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BMAL1-NPAS2–dependent transcription activation (19–27) while enhancing Rev-erba-mediated transcription repression, providing a potential means of maintaining the amplitude of circadian rhythms.

Expression of the gene encoding ALAS1, the rate-limiting enzyme in heme biosynthesis, increased in response to peroxisome proliferator activated receptor coactivator–1α (29), a regulator of mitochondrial biogenesis that increases flux through the Krebs cycle (30). This first and rate-limiting enzyme in heme biosynthesis requires succinyl CoA, a Krebs cycle intermediate (17, 29). Gluconeogenesis competes with the Krebs cycle for metabolic intermediates whose depletion compromises heme biosynthesis as well as mitochondrial oxidative metabolism (fig. S13).

The ability of Rev-erba to function as a receptor for heme could provide a general mechanism for coordinating these processes.

References and Notes

7. N. Preitner et al., Cell 110, 251 (2002).
31. We thank R. Rampe, D. Steger, T. Stanley, M. Walker, J. Williams, and T. Willson for helpful discussions. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grant R01 DK55586 (M.A.L.).

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The Arabidopsis Circadian Clock Incorporates a cADPR-Based Feedback Loop

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Transcriptional feedback loops are a feature of circadian clocks in both animals and plants. We show that the plant circadian clock also incorporates the cytosolic signaling molecule cyclic adenosine diphosphate ribose (cADPR). cADPR modulates the circadian oscillator’s transcriptional feedback loops and drives circadian oscillations of Ca2+ release. The effects of antagonists of cADPR signaling, manipulation of cADPR synthesis, and mathematical simulation of the interaction of cADPR with the circadian clock indicate that cADPR forms a feedback loop within the plant circadian clock.

Circadian clocks are adaptations to the daily rhythm of the planet. In plants and cyanobacteria, benefits occur when the clock is resonant with the environment (1–3). This requires the oscillator to be robust yet flexible, which may explain the evolution of molecular clocks with multiple feedback loops (4–6). We tested the hypothesis that plant circadian oscillators also incorporate cytosolic signaling molecules because there are circadian rhythms in the
concentration of cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{cyt}\)] (7, 8). We investigated the function within the plant circadian system of cyclic adenosine diphosphate ribose (cADPR), a cytosolic ligand that promotes the release of Ca\(^{2+}\) into the cytosol from internal stores through cation channels (9).

To identify potential interactions between plant circadian and Ca\(^{2+}\) signaling pathways, we examined the overlap between a circadian transcriptome and transcriptomes for known regulators of [Ca\(^{2+}\)\(_{cyt}\)] (Fig. S1) (10). We obtained a new near whole-genome transcriptome in constant light (LL) under conditions allowing circadian [Ca\(^{2+}\)\(_{cyt}\)] oscillations (10, 11). This was necessary because previous circadian transcript analyses used plants grown on 3% sucrose (12, 13), which abolishes circadian [Ca\(^{2+}\)\(_{cyt}\)] oscillations (7). On the basis of a threshold multiple measures–corrected probability of rhythmicity (pMMC) of 0.15, 2282 (12.08%) transcripts were circadian-regulated (spreadsheet S1 and fig. S1) (12, 14).

A cADPR-regulated transcript set (15) had the most statistically significant overlap with our circadian transcriptome ([Fig. 1A and table S1] \(P = 5.15 \times 10^{-52}\)). The overlap was significantly larger than the 93 transcripts expected for a chance overlap of two similarly sized datasets selected randomly from the Arabidopsis genome. We noted that 252 transcripts were both circadian- and cADPR-regulated (Fig. 1A and spreadsheet S2). There was a phase relation between circadian- and cADPR-regulation of transcript abundance: The majority of cADPR–up-regulated rhythmic transcripts reached peak abundance at zeitgeber time (ZT) ZT8 or ZT12, and the majority of cADPR-repressed rhythmic transcripts reached peak abundance between ZT20 and ZT4 (Fig. 1B).

Because a phase relation existed between circadian- and cADPR-regulated genes and the circadian-regulated evening element was significantly overrepresented in cADPR–up-regulated genes (spreadsheet S3), we reasoned that cADPR signaling might be circadian-regulated (12, 15). We measured cADPR in extracts of aerial tissues of seedlings in LL (10). [cADPR] was circadian-regulated, because cADPR was significantly higher during subjective light (mean ± SEM, 0.27 ± 0.04 pmol · μg protein\(^{-1}\)) than subjective dark (0.16 ± 0.02 pmol · μg protein\(^{-1}\)) (Fig. 2, A and B, and fig. S2), cADPR variations persisted through the second and third days of LL and were eliminated in plants overexpressing CIRCADIAN CLOCK ASSOCIATED1 (CCA1-ox) (Fig. 2A). CCA1-ox abolishes all known plant circadian rhythms (2, 16).

We tested whether circadian [cADPR] variations cause circadian rhythms of [Ca\(^{2+}\)\(_{cyt}\)]. Under LL, we monitored [Ca\(^{2+}\)\(_{cyt}\)] in seedlings treated every 3 hours with Ca\(^{2+}\) signaling antagonists (Fig. 2, C and D). Nicotinamide (10 mM) reduced the amplitude of the [Ca\(^{2+}\)\(_{cyt}\)] oscillation, and 50 mM nicotinamide abolished the oscillation (Fig. 2C) (10). Nicotinamide is an antagonist of cADPR signaling that, at 50 mM, inhibits the synthesis of cADPR from NAD\(^{+}\) by adenosine diphosphate ribosyl cyclase (ADP-RCs) (17). Circadian [Ca\(^{2+}\)\(_{cyt}\)] oscillations were unaltered by GdCl\(_3\), which inhibits extracellular Ca\(^{2+}\) influx (18) (Fig. 2D), and the phospholipase C (PLC) inhibitor U73122, which inhibits production of inositol 1,4,5-trisphosphate (IP\(_3\)) at 1 μM (19) (Fig. 2D). Arabidopsis ADP-RC activity (10, 20) was inhibited in vitro by 40 mM nicotinamide (fig. S3), which suggests that nicotinamide prevents cADPR synthesis in plants. Because 50 mM nicotinamide abolished circadian [Ca\(^{2+}\)\(_{cyt}\)] oscillations, and circadian [cADPR] and [Ca\(^{2+}\)\(_{cyt}\)] alterations had a similar phase, circadian cADPR oscillations are likely to drive circadian [Ca\(^{2+}\)\(_{cyt}\)] rhythms.

Several genes encoding components of the circadian clock are cADPR-regulated (15). GIGANTEA (GI), CRYPTOCHROME2 (CRY2), GLYCINE-RICH BINDING PROTEIN7 (GRP7), and PSEUDO-RESPONSE REGULATOR5 (PRR5) and PRR7 are cADPR–up-regulated, and LATE ELONGATED HYPOCOTYL (LHY) and CCA1 are down-regulated. TIMING OF CHLOROPHYLL A/B BINDING PROTEIN (TOC1) is unaffected (spreadsheet S2) (15). To understand how circadian function might be affected by cADPR-induced changes in transcript abundance, we performed a simulation using a previously described mathematical model (21). In that model, 13 equations define an approximation for the Arabidopsis circadian oscillator. We controlled the parameters of this model to constrain CCA1/LHY, GI, and TOC1 transcripts to adopt the fold-changes that are caused by cADPR in plants (figs. S4 and S5) (10, 15). First, we constrained the parameters transiently, because increasing cADPR synthesis by inducing the Aplysia ADP-RC gene, fused to a β-estradiol–inducible promoter (XVE:ADP-RC), causes transient alterations in clock transcripts although [cADPR] remains elevated (15). Depending on the parameters that were constrained, there was a short disruption to the modeled oscillator that sometimes caused phase changes, but the period remained 24 hours (Fig. 3A and fig. S5C). Next, we constrained the parameters continuously to evaluate the effects of continuous cADPR-induced alteration in clock transcripts. Depending on the parameters that were constrained, the circadian period often changed and remained altered throughout the simulation (Fig. 3B). Finally, we inverted the permanent constraint of the parameters to understand the possible effects of removal of cADPR-regulation of clock transcripts. Most of the parameters that did not lead to arrhythmia caused the simulations to run with a longer period (Fig. 3C). These simulated outcomes provided a framework with which to interpret the effects of cADPR on the plant circadian clock.

We experimentally tested how transient [cADPR]-induced alterations in circadian clock transcripts affect circadian behavior. We elevated [cADPR] in two independent lines by inducing the XVE:ADP-RC transgene (15) with 100 μM β-estradiol. [cADPR] increased 13.5-fold (line 1) and 27-fold (line 2) relative to controls (fig. S6). The circadian period and phase of leaf position were unaltered when XVE:ADP-RC were induced at ZT2 under LL (Fig. 4A and fig. S7, A, B, and G), or at two times during the final LD cycle before LL (fig. S7, C to G). Circadian [Ca\(^{2+}\)\(_{cyt}\)] oscillations were unaltered by XVE:ADP-RC induction (Fig. 4B). The invariance of circadian period may be explained by the simulated evidence that transient cADPR-induced alterations in oscillator transcripts do not alter circadian period. This invariance also reveals remarkable stability of the oscillator.

We investigated whether continuous [cADPR] synthesis alters circadian behavior. We overexpressed Aplysia ADP-RC using the constitutive 35S promoter (3SS:ADP-RC) (10). This approximately doubled [cADPR] (0.38 ± 0.09 pmol · μg protein\(^{-1}\)) (line 1); 0.45 ± 0.13 pmol · μg
protein−1 (line 2), 0.23 ± 0.05 pmol · µg protein−1 (wild type); n = 5). [cADPR] was not measured over a circadian time course because of poor 35S:ADPRc plant growth, which was consistent with a lesion to stress and circadian signaling (2, 3). The mean period of circadian rhythms in leaf position was unaltered in 35S:ADPRc [P = 0.51 by two-sample t test (fig. S8, A and B)]. However, there was a broader period length distribution compared with the wild type when all replicates with all lines were considered, as indicated by both the spread and significantly different standard deviation (fig. 4C) and the median period, SD = 24.4 hours, spread = 22.5 to 25.8 hours, n = 45; 35S:ADPRc: SD = 0.96 hours, spread = 21.9 to 26.3 hours, n = 106; test of homogeneity of variances Levene statistic = 5.81, P = 0.017**]. ADPRc overexpression disturbed the sinusoidal circadian [Ca2+]cyt oscillations (fig. S9) and increased the relative amplitude error (RAE) (22) of the oscillations (fig. 4D) wild-type RAE, 0.28 ± 0.02; line 1, 0.42 ± 0.03; line 2, 0.55 ± 0.06]. The greater RAE indicated a poorer Fourier fit to the data. Because [Ca2+]cyt images were obtained from groups of 15 to 20 seedlings (23), the increased RAE of circadian [Ca2+]cyt oscillations could reflect increased variability of the [Ca2+]cyt oscillation period between individuals. The simulations suggested that oscillator period is affected by high [cADPR] (Fig. 3B). We found that 35S:ADPRc affected oscillator period by increasing variability, which might be a consequence of differing levels of cADPR between seedlings and cells having different effects on period.

Finally, we investigated how continuously suppressed cADPR signaling affected the clock by treating seedlings with nicotinamide. The circadian period of rhythms of leaf position was significantly longer with 10 mM or 50 mM nicotinamide compared with water and osmotic controls. The period increase was nicotinamide concentration-dependent (fig. 4, E and F, and table S2) control period 23.6 ± 0.3 hours; 10 mM nicotinamide, 24.5 ± 0.3 hours, P = 0.001; 50 mM nicotinamide, 26.6 ± 0.4 hours, P = 0.002; comparisons by two-sample t tests). Circadian rhythms of leaf position were unaltered by other chemical modifiers of Ca2+ signaling [CaCl2, GdCl3, LaCl3, EGTA, or U73122 (table S2)]. At 50 mM, nicotinamide also lengthened circadian period of CAB2:luciferase luminescence [nicotinamide, 25.3 ± 0.4 hours; 50 mM mannitol, 26.6 ± 0.5 hours (Fig. 4G and fig. S10)], the

Fig. 2. [cADPR] oscillates with a circadian rhythm and a cADPR signaling antagonist inhibits circadian [Ca2+]cyt oscillations. (A) [cADPR] during 48 hours of constant light in Col-0 wild type and arrhythmic CCA1-ox (n = 5; ± SEM). Seedlings were transferred to LL 24 hours before sampling. (B) Data from (A) binned according to subjective day and night, P values from two-sample t tests indicated. (C and D) Circadian [Ca2+]cyt oscillations in 11-day-old seedlings dosed every 3 hours with (C) nicotinamide, mannitol (osmotic control), or water; (D) GdCl3 and U73122. n = 12; greatest SEM for each panel at top right. On x axes are black bars, darkness; white bars, light, hatched bars, subjective dark under LL.

Fig. 3. Constraint of a mathematical model for the Arabidopsis circadian clock (21) to explore how cADPR-induced alterations in CCA1/LHY, GI, and TOC1 transcript abundance alter clock function. (A) Transient constraint of model parameters to impose cADPR-induced alterations in clock transcript abundance caused variable phase changes that depended on parameters constrained. (B and C) For uniform parameter constraints analogous to continuous cADPR signaling (B) and repression (C), the period changed by a magnitude that depended on the parameters constrained. (A to C) Each point represents a different parameter pair that correctly constrained simulated transcripts when cADPR synthesis was induced between ZT20 to 6 or ZT20 to 24 (crosses) and ZT11 to 19 (circles); explained in fig. S5B. Broken lines indicate period or phase of unperturbed simulated oscillator. Phase and period calculated after simulated 480 hours in LL; arrhythmic simulations excluded from plots because perturbation of cADPR signals in plants (Fig. 4) did not cause arrhythmia.
The circadian clock and its outputs are altered by continuous disruption of cADPR signaling. (A) Circadian rhythm of leaf position (n = 30 to 32) and (B) XVE:ADPRc induction on day 1 of LL. (A) Circadian rhythm of leaf position (n = 26.0 hours; H2O control, 23.3 hours; 50 mM mannitol, 22.3 hours), and CAB2, luciferase transcript abundance (Fig. 4H) (10). Inhibition of cADPR synthesis, therefore, lengthened the period of the clock and its outputs.

Our data demonstrate the existence of a feedback loop within the plant circadian clock that incorporates cADPR, because [cADPR] is regulated by the circadian oscillator and cADPR, in turn, regulates the abundance of clock gene transcripts. Circadian oscillations in [cADPR] also regulate circadian [Ca2+]cyt oscillations. It has been suggested that diurnal variations in [Ca2+]cyt are regulated by the Ca2+ sensor CAS through an IP3-mediated system (24). Our data indicate that IP3 does not contribute to the control of circadian [Ca2+]cyt oscillations (Fig. 2D). The circadian feedback loop incorporating cADPR may interface the clock with exogenous stimuli that transiently elevate intracellular [cADPR] and so optimize the adaptive value of circadian control during unpredictable short-term environmental variations. We have established that cytosolic signaling molecules represent a hitherto unrecognized class of circadian clock components.

References and Notes
10. Materials and methods are available on Science Online.
11. Microarray data deposited at ArrayExpress (www.ebi.ac.uk/arrayexpress), accession E-TABM-331.
25. The authors thank N.-H. Chua for inducible XVE:ADPRc lines; H. Okamoto, S. Takasawa, H.-C. Lee, and R. Graeff for Aplysia ADPRc; H.-C. Lee, R. Graeff, E. Zocchi, and S. Brazzone for advice; B. Handley for programming assistance; and J. C. Gray, E. A. C. MacRobbie, and J. Carr for critical reading. Research funded by the U.K. Biotechnology and Biological Sciences Research Council (BBSRC), the Royal Society of London, the Broadband Foundation Cambridge, the Gates Foundation, the Danish Research Council, the Danish Research Council, and the Danish Research Council.

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