Comment on “The Arabidopsis Circadian Clock Incorporates a cADPR-Based Feedback Loop”

Xiaodong Xu,1,4 Richard Graeff,2 Qiguang Xie,1,4 Karen L. Gamble,1 Tetsuya Mori,1 Carl Hirschie Johnson1†

Dodd et al. (Reports, 14 December 2007, p. 1789) reported that the Arabidopsis circadian clock incorporates the signaling molecule cyclic adenosine diphosphate ribose (cADPR). In contrast, we found that there is no rhythm of cADPR levels nor are there any significant effects on the rhythm by cADPR overexpression, thus raising questions about the conclusions of Dodd et al.

Circadian (daily) rhythms regulate many biological processes to enhance fitness (1). In plant and animal cells, there is a daily oscillation in the level of cytosolic free calcium (Ca2+) that is likely to rhythmically modulate the activities of myriad Ca2+-regulated cellular processes (2–4). A question of central importance is what factor or factors couple the rhythm of Ca2+ to the core circadian pacemaker.

From their experiments on the plant Arabidopsis thaliana, Dodd et al. (5) contend that this Ca2+-regulating factor is the intracellular level of cyclic adenosine diphosphate ribose (cADPR). Cyclic ADPR would seem to be an excellent potential candidate for a regulator of Ca2+ rhythms, because it is known to promote release of Ca2+ from internal stores in plants and animals (6, 7). Moreover, cADPR mediates hormone responses in Arabidopsis (8, 9). Dodd et al. (5) reported that cADPR levels undergo significant circadian oscillations in Arabidopsis and, further, that manipulations of cADPR levels have significant effects on circadian rhythms. Our attempts to confirm a circadian rhythm of cADPR levels or to detect an impact of changes in cADPR levels upon the circadian system in Arabidopsis were unsuccessful. First, Dodd and colleagues reported a circadian oscillation of cADPR with higher levels in the subjective daytime (5). We repeated these measurements using the same assay as that used by Dodd and colleagues, which had been previously developed and applied to both plant and animal tissue (fig. S1) (10). The first discrepancy we noted was that Dodd and colleagues reported basal cADPR levels in wild-type Arabidopsis seedlings to range between 0.1 and 0.5 pmol/µg protein (100 to 500 pmol/mg protein), which is ~1000 times as high as the levels we measured (Fig. 1 and fig. S2) and 100 to 500 times as high as those measured by an independent group (9). More important, we found no evidence for a circadian oscillation in cADPR levels. We performed two separate 48-hour time course experiments in constant light (LL) using the same Arabidopsis accession line as that of Dodd et al. (Col-0) and a third 48-hour experiment using a different Arabidopsis accession (Ws) (Fig. 1, A and B and fig. S2) (11). Cosinor analyses (12) on the data depicted in Fig. 1A revealed that less than 3% of the variance in cADPR levels could be attributed to a 24-hour oscillation and is therefore insignificant (R2 = 0.029) (11). Moreover, when all three separate experiments were analyzed in a linear mixed model design (11), the 24-hour component to the data was not significant (fig. S3).

We conclude that there is no consistent or reproducible circadian oscillation of cADPR levels. A major experimental support to the argument of Dodd et al. of the importance of cADPR in the plant circadian system was that 10 to 50 mM nicotinamide suppressed the amplitude of their cADPR rhythm and that 50 mM nicotinamide lengthened the period of the leaf movement and

Fig. 1. [cADPR] fluctuation shows no circadian rhythm, and circadian [Ca2+]cyt oscillates independently of the elevated cADPR level. (A) cADPR concentration in constant light in Col-0 wild type. For cADPR analysis, aerial tissues were collected 24 hours after the entrained seedlings were released to constant light (LL). Each point represents data from three to five assays of an extract taken at the indicated time point, plotted as mean ± SEM. (B) Mean ± SEM of cADPR from subjective day and night separately. By Student’s t test, there was no statistically significant difference between the mean day versus night levels in either the second day (P = 0.453) or the third day (P = 0.567) of LL. (C) Mean traces of circadian [Ca2+]cyt oscillation as monitored by aequorin luminescence (2) in 8-day-old Arabidopsis seedlings treated with 30 µM 17β-estradiol at CT 13 on the first day in LL. Filled circles, empty vector control; open triangles and gray squares, overexpression of Aplysia ADPR cyclase, lines 1 and 4 respectively. Mean for 8 to 11 samples per point (the same data are shown in fig. S5 with error bars). (D) cADPR levels in transgenic lines expressing Aplysia ADPR cyclase. Sampling was done after 0, 6, 24, and 48 hours induction with 30 µM 17β-estradiol. Open bars, empty vector control; expression of Aplysia ADPR cyclase, lines 1 (striped bars) and 4 (cross-hatched bars). The extent of the cADPR increase was 4.6-fold in line 1 and 7.8-fold in line 4 at 48 hours of treatment. Mean values of five independent induction treatments are plotted with SEM bars.

1Department of Biological Sciences, Vanderbilt University, Nashville, TN 37235, USA. 2Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455, USA.
*Present Address: Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA.
†To whom correspondence should be addressed. E-mail: carl.h.johnson@vanderbilt.edu

References

1. Dodd et al. (Reports, 14 December 2007, p. 1789) reported that the Arabidopsis circadian clock incorporates the signaling molecule cyclic adenosine diphosphate ribose (cADPR). In contrast, we found that there is no rhythm of cADPR levels nor are there any significant effects on the rhythm by cADPR overexpression, thus raising questions about the conclusions of Dodd et al.
**CAB2** promoter activity rhythms (5). However, extracellular nicotinamide treatment has been reported to have many cellular effects, including affecting pyridine nucleotide levels and redox status, inhibiting key enzymes in multiple pathways (e.g., PARP, Sir2, and ADP-ribosyl transferases), increasing plant growth rate, altering plant hormone levels (IAA, GA3, cytokinins, and ethylene), stimulating plant cell apoptosis, and reducing insulin secretion by β cells (11). In particular, the high concentrations used by Dodd et al. (10 to 50 mM) will be expected to have many nonspecific effects. Therefore, we argue that a more specific modulator of cADPR levels is needed before an effect of cADPR on the circadian system can be inferred.

Dodd et al. (5) sought to provide a more specific manipulation of cADPR levels by constitutively expressing *Aplysia* ADP ribosyl cyclase. This treatment would be expected to clamp cADPR levels at a high level, thereby swamping rhythmic expression of processes under putative circadian control by cADPR. Nevertheless, Dodd et al. reported only a modest effect on rhythms by cADPR elevation; there was no significant effect on the circadian rhythm of leaf movement at CT4 or CT13 (fig. S6B). In contrast to the findings of Dodd et al., cADPR elevation did not reduce the robustness of the circadian rhythm; the RAE was not reproducibly different between the controls and cyclase overexpression for treatment at CT4 or CT13. In fact, the rhythms for cADPR elevation appear to be more robust (lower RAE) than for the controls (fig. S6A). Finally, not only does cADPR elevation have no significant effect upon the Ca^{2+} rhythm, it also does not alter circadian rhythms of Ca^{2+} (neither period, amplitude, nor phase), or indeed basal cytosolic Ca^{2+} levels. We therefore conclude that the circadian clock does not incorporate a cADPR-based feedback loop and that the basis for the plant Ca^{2+} rhythm must be sought elsewhere.
Comment on "The Arabidopsis Circadian Clock Incorporates a cADPR-Based Feedback Loop"
Xiaodong Xu, Richard Graeff, Qiguang Xie, Karen L. Gamble, Tetsuya Mori and Carl Hirschie Johnson

Science 326 (5950), 230.
DOI: 10.1126/science.1169503

http://science.sciencemag.org/content/326/5950/230.2
http://science.sciencemag.org/content/suppl/2009/10/21/326.5950.230-b.DC1
http://science.sciencemag.org/content/326/5950/230.2#BIBL
http://www.sciencemag.org/help/reprints-and-permissions

Use of this article is subject to the Terms of Service