°C and shaken at 175 rpm until OD₆₀₀ reached between 0.6 and 0.7 (several dilutions were started to assure that a suitable culture was available at the time of treatment). To infect plants, the *Pst* DC3000 cell suspension inoculum was prepared. Bacteria from a liquid culture (OD₆₀₀ between 0.6 and 0.7) were harvested by centrifugation at 3220 x g for 2 min., resuspended in sterile water (LabChem), and harvested by centrifugation at 3220 x g for 3 min. The bacterial pellet was resuspended in water (LabChem) adjusting OD₆₀₀ to 0.2 (10^8 colony forming units), and Silwet L77 (Lehle seeds) was added to a final concentration of 0.025%. Half of a single true leaf was dipped into this *Pst* DC3000 cell suspension for 1 min. After treatment, the excess inoculum was blot-dried from the leaf surface using a sterile filter paper strip and plants were returned to constant light for mRNA time course organ collection.

RNA isolation, mRNA-sequencing library construction, and sequencing

Seedlings were grown in soil with standard light-dark cycles for 14 days, and then transferred to constant light. Harvesting occurred at 6 timepoints after transition to constant light: 90, 94, 98, 102, 106, and 110 hours. For each sample collection, 6-10 seedlings were snap frozen in liquid nitrogen and then cryodissected to isolate the shoot apex and true leaves. Two independent replicate experiments (“replicate 1” and “replicate 2”) were performed, rendering a total of 24 samples. Total RNA was isolated from each sample using the RNeasy Plant Mini kit (Qiagen, Cat# 74904) and mRNAs were purified from 0.46µg of total RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Cat# E7490) before library preparation with the NEBNext UltraII RNA Library Prep Kit (New England Biolabs, Cat# E7770). Sera-Mag Magnetic SpeedBeads (Thermo Scientific, Cat# 65152105050250) were used for all size selection and clean-up steps and the resulting libraries were pooled and sequenced (single end 50 bp, SE50) on a HiSeq 2500 machine (Illumina).

mRNA-seq mapping and quantification of expression levels

mRNA-seq reads were mapped to the TAIR10 reference genome using STAR v2.5.0c (18) with the following options: “—outFilterMismatchNmax 2 —outFilterMultimapNmax 1”. Sorted .bam files were used to generate HOMER (19) Tag Directories using the makeTagDirectory script with the “-mis 2 -unique” option. UCSC format tracks were generated using the makeUCSCfile script with the “tair10 -fragLength given -style rnaseq” options. Raw read counts and normalized values (fragments per kilobase of transcript per million mapped reads, FPKM) for each sample were obtained using the HOMER analyzeRepeats.pl program with the “-noadj” or “-fpkm” options, respectively, and mapped to the TAIR10 genome annotation (20) with isoforms collapsed by “-condenseGenes”.

Principal component and clustering analysis

Unnormalized, rlog-transformed read counts with blind dispersion estimation were used to perform principal component analysis with the DESeq2 R package (21).

Differential gene expression analysis

Unnormalized read counts were used to perform differential gene expression analysis separately for the apex and leaf data using the DESeq2 R package (21). A design of “~ Collection time + Replicate + Infection condition” was used. Significant genes were filtered using a threshold of $\log_2$ -fold change in expression >1.5 and P value <0.05 .

Gene ontology (GO) analysis

To find GO biological process terms enriched in specific gene sets compared to the genomic background, we used the “elim” method from the topGO R package deposited in Bioconductor (22) and reported those with raw $P < 0.001$ using Fisher’s exact test (Supplemental Table 2.4). Then, to summarize and remove redundant GO terms, we used the Revigo tool (23) with a semantic similarity cutoff value of $C = 0.5$ and visualized the results using the ggplot2 R package, following previously reported recommendations (24).

Analysis of circadian gene expression

For optimal downstream statistical detection of rhythmic genes we followed previously described recommendations to first define the set of “expressed” genes (25). For that, we filtered the mRNA-seq dataset of normalized reads by applying an “expression” threshold that retained

only those genes that had >3 FPKM in both replicates of at least one time point. To identify genes that cycled with a 24-hour period, we analyzed the filtered dataset of normalized reads using the Metacycle package for R (26). We used the integrated meta2d output values for downstream analysis and considered a gene to be “rhythmic” if it passed a false discovery rate threshold of Benjamini-Hochberg $Q < 0.05$.

Motif enrichment analysis

The findMotifsGenome.pl script from HOMER v4.11.1 (19) was used to identify enriched motifs in the cis-regulatory regions of specific gene sets. The queried genomic promoter sequence for each locus was defined as 750 bp upstream plus 500 bp downstream of the 5’ end of the representative gene model annotation from Araport11 (27). The target gene sets were: (1) all upregulated DEGs, (2) all upregulated DEGs in the shoot apex, (3) all upregulated DEGs in the leaves, (4) DEGs upregulated only in the shoot apex, (5) DEGs upregulated only in the leaves, (6) DEGs upregulated in both the shoot apex and the leaves, (7) all downregulated DEGs, (8) all downregulated DEGs in the shoot apex, (9) all downregulated DEGs in the leaves, (10) DEGs downregulated only in the shoot apex, (11) DEGs downregulated only in the leaves, (12) DEGs downregulated in both the shoot apex and the leaves, (13) all genes gaining rhythmicity after infection, (14) all genes gaining rhythmicity in the shoot apex after infection, (15) all genes gaining rhythmicity in the leaves after infection, (16) genes gaining rhythmicity only in the shoot apex after infection, (17) genes gaining rhythmicity only in the leaves after infection, (18) genes gaining rhythmicity in both the shoot apex and leaves after infection, (19) all genes losing rhythmicity after infection, (20) all genes losing rhythmicity in the shoot apex after infection, (21) all genes losing rhythmicity in the leaves after infection, (22) genes losing rhythmicity only

in the shoot apex after infection, (23) genes losing rhythmicity only in the leaves after infection, and (24) genes losing rhythmicity in both the shoot apex and leaves after infection. The background gene sets were all genomic loci for the analyses of DEG sets and all expressed loci passing the 3 FPKM “expression” threshold (see previous section) for the gene sets gaining or losing rhythmicity. We reported motifs significant at a level of $P < 10^{-12}$.

Accession numbers

Gene models in this article can be found in The Arabidopsis Information Resource (TAIR) (<https://www.arabidopsis.org>) with the following accession numbers: *BOA*, AT5G59570; *CCA1*, AT2G46830; *CHE/TCP21*, AT5G08330; *COR27*, AT5G42900; *COR28*, AT4G33980; *ELF3*, AT2G25930; *ELF4*, AT2G40080; *GI*, AT1G22770; *LHY*, AT1G01060; *LNK1*, AT5G64170; *LNK2*, AT3G54500; *LNK3*, AT3G12320; *LNK4*, AT5G06980; *LUX*, AT3G46640; *LWD1*, AT1G12910; *LWD2*, AT3G26640; *PP2A*, AT1G13320; *PRR3*, AT5G60100; *PRR5*, AT5G24470; *PRR7*, AT5G02810; *PRR9*, AT2G46790; *RVE4*, AT5G02840; *RVE6*, AT5G52660; *RVE8*, AT3G09600; *TCP20*, AT3G27010; *TCP22*, AT1G72010; *TOC1*, AT5G61380; *ZTL*, AT5G57360.

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Chapter 4 DISCUSSION OF THE FINDINGS

The circadian clock is a timing mechanism intrinsic to many organisms that orchestrates daily rhythmic activity, including gene expression. We performed time course mRNA sequencing (mRNA-seq) experiments of Arabidopsis shoot apices and leaves to reveal the extent of clock regulation across the plant shoot system in both basal conditions (Chapter 2) and after a single-leaf bacterial infection (Chapter 3).

In basal conditions, we observed that, overall, there were more genes rhythmic in leaves than the shoot apex (Figure 2.1C). This was apparently not due to an inherent lack of rhythmicity in the shoot apex, as the amplitudes of rhythmic genes were similar between tissues (Figure 2.1F). Additionally, the expression of most organ-specific rhythmic genes was not turned off in the non-rhythmic organ (Figure 2.1D). Rather, it appears that organ-specific mechanisms damp the amplitudes of a subset of genes to determine the rhythmic gene set of each organ. While disparities in rhythmic gene sets between organs have been observed in mammals (1–5), flies (6), and plants (7) among other organisms, researchers have provided different explanations and speculations as to what drives these differences. Mure *et al.* (5) (working with baboons in diurnal conditions) and Storch *et al.* (4) (working with free-running mice) arrived at a similar conclusion as in our present study, namely that “Tissue-specific rhythmic expression is not a function of tissue-specific gene expression” (5). Rather it is a consequence of tissue-specific rhythmic transcriptional regulation of certain sets of genes, many of which may be well-expressed but non-rhythmic in other tissues. However, at least one group working with free-running mice (3) observed that tissue-specific rhythmic genes largely do appear to be shut off in the non-rhythmic tissue, while another group working with free-running flies (6) found evidence for a combination of both mechanisms. These observations raise the question of whether differences in rhythmic

gene composition between organs could be driven by differences in the expression of components of the core clock itself. Our dataset revealed that the composition of the known core circadian clock was similar between organs (Figure 2.2A), as has also been reported in most of the other spatial transcriptomic studies mentioned (1, 2, 4, 6), although Mure *et al.* (5) and de Bares *et al.* (7), both working in driven conditions, found some degree of heterogeneity in clock composition by tissue in their data. Overall, our results suggest that the core clock remains similar between shoot apex and leaf, and that rhythmic gene set identities are controlled downstream of the clock by organ-specific modulators of amplitude that reduce rhythmicity with little effect on general expression levels. It would be interesting to search for signatures of regulation in the promoters of genes that are expressed in both organs, but only rhythmic in one, in order to identify potential amplitude regulators downstream of the core clock.

Furthermore, our dataset revealed a widespread offset in the peak expression times of rhythmic genes (Figure 2.1, H and I), with greater than three quarters of rhythmic genes peaking in the shoot apex before the leaf (Figure 2.1J). This suggested a coordinated regulation of the phase offset between organs, and indeed the core clock components themselves exhibited a shoot apex-first pattern as well (Figure 2.2C). While several studies report spatially-specific differences in transcript phases (1–8), our dataset is unique in linking a widespread organ phase offset with a corresponding offset in core clock components. We confirmed the core clock phase offset with qPCR (Figure 2.2F) and a luciferase reporter of *CCA1* promoter activity (Figure 2.2G), which notably had a much higher time resolution and longer duration than our mRNA-seq dataset, allowing for more precise measurement of circadian characteristics. Furthermore, we hypothesized that different organs may be set up with different phases of rhythmic activity in order to coordinate specific biological functions. Accordingly, we explored gene ontologies

associated with rhythmic gene sets in shoot apex and leaf (Figure 2.2K). In brief, we found that genes rhythmic in the shoot apex were enriched for terms related to chloroplast development (which is known to begin in the shoot apex (9, 10)), while genes rhythmic in the leaf were associated with metabolism, protein synthesis and other biosynthetic pathways (which is consistent with the central role of leaf metabolism in plant physiology). Meanwhile, genes rhythmic in both organs were enriched for a wide variety of terms including energy production and nutrient assimilation, perception of light and temperature, and photoperiodic flowering, which appear to be important targets of circadian control across the shoot system broadly. These results suggest that the circadian clock plays a specialized role in controlling rhythmic processes depending on organ context, and this specialization is likely generated downstream on the core clock by organ-specific mechanisms. It may also be fruitful in the future to explore how biological processes are coordinated according to phase groupings, as this may suggest time-of-day specific coordination of physiological functions.

Next we turned our attention toward identifying potential upstream regulators of the core clock that contribute to establishing the phase offset between organs. We focused on the morning-expressed core clock component CCA1 and screened its promoter sequence against a library of transcription factors (TFs) in a yeast 1-hybrid assay, a method which has previously been successful in identifying an important CCA1 regulator (11). However, we focused our analysis only on TFs that were enriched in a particular organ, and thus might control CCA1 phase in an organ-specific manner. We identified three shoot apex-specific potential regulators of *CCA1* (Figure 2.3B) and focused on one of them – TCP19 – because at least three other members of the TCP family have been reported to control aspects of clock function (11, 12). We confirmed the shoot apex-specific expression of TCP19 (which had been suggested previously in

gene-trap experiments (13)) using reporters of promoter activity and direct protein detection (Figure 2.3, D-G). Finally, to explore the genetic interaction of *TCP19* and *CCA1* we performed flowering time experiments and saw that an early flowering phenotype caused by *TCP19* overexpression was reverted to wildtype by the *cca1-1* mutation, indicating a genetic dependence of the phenotype on *CCA1* (Figure 2.3I). Together, these experiments validated our approach for identifying organ-specific TFs that may play a role in organ-specific clock regulation, and there is room for future exploration of organ-enriched TFs, including the two others identified in Figure 2.3B.

After identifying *TCP19* as a shoot apex-specific TF that can interact with the *CCA1* promoter, we investigated its role in regulating the dynamics of clock function. Using a reporter of *CCA1* promoter activity, we determined that mutating the *TCP19* locus causes a phase delay specifically in the shoot apex region (Figure 2.4C). This indicated that *TCP19* may play an indirect role in establishing the phase offset we observed in the mRNA-seq dataset (Figure 2.3H). Conversely, overexpressing *TCP19* had the opposite effect, namely a phase advance in the leaves – an organ where *TCP19* is not typically expressed (Figure 2.4F). Finally, we looked for flowering phenotypes associated with the *tcp19-2* mutation but found that only in a sensitized *lhy* mutant background in short day conditions could we observe an effect of mutating the *TCP19* locus (Figure 2.4I). This is not without precedent, as flowering phenotypes in short days are much more apparent when the *cca1* and *lhy* mutations are combined than in their respective single mutants (14). Tangentially, the period shortening effect of another TCP mutant, *che-1*, was not revealed until combined with the *lhy* mutation (11). These results indicate that wild-type levels of LHY may mask the effects of *TCP19* on *CCA1*-controlled photoperiodic flowering. Interestingly, a short day-specific flowering phenotype is a rarity in previous reports, but given

that TCP19 appears to be restricted to the shoot apex, it is logical to expect that its mutation would not play a role in long day flowering, which operates primarily through regulation of FLOWERING LOCUS T in the leaves (15).

In summary of Chapter 2, our data supports a model in which TCP19 acts upstream of the core clock in the shoot apex to control the phase of CCA1. This establishes a phase offset between the rhythmic transcriptomes of shoot apex and leaves, which is important for physiological processes including photoperiodic flowering. Downstream of the clock, the amplitudes of a subset of genes are modulated to reduce rhythmicity and thus define organ-specific rhythmic gene sets (Figure 2.4J). Although the factors orchestrating this amplitude modulation remain unidentified, our proposed mechanism is supported by a recent report from mouse studies that identified two possible amplitude output modulators downstream of the core clock (16). However, mechanisms for amplitude modulation of the clock itself are also known to exist (17). Additionally, it remains unclear how TCP19, whose expression apparently does not oscillate in free-running conditions, is able to confer time of day specific expression information to the *CCA1* locus. There are several possible explanations: TCP19 may oscillate at the protein level, it may have oscillating binding partners (TCPs are known to heterodimerize (18)), or it may compete for occupancy of target promoters with other rhythmic factors (19, 20). Additionally, TCP19 appears to oscillate in driven conditions, which suggests an additional role in diurnal regulation of the clock (21). Overall, we provided a comprehensive view of the shoot apex and leaf circadian transcriptomes, identified a novel organ-specific regulator of clock function, and provided an example of a mechanism through which clocks from diverse organisms may be able to achieve spatially-specific control of rhythmic outputs.

In Chapter 3, we turned our attention to the transcriptomes of shoot apices and leaves from plants that had been subjected to a distal, localized single-leaf *Pst* DC3000 infection. We saw that hundreds of genes were differentially expressed in these untreated organs as a result of single-leaf infection when compared to uninfected individuals (Figure 3.3C). Interestingly, most of these genes were specific to either the shoot apex or leaves, indicating a strong component of organ-specificity in this transcriptional response. In addition, there were many more DEGs in the shoot apex than in the leaf, indicating that the shoot apex is more responsive to the treatment than the leaves. This may explain why significant defects were seen in the shoot apices of plants treated with single leaf infection (22). Furthermore, it would be worth exploring to what degree the DEGs we identified overlap with existing lists of infection-responsive genes as well as more general “core” or “universal” stress response genes (23, 24). It is also interesting to note that many GO terms associated with the identified upregulated genes correspond to non-bacterial responses, including responses to wounding, insects, oomycetes, molecules of fungal origin, hypoxia, and so on (Figure 3.4). These may reflect the involvement of genes from the aforementioned “universal” stress response, or it may indicate that there is more specific interplay happening between these stress response pathways as has been suggested elsewhere (25). These results lead us to hypothesize that a single-leaf *Pst* DC3000 infection may have consequences for subsequent stresses applied to other areas of the plant; *i.e.*, a single-leaf infection may lead to a protective effect on distal leaves against future herbivory. Further investigations into this hypothesis may prove fruitful at uncovering this interplay between stress response pathways. Finally, our search for enriched regulatory motifs in the promoters of DEGs led to the identification of known and previously uncharacterized elements associated with stress responses. WRKY binding sequences as well as elements resembling G-boxes appeared several

times among these gene sets (Figure 3.5). WRKYs are well-established to be involved in biotic defense responses and they may function as “master regulators” that balance these responses with the growth needs of plants (26, 27). Meanwhile, the G-box motif is associated with binding of bZIPs and bHLH transcription factors, and may also integrate information from multiple signal perception pathways including defense responses, light perception, and circadian rhythms among others (28, 29). The role of specific instances of G-box motifs in DEGs from our dataset may be further elucidated using prediction tools that ascribe more specific functions to G-boxes based on their flanking sequences (30).

Next, we characterized the rhythmic nature of gene expression in shoot apices and leaves distal to the single-leaf infection. There were far more leaf-specific rhythmic genes than shoot apex-specific rhythmic genes (Figure 3.6), mirroring what was observed in uninfected plants (Figure 2.1C). However, when compared to uninfected plants, the number of rhythmic genes fell in the shoot apex but rose in the leaves (Figure 3.7A). The loss of rhythmicity in the shoot apex may contribute to the significant growth and development defects seen in the shoot apices of plants treated with single leaf infection, and may also reflect the shoot apex-specific loss of *CCAI* promoter activity as a result of this treatment (22). Meanwhile, an increase in rhythmic gene expression in the leaves may reflect intensified circadian regulation of processes critical for continued growth and survival in the face of pathogen attack. These hypotheses were also supported by the loss of rhythmic shoot apex-enriched GO terms associated with growth and development and the gain of rhythmic leaf-enriched GO terms associated with metabolism and protein regulation (Figure 3.9). However, it was surprising to find that there were no GO terms enriched in genes that gained rhythmicity in both the shoot apex and leaves as a result of the

distal infection. This highlights the highly organ-specific nature of the rhythmic responses to the *Pst* DC3000 treatment.

The timing of rhythmic gene expression was also affected due to single leaf infection. Gene expression phases appeared to be delayed compared to uninfected individuals, particularly in the shoot apex, and there was no bias for genes to peak first in one or the other organ (Figure 3.8). It remains to be explored whether the change in the distributions of rhythmic genes as a result of single leaf infection is driven by a time-of-day-specific loss/gain of rhythmicity or gene-specific phase shifts (or both). However, it was clear that there was also a shift in the phases of core clock gene expression as a result of the *Pst* DC3000 treatment, with a tendency for genes to be close in phase between organs and, more often than not, peaking in the leaf first (Figure 3.11). This is in stark contrast to the shoot apex-first pattern observed in uninfected plants (Figure 2.2C). We conclude that the shift in clock gene phases contributes to the shift in the overall phase distributions observed as a result of single leaf bacterial infection (Figure 3.8), and adds support to our model of the clock network wherein the phase of rhythmic outputs is controlled by organ-specific mechanisms upstream of the core clock (Figure 2.4I).

In conclusion, the work presented here provides a basic model for the circadian clock network in the *Arabidopsis* shoot system, emphasizing a conserved systemwide mechanism that drives circadian rhythms across different organs while highlighting the critical role of organ-specific input and output pathways in modulating rhythmic gene expression. Our comparative analysis of clock-regulated transcriptomes in the shoot apex and leaves revealed distinct rhythmic genes unique to each organ, with a notable earlier peak in the shoot apex in uninfected conditions (Chapter 2). This organ-specific phase regulation, validated by the identification of the shoot apex-specific regulator TCP19, underscores the importance of localized control in

coordinating photoperiodic flowering and other physiological processes. Furthermore, our investigation into the systemic effects of a localized single-leaf bacterial infection demonstrated significant alterations in the rhythmic transcriptome, including phase shifts and changes in rhythmic gene identities, reinforcing our model's robustness (Chapter 3). These findings suggest that organ-specific control mechanisms enable dynamic modulation of clock-controlled processes in response to both local and systemic signals, thus providing a critical homeostatic mechanism for maintaining optimal organismal fitness amidst varying endogenous and environmental cues.

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