Plant Circadian Clocks Increase Photosynthesis, Growth, Survival, and Competitive Advantage

Antony N. Dodd, Neeraj Salathia, Anthony Hall, Eva Kével, Réka Tóth, Ferenc Nagy, Julian M. Hibberd, Andrew J. Millar, Alex A. R. Webb

Circadian clocks are believed to confer an advantage to plants, but the nature of that advantage has been unknown. We show that a substantial photosynthetic advantage is conferred by correct matching of the circadian clock period with that of the external light-dark cycle. In wild type and in long- and short-circadian period mutants of Arabidopsis thaliana, plants with a clock period matched to the environment contain more chlorophyll, fix more carbon, grow faster, and survive better than plants with circadian periods differing from their environment. This explains why plants gain advantage from circadian control.

Circadian clocks produce an internal estimate of time that synchronizes biological events with external day-night cycles (T). Clocks with

similar properties and regulatory architecture have evolved at least four times, indicating that circadian rhythms confer a selective advantage. In plants, circadian rhythms control gene expression, stomatal opening, and the timing component of photoperiodism, which regulates seasonal reproduction, but the basis for their contribution to fitness during vegetative growth remains understood. Indirect evidence suggests a physiological benefit from circadian rhythms during growth under unnatural short photoperiods. Cyanobacteria and higher plants gain an advantage when the endogenous period is matched to the light-dark cycle. Rhythmic growth inhibitor secretion might provide an advantage by optimizing the phase relationship between clock-controlled biology and exogenous day-night cycles. Plants having clocks that are dissonant from the environment, therefore, may be disadvantaged. To test this hypothesis, we compared the performance of wild-type plants with lines having mutations that alter clock period length, in a range of environmental period lengths (“T cycles”) that were either matched or unmatched to the endogenous clock period.

We used three experimental approaches to test this hypothesis. First, wild-type plants with a circadian period of about 24 hours were grown in 10 hours light–10 hours dark (T20), 12 hours light–12 hours dark (T24) and 14 hours light–14 hours dark (T28) cycles. Second, we grew the long- and short-period mutants zfl-1 [T = 27.1 hours–32.5 hours; (10)] and toc-1 [T = 20.7 hours; (11)] in T cycles that were similar to, or dissimilar from, their endogenous clock periods (T20 and T28). In these T-cycle experiments, relative performance was measured within, but not between, genotypes, which specifically quantified the benefit when correctly tuned, the Arabidopsis circadian system enhances chlorophyll content, photosynthetic carbon fixation, and growth. We also show that circadian enhancement of photosynthesis leads to improved survival and competitive advantage.

References and Notes

1. Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge, CB2 3EA, UK. 2Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, UK. 3Plant Biology Institute, Biological Research Centre of the Hungarian Academy of Sciences, Post Office Box 521, H-6701 Szeged, Hungary. 4Present address: Bauer Center for Genomics Research, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA. 5Present address: School of Biological Sciences, University of Liverpool, Crown Street, Liverpool L69 7ZB, UK. 6Present address: Institute of Molecular Plant Sciences, Mayfield Road, University of Edinburgh, Edinburgh EH9 3JH, UK. 7To whom correspondence should be addressed.

E-mail: alex.webb@plantsci.cam.ac.uk
of circadian resonance and excluded effects not associated with the clock. Last, the effect of circadian arrhythmia on growth and physiology was investigated in well-characterized arrhythmic plants overexpressing the molecular oscillator component CCA1 (CCA1-ox), and compared with rhythmic wild types (8, 12). Experiments were conducted during vegetative growth, to assess the contribution of circadian resonance to growth and fitness, and to eliminate pleiotropic effects on life history due to the flowering time alterations that arise in circadian period mutants (13). We assessed the contribution of circadian resonance to carbon fixation, biomass, and leaf chlorophyll. Carbon fixation rates (14–16) and biomass (17) are traits associated with plant fitness; therefore, our study provides information concerning specific mechanisms by which the clock contributes to fitness.

In wild type and in short- and long-period mutants, leaves contained more chlorophyll when the oscillator period matched that of the environment. Leaves of Columbia-0 (Col-0) wild-type plants grown for 30 days in T24 contained more chlorophyll than Col-0 grown in T20 or T28 (Fig. 1A). When ztl-1 and toc1-1 were grown under T20 and T28, the long-period mutant ztl-1 contained more chlorophyll after growth in T28 than T20, whereas the short-period mutant toc1-1 contained more chlorophyll after growth in T20 than T28 (Fig. 1B). Therefore, correct matching of the circadian period with the external period increases chlorophyll accumulation. When the Col-0 clock was stopped by CCA1 overexpression, less chlorophyll was present compared with wild-type Col-0 under T24, which confirmed the dependence of chlorophyll accumulation on clock function (Fig. 1A).

There are circadian rhythms in transcript abundance of genes associated with chlorophyll synthesis, heme production, chlorophyll accumulation, and synthesis of chlorophyll-binding proteins (11, 18–20). Virtually all clock-controlled genes associated with chlorophyll synthesis and the light-harvesting apparatus exhibit peak circadian transcript abundance 4 hours after subjective dawn (18), which suggests that circadian expression of these genes could be important for enhancing light-harvesting capacity. Although the amount of light-harvesting complex pigments and proteins remains uniform over the diel cycle (19), the clock might sustain steady-state levels of proteins that exhibit light-induced degrada-

Fig. 1. Leaves contain more chlorophyll when their clock period matches the environmental period. (A) Total chlorophyll in Col-0 wild type grown under T20, T24, and T28, and in arrhythmic line CCA1-ox under T24. (B) Total chlorophyll in ztl-1 (long-period mutant) and toc1-1 (short-period mutant) grown in T20 and T28. For all groups, n = 5; data are means ± SEM. In two-sample t tests comparing chlorophyll concentrations to the line having the period matched to the environment, significance of results: *P < 0.05, **P < 0.01.

Fig. 2. The circadian clock enhances photosynthetic carbon fixation. (A) Mean C fixation per hour in ztl-1 and toc1-1 grown in T20 and T28. (B) Mean C fixation per hour in Col-0 wild type and arrhythmic CCA1-ox, in T24. (C) CCA1 overexpression (open circles) abolishes the circadian rhythms of CO2 fixation and stomatal opening that occur in Col-0 wild type (filled circles). (D) CO2 assimilation and stomatal conductance in CCA1-ox (open circles) and Col-0 wild type (filled circles) under light-dark cycles (indicated by bars on the x-axis). For these experiments, n = 6; data are means ± SEM (A and B) or largest standard error (C and D). In two-sample t tests comparing net C fixation per hour to the line with the clock period matched to the environment, significance of results: *P < 0.05, **P < 0.01.
of continuous light, but during prolonged constant light, assimilation was higher in CCA1-ox than wild type (Fig. 2C). This was reminiscent of the overexpression of rhythmic cyanobacterial lines by the arrhythmic line CLA (under continuous light (8). CCA1 overexpression abolished circadian rhythms of CO₂ fixation and stomatal opening in constant conditions. Fourier analysis (22) estimated period lengths of 24.1 ± 0.6 hours and 23.5 ± 0.3 hours for stomatal conductance and carbon assimilation, respectively, in Col-0 wild type, but failed to detect circadian regulation for CCA1-ox. Similarly, the tocl-1 and ztl mutations cause respective shortening and extension of the circadian period of CO₂ fixation and stomatal opening rhythms that occur in continuous light (23, 24).

Under light-dark cycles, rhythmic stomatal opening and closure was restored in CCA1-ox, but anticipation of dawn and dusk was absent, which demonstrated that the clock remained stopped in CCA1-ox, even in light-dark cycles (Fig. 2D). The stomata continued to open for the entire photoperiod in CCA1-ox, whereas in Col-0, stomatal opening ceased around midday. CCA1-ox, therefore, had higher total transpiration than Col-0 during the light period (Fig. 2D). Thus, the clock allows stomata to anticipate dusk and might participate in enhancement of water-use efficiency.

Because photosynthesis increased when exogenous and endogenous periods were similar (Fig. 2) and because long-term carbon fixation is correlated to leaf chlorophyll content (25), we reasoned that circadian resonance might increase vegetative growth. Col-0 wild type grown under T20, T24, and T28 had greatest vegetative biomass in T24 (Fig. 3, A and B). Under T20, aerial biomass was reduced by 4% in T24, and growth under T28 resulted in 42% less biomass relative to T24. When the long- and short-period mutants ztl-1 and toc1-1 were grown under T20 and T28, ztl-1 had maximum aerial biomass and leaf area under T28. In toc1-1, aerial biomass and leaf area were maximal under T20 (Fig. 3, C and D). These measures of growth were lower in toc1-1 than ztl-1 under all conditions. Separately, we compared growth of Col-0 and CCA1-ox under T24. After 32 days, the aerial biomass of CCA1-ox was 53% lower than that of Col-0 (Fig. 3, E and F). We did not compare growth of CCA1-ox and Col-0 in continuous light, because wild-type plants become arrhythmic under extended continuous light (26).

Therefore, circadian resonance enhances growth in wild-type plants and mutants with altered circadian period, and stopping the clock further reduces growth. Enhancement of biomass and photosynthesis by the circadian clock consequently indicates processes by which the clock increases fitness (14-17).

Fitness arising from circadian resonance might be reported by seedset (5). We compared seedset from short- and long-period mutants and wild type, under T24. In a single experiment, there were no large or consistent differences in mean seed production (toc1-1, 16200 seeds per plant; wild type, 15757 seeds per plant; n = 15). Because the clock determines flowering time, which becomes altered in period mutants (13), seedset is likely to be an ambiguous marker for the fitness implications of circadian resonance. However, we have demonstrated that circadian resonance enhances the established fitness traits of photosynthesis and biomass (Figs. 1 to 3) (14-17).

We performed reciprocal competitions between long- and short-period plants (7), using two short-period mutants of TOC1 [toc1-1 and toc1-2, τ = 20 hours (27)] and two long-period mutants of ZTL [ztl-1 and ztl-27, τ = 28 hours (9, 10)]. Mixed populations of toc1 and ztl were grown under T20 and T28, which generated a crowded lawn of plants. In these conditions, interactions among neighboring plants of differing genotypes affect physiological outcomes, in addition to the interaction between each plant and the light-dark cycle that we tested previously. We grew monocultures of each line, to assess the importance of differing growth rates among neighbors compared with growth rates among lines. Testing for competitive advantage derived from circadian resonance, using reciprocal competition, requires a mixed population of two period-length genotypes; therefore, it cannot be assessed in just wild type. The period-length mutants were appropriate for this experiment because the growth disadvantage that occurred when T ≠ τ in wild type also occurred with the period mutants (Figs. 1 to 3).

In two separate competition experiments, using different ZTL and TOC1 mutants, under T20, TOC1 mutants grew more successfully than ZTL mutants, as indicated by multiple parameters including chlorophyll content, leaf number, rosette diameter, and aerial biomass (Fig. 4). Conversely, under T28, growth of ZTL mutants was enhanced compared with TOC1. This was similar to results obtained when plants were grown without competition (Figs. 1 to 3). However, competition caused mortality of some plants, which did not occur in the absence of competition. Mortality was greater in ztl-27 under T20, and greater in toc1-2 under T28 (Fig. 4B). Circadian resonance, therefore, en-
hanced growth and survival, and this was more pronounced under competition than during monoculture (Fig. 4A). In two separate monoculture experiments, there was no consistent pattern of T cycle-dependent mortality (Fig. 4C). Therefore, both poor individual growth and outcompetition confer a disadvantage under dissonant T cycles. Our data underestimate the true growth advantage that occurs under light-harvesting complex proteins and chlorophyll, both of which are unstable in their unbound state (18). Incorrect matching of endogenous rhythms to environmental rhythms reduced leaf chlorophyll content, reduced assimilation, reduced growth, and increased mortality. Optimization of these parameters by circadian resonance could represent one of the mechanisms that has selected for circadian clock function during plant evolution. We suggest that selective plant breeding for enhanced crop performance must be performed carefully, because phase and period changes could arise from the close genetic linkage of phase and period loci (31) to the trait under selection, and cause alterations to clock function that might reduce vegetative yield. Clock manipulation could enhance food production during exploration of space and other planets, where the light-dark cycle may differ from the terrestrial 24-hour period. Circadian resonance is likely to provide an advantage in all kingdoms, because resonance of the internal clock with the external light-dark cycle ensures an optimal phase relation between physiology and the day-night cycle and provides the basis for anticipation of changes in environmental conditions.

References and Notes

6. H. R. Highkin, J. B. Hanson, Plant Physiol. 29, 301 (1954).
9. Materials and methods are available on Science Online.
28. N. Salatia et al., unpublished observations.
32. Authors E.K., R.T., A.H., and A.M. identified ztl-27; N.S. and A.M. conceived and performed competition and seedset experiments with A.H.; A.N.D., J.M.H., and A.A.R.W. performed all other experiments. We thank the U.K. Biotechnology and Biological Sciences Research Council (A.A.R.W., J.M.H., and A.M.); the Royal Society of London and the Isaac Newton Trust (A.A.R.W.); and the Howard Hughes Medical Institute (F.N.). We are grateful to E. Tobin (UCLA) and S. Kay (Scripps) for collaboration and donation of CCA1-ox and toc1-2, respectively.

Supporting Online Material

www.sciencemag.org/cgi/content/full/309/5734/630/DC1
Materials and Methods
References and Notes

1 April 2005; accepted 7 June 2005
10.1126/science.1115581

www.sciencemag.org SCIENCE VOL 309 22 JULY 2005 633
Plant Circadian Clocks Increase Photosynthesis, Growth, Survival and Competitive Advantage

Antony N. Dodd, Neeraj Salathia, Anthony Hall, Eva Kévei, Réka Tóth, Ferenc Nagy, Julian M. Hibberd, Andrew J. Millar, Alex A. R. Webb

Materials and Methods

Plant growth for period length manipulations and gas exchange. Surface sterilized Arabidopsis thaliana seeds were germinated on 0.5× Murashige and Skoog (MS) nutrient mix dissolved in 0.8 % (w/v) agar. Seedlings (10 days old) were transferred to a 1:1 mix of potting compost and vermiculite, and grown under a photon flux density of 160 mmol/m² per s, 65% relative humidity, and 20°C by day and night. Period lengths were manipulated by repeated advancement or reversal of the growth chamber timer clocks. During the growth period leaf area was measured. Leaf rosettes were imaged at regular intervals using a digital camera, and visible leaf area calculated from images using the ImageJ software package (rsb.info.nih.gov/ij/). Plants were selected for gas exchange measurements after 3 weeks of growth, and the growth experiment was terminated upon appearance of the first nascent inflorescence. At this point, plant material was harvested and dried for 24 hours for biomass measurement.

Total chlorophyll measurement. Chlorophyll was extracted from mature leaves in 96% ethanol, and measured (S1), using a minimum of five replicates for each measurement.

Photosynthetic gas exchange. Leaf gas exchange was measured using a six-cuvette custom-built infra-red gas analyzer [Arabidopsis Special System, PP Systems, Hitchin, UK; (S2)]. Cuvettes enclosed the entire leaf rosette, and conditions within the cuvette were automatically controlled so that relative humidity remained within 1% of 65% and [CO₂] was 365 ± 5 µl/liter. Each cuvette was measured for 10 min, at hourly intervals. Measurements represent the mean from 3 min of continuous sampling. When required, Fast Fourier Transform (nonlinear least squares method) was performed (S3). Results from each experiment, which were side-by-side comparisons of two lines, were independently verified by conducting two identical repeats of each experiment.

Reciprocal competition experiment. A. thaliana was germinated on MS agar in constant dim light (17–20 mmol/m² per s). Seedlings were transferred after 10 days to a 1:1 mix of vermiculite and compost, and grown in an array of alternating toc1-2 and ztl-27 genotypes (or single genotype for monocultures) at 1–cm spacing (9 plants × 11 plants array), with a row of guard plants. Plants were grown under cool white fluorescent lights (40 µmol/m² per s) at 19°C–21°C, under 10 hours light/10 hours dark or 14 hours light/14 hours dark cycles and harvested for morphological analysis after 3 weeks. Chlorophyll concentrations were quantified, and plants scored as "dead" when they had no visible chlorophyll.

ztl-27 was identified as a late-phase EMS mutant of the CAB:LUC transgenic line in the C24 ecotype, essentially as described previously for ztl-1 (S4, S5), and back-crossed
twice to the *CAB:LUC* parent. The mutation is predicted to change Gly\(^{452}\) to Asp in the fourth kelch repeat of ZTL. *ztl-27* has a long-period phenotype very similar to *ztl-1*, which will be described in detail elsewhere.

**References and Notes**