
Peer reviewed version

Link to published version (if available):
10.1111/nph.12489

Link to publication record in Explore Bristol Research
PDF-document

University of Bristol - Explore Bristol Research
General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
http://www.bristol.ac.uk/pure/about/ebr-terms.html
The circadian clock has transient plasticity of period and is required for timing of nocturnal processes in Arabidopsis

Antony N. Dodd1, Neil Dalchau2, Michael J. Gardner3, Seong-Jin Baek4 and Alex A. R. Webb4

1School of Biological Sciences, University of Bristol, Bristol, BS8 1UG, UK; 2Microsoft Research, 21 Station Road, Cambridge, CB1 2FB, UK; 3School of Life and Environmental Sciences, Deakin University, Geelong, Vic., 3220, Australia; 4Department of Plant Sciences, University of Cambridge, Cambridge, CB2 3EA, UK

Summary

- A circadian rhythm matched to the phase and period of the day–night cycle has measurable benefits for land plants. We assessed the contribution of circadian period to the phasing of cellular events with the light : dark cycle. We also investigated the plasticity of circadian period within the Arabidopsis circadian oscillator.
- We monitored the circadian oscillator in wild-type and circadian period mutants under light : dark cycles of varying total duration. We also investigated changes in oscillator dynamics during and after the transition from light : dark cycles to free running conditions.
- Under light : dark cycles, dawn and dusk were anticipated differently when the circadian period was not resonant with the environmental period (‘T cycle’). Entrainment to T cycles differing from the free-running period caused a short-term alteration in oscillator period. The transient plasticity of period was described by existing mathematical models of the Arabidopsis circadian network.
- We conclude that a circadian period resonant with the period of the environment is particularly important for anticipation of dawn and the timing of nocturnal events; and there is short-term and transient plasticity of period of the Arabidopsis circadian network.

Introduction

Circadian regulation provides a cellular estimate of the time of day. This is thought to co-ordinate physiology and metabolism with day–night cycles, and anticipate daily changes in the environment. Plant circadian clocks also tune signalling pathways so that stimulus-induced responses are appropriate for the time of day (Hotta et al., 2007). In Arabidopsis (Arabidopsis thaliana), the circadian clock optimizes productivity, starch utilization, seed yield and seed viability (Green et al., 2002; Dodd et al., 2005; Graf et al., 2010). Circadian regulation also contributes to the performance of barley, rice and soybean (Izawa et al., 2011; Faure et al., 2012; Preuss et al., 2012). Understanding how circadian regulation improves plant performance is therefore an agriculturally relevant topic that can be used to guide future crop improvement.

In Arabidopsis, the circadian oscillator is formed from a series of interlocked transcription–translation feedback loops (Pokhilko et al., 2012). Entrainment of the oscillator sets circadian phase to match that of the light : dark cycle. This occurs through the action of cryptochrome and phytochrome photoreceptors, and in response to exogenous temperature cues (Salomé & McClung, 2005; Harmer, 2009; Thines & Harmon, 2010). The oscillator produces output timing signals that communicate an estimate of the time of day from the oscillator to circadian regulated processes within the cell. Output signalling involves the circadian regulation of transcription (Harmer et al., 2000; Covington et al., 2008), Ca2+ signalling (Dodd et al., 2007), epigenetic mechanisms (Jones et al., 2010), modulation of phytohormone signalling (Covington & Harmer, 2007; Legnaioli et al., 2009) and alternative splicing (Sanchez et al., 2010). Many oscillator components including CIRCADIAN CLOCK ASSOCIATED1 (CCA1), TIMING OF CAB2 EXPRESSION1 (TOC1), ZEITLUPE (ZTL), GIGANTEA (GI) and LATE ELONGATED HYPOCOTYL (LHY) were identified on the basis of altered circadian rhythm or development phenotypes in genetic screens or overexpression studies (Millar et al., 1995; Schaffer et al., 1998; Wang & Tobin, 1998; Fowler et al., 1999; Somers et al., 2000). Oscillator function is often analysed in terms of emergent properties such as free running period (FRP) and phase. These are genetically encoded, but can be modulated by signalling, in addition to genetic or pharmacological manipulation (Millar et al., 1995; Dodd et al., 2007). It has been proposed that the complexity of circadian clocks is an adaptation that confers robustness against perturbation by environmental noise (Troein et al., 2009).

In cycles of light and dark, many outputs from the circadian oscillator combine circadian timing signals with light signals to regulate the phasing of cellular events (Dalchau et al., 2010; Noordally et al., 2013). Because photoperiod length modulates phase (Love et al., 2004; Sawa et al., 2007), we wished to determine the contribution of circadian regulation to cellular timing.

doi: 10.1111/nph.12489

Key words: Arabidopsis thaliana, circadian rhythms, environmental adaptation, signal transduction, systems biology.
under cycles of light and dark. This is important for understanding the function of circadian rhythms in whole plants. For example, phenotypes arising from mutations to the circadian oscillator are often interpreted in the context of mistiming of cellular events. We therefore investigated the role of circadian period for anticipation of daily changes in the environment. We performed experiments where the circadian period resonated with the period of the environment, and where the circadian oscillator was non-resonant (or ‘dissonant’) with the environment because the FRP differed from the environmental period by several hours (Ouyang et al., 1998). We found that a circadian period that is resonant with the period of the environment is particularly important for anticipation of dawn. We also investigated the changes in oscillator properties that are caused by forced entrainment of the oscillator to light : dark cycles (‘T cycles’) that differ from the FRP. We found that the oscillator has transient plasticity of period. We found that period is altered temporarily by entrainment to a T cycle differing from FRP. Existing mathematical models of the circadian network broadly capture the timing of this transition.

Materials and Methods

Plant culture and entrainment conditions

Surface-sterilized seeds of Arabidopsis thaliana (L.) Heynh., were germinated and grown under sterile culture as described elsewhere (Noordally et al., 2013). Seeds were stratified in darkness for 48 h before germination and growth in Panasonic MLR-352 growth chambers. Seedlings were cultivated in clusters of 15–20 plants, within sterile plastic rings, to produce six replicate regions of luciferase bioluminescence per treatment or genotype (Love et al., 2004). Plants were entrained to square wave cycles of light (110 μmol m⁻² s⁻¹) and dark at 19°C for 10 d before timecourse imaging. Entrainment regimes comprised light and dark periods of equal duration (e.g. 10 h : 10 h, light : darkness, for 20 h T cycle). Seedlings were treated with 5 mM luciferin (D-luciferin potassium salt, Melford Laboratories, Suffolk, UK) 24 and 12 h before the start of imaging. Growth chambers were configured so that dawn occurred on day 10, 1 h before the start of timecourse imaging, irrespective of entrainment regime.

Timecourse imaging of luciferase bioluminescence

Bioluminescence imaging was performed using a Photek ICCD218 high resolution intensified CCD photon counting camera system (Photek, East Sussex, UK). Luciferase bioluminescence was integrated every 2 h, using the camera in photon counting mode. Agar plates were illuminated between integrations using an equal mix of red and blue LED light (75 μmol m⁻² s⁻¹ total photon flux density). For light : dark cycle experiments, lights were configured to automatically create T20, T24 and T28 regimes. During imaging the temperature was 19°C. The first 120 s of each integration was removed to eliminate delayed chlorophyll fluorescence from the data. For experiments under constant light (LL), oscillation properties were quantified using the BRASS software, with phase values determined using the Mfourfit algorithm (millar.bio.ed.ac.uk). The first 24 h of LL is considered transitory, and consequently not a true circadian cycle, so these data were discarded before BRASS analysis.

Poincaré analysis

Oscillatory behaviours were analysed using a methodology based on the theory of the Poincaré map (Moon, 1992 and Wikipedia entry for a brief history and description). Our usage was motivated by the ‘phasegram’ methodology (Herbst et al., 2013). First, a two-dimensional ‘phase plot’ of the data (measured or simulated) was constructed. The data points were plotted as a function of the data points at a fixed interval in the past (as close to one quarter-cycle as possible, i.e. y(t) vs y(t + τ)). For example, the measurement at t = 30 h was compared with the measurement at t = 24 h, t = 32 h compared with t = 26 h, etc. τ was chosen as the nearest interval to one quarter of T. As the measurements were collected at a 2-h sample interval, this equated to 6 h for T20 and T24, and 8 h for T28. Next, ‘Poincaré sections’ were drawn that intersect the phase plots, producing two groups of intersections per cycle. In graphical representations of the data, the sections were indicated as dashed lines, and groups of intersection points indicated by different colours. We used two lines throughout, one defined by y = x, and the other defined by y = c−x, where c was chosen to approximately intersect the centre of the circular phase plots. The intersection points were calculated using the ‘Fast and Robust Curve Intersections’ software contributed to Matlab Central (Schwarz, 2012). Each group of intersection points represents equivalent positions through successive cycles. Therefore, the time since last intersection was calculated for each point, giving an approximation of cycle period as a function of time.

Simulation of forced oscillator dynamics

Mathematical models of the Arabidopsis circadian network were simulated under entrained T cycles of between 20 and 28 h duration (Locke et al., 2006; Pokhilko et al., 2010, 2012). The equations were solved numerically using the ode15s stiff equation solver in Matlab (Shampine & Reichelt, 1997). Each model was simulated for 1200 h in entrained T cycles to ensure convergence to a stable limit cycle, although only two cycles of light and dark are shown in figures. The light input parameter was then switched to simulate LL and enable analysis of the transition from forced cycles to free run.

Results

Oscillator dynamics under light : dark (LD) cycles similar to, and different from, the free running period

In order to investigate the contribution of circadian period length to cellular timing in LD cycles we monitored the functioning of the circadian oscillator under light : dark cycles with a period similar to, or different from, the FRP. We used a variety of
promoter-luciferase reporters and genotypes to distinguish general features of circadian regulation from reporter- or genotype-specific phenotypes. First, we studied rhythms of activity of the promoter of CHLOROPHYLL A/B-BINDING PROTEIN 2 \((\text{CAB2})\) fused to recombinant luciferase \((\text{LUC})\) under LD cycles that were similar to and different from the FRP of \(C24\) wild-type and the period mutants \(\text{toc1-1}\) and \(\text{ztl-1}\) (FRP c. 24, 21 and 28 h, respectively; Millar \textit{et al.}, 1995; Somers \textit{et al.}, 2000). In LD cycles with a 20 h period (‘\(T20\)’), \(T24\) and \(T28\), there were oscillations in \(\text{CAB2::LUC}\) bioluminescence in the \(C24\) wild-type, \(\text{toc1-1}\) and \(\text{ztl-1}\) (Fig. 1a–c).

The timing of the trough and reactivation of \(\text{CAB2::LUC}\) activity during the dark period provides a measure of the anticipation of dawn by the circadian oscillator. The timing of the nocturnal trough of \(\text{CAB2::LUC}\) changed substantially when circadian period differed from \(T\). Under \(T24\), minimum \(\text{CAB2::LUC}\) activity in \(C24\) wild-type was \(6.4 \pm 0.3\) h before dawn, compared with \(8.3 \pm 0.5\) and \(4.2 \pm 0.3\) h before dawn in \(\text{toc1-1}\) and \(\text{ztl-1}\) respectively (Fig. 1b, right-hand detail). In \(T20\), the trough of \(\text{CAB2}\) promoter activity occurred closer to dawn in \(\text{toc1-1}\) (\(2.4 \pm 0.5\) h before dawn) and \(C24\) (\(2.8 \pm 0.4\) h before dawn) compared with \(\text{ztl-1}\) (\(4.0 \pm 0.4\) h before dawn; Fig. 1a). In \(T28\), nocturnal anticipation of dawn varied more substantially between genotypes. In \(\text{toc1-1}\) and \(C24\), minimum \(\text{CAB2}\) promoter activity was \(13.1 \pm 0.3\) and \(13.4 \pm 0.3\) h before dawn, whereas in \(\text{ztl-1}\), the minimum was \(6.9 \pm 0.3\) h before dawn (Fig. 1c). There was a pre-dawn increase in \(\text{CAB2}\) promoter activity that occurred earlier in \(\text{toc1-1}\) and \(C24\) than \(\text{ztl-1}\) (Fig. 1c, right-hand detail). Therefore, when endogenous period differs considerably from the \(T\) cycle, circadian timing becomes progressively desynchronized with the environment the more time that has elapsed since dawn. This suggests a circadian clock with a period well-matched with the environment is important for the timing of nocturnal processes and anticipation of dawn.

Circadian regulation was also important for anticipation of dusk, but to a smaller degree than for anticipation of dawn. Anticipation of dusk was mis-timed when the circadian period was longer than the \(T\) cycle. For example, when \(\text{ztl-1}\) was under \(T24\) an elevated shoulder of \(\text{CAB2}\) promoter activity extended across dusk, compared with pre-dusk deactivation of \(\text{CAB2::LUC}\) in the wild-type (Fig. 1b). Similarly, in \(T20\) \(\text{CAB2::LUC}\) activity persisted later into the dark period in \(C24\) and \(\text{ztl-1}\), compared with \(\text{toc1-1}\) (Fig. 1a, right-hand detail). Under \(T20\), the mean time of peak \(\text{CAB2::LUC}\) bioluminescence did not vary significantly between \(C24\), \(\text{ztl-1}\) and \(\text{toc1-1}\) (Fig. 1a). Under \(T28\), \(\text{CAB2::LUC}\) bioluminescence peaked \(4.2 \pm 0.2\) h after dawn in \(\text{ztl-1}\). This was significantly later than the other genotypes (\(P < 0.001\) in Tukey pairwise multiple comparison after ANOVA) and is consistent with the long circadian period of \(\text{ztl-1}\) (Somers \textit{et al.}, 2000; \(3.4 \pm 0.2\) and \(2.5 \pm 0.3\) h after dawn in \(\text{toc1-1}\) and wild-type, respectively; Fig. 1c). The slightly later \(\text{CAB2::LUC}\) peak (\(0.9\) h) in \(\text{toc1-1}\) relative to wild-type under \(T28\) is moderately significant (\(P = 0.04\) in Tukey pairwise multiple comparison) and may arise from rounded peaks of \(\text{CAB2::LUC}\) bioluminescence compared with \(C24\) under \(T28\) (Fig. 1c).

We used reporters of the core circadian network to investigate oscillator function under LD cycles that were resonant with and dissonant from FRP. Under \(T24\), \(\text{CCA1}\) promoter activity peaked immediately after dawn, with minimum activity at the end of the dark period (Fig. 2a). Under \(T28\), \(\text{CCA1::LUC}\) activity started to increase well before dawn (\(9.3 \pm 0.3\) h; Fig. 2a). The early activity increase was considerably larger than the \(4\) h difference between the \(T\) cycle duration of \(T24\) and \(T28\), suggesting that oscillation waveform is modified in \(T28\). Peak \(\text{CCA1::LUC}\) activity varied little irrespective of \(T\) cycle duration, presumably because the oscillator was reset by dawn (Fig. 2a). We also investigated this by monitoring rhythms of \(\text{TOC1}\) and...
COLD, CIRCADIAN RHYTHM, AND RNA BINDING2 (CCR2) promoter activity, which have reversed phases relative to CAB2 and CCA1. Under T24, TOC1::LUC and CCR2::LUC bioluminescence peaked immediately after dusk (1 h in both cases), reaching a minimum during the second half of the photo-period (Fig. 2b,c). In T28, maximum TOC1 and CCR2 promoter activity occurred earlier relative to dusk than under T24 (2.8 ± 0.2 h (TOC1) and 3.0 ± 0.3 h (CCR2) before dusk; Fig. 2b,c). Under T20, the dusk peak of TOC1 promoter activity was attenuated and appeared arrhythmic or biphasic (Fig. 2b). A T cycle shorter than the FRP therefore prevented the rhythmic diel programme of promoter activity of a dusk-phased oscillator component. The data from the diurnal-phased (Figs 1, 2) and nocturnal-phased reporters (Fig. 2) both indicate that a circadian period that is dissonant from the environmental period causes a progressively increasing phase mismatch through the diel cycle. This suggests that a mismatch between FRP and environmental period has an increasingly disruptive impact as time from dawn increases. This is likely to be because the primary resetting signal for the oscillator is dawn.

Together with our experiments using period mutants (Fig. 1), this also indicates that a circadian oscillator that resonates with the environment is particularly relevant for the timing of nocturnal events and anticipation of dawn. This is because the amount of time before dawn that the trough of CAB2::LUC occurred was dependent upon T cycle duration (Fig. 3b), which was statistically significant. There was also a strong relationship between T cycle duration and the amount of time before dawn of the CCA1 promoter activity minimum, and the time before dawn of the TOC1::LUC and CCR2::LUC peaks (Fig. 3d). By contrast, there was limited variation in the time after dawn of the peak in CAB2::LUC bioluminescence across all T cycles and genotypes investigated (Fig. 3a). Similarly, there was little dependency upon T cycle length on the timing of the post-dawn peak of CCA1::LUC activity or the midday trough of TOC1::LUC and CCR2::LUC (Fig. 3c).

For the relationships in Fig. 3, a gradient of −1 indicates that the time of the peak or trough of promoter activity was defined by the previous zeitgeber (dawn), and a gradient of 0 indicates that the peak/trough was controlled by the T cycle duration, dark period duration or timing of dusk. The mean gradients of −1.3 for both toc1-1 and C24 indicate that for longer T cycles, the trough of CAB2 promoter activity occurred earlier in the night relative to dawn, so dawn was anticipated earlier than under resonant conditions (Fig. 3b; Table 1). Similarly, the mean gradients of −1.1 (CCA1::LUC) and −0.8 (TOC1::LUC and CCR2::LUC) indicate a strong but not total dependency of the timing of the trough of bioluminescence upon the time elapsed since dawn in Col-0 (Fig. 3d; Table 2). In ztl-1, the CAB2 minimum was a similar amount of time before dawn irrespective of T cycle length.

**Fig. 2** Rhythms of (a) CCA1::LUCIFERASE, (b) TOC1::LUCIFERASE and (c) CCR2::LUCIFERASE bioluminescence in total light: dark cycles of 20 h (T20), 24 h (T24) and 28 h (T28). Data are mean background-subtracted bioluminescence from a 650 s integration of Arabidopsis thaliana seedlings (n = 6 ± SEM). White and black bars on x-axes indicate light and dark periods, respectively.
circadian oscillator to simulate the transition of the oscillator on current understanding, we used mathematical models of the cycles that differ from the FRP. To provide a perspective based for the circadian oscillator incorporate properties that explain the cycles Simulated oscillator behaviour after forcing to dissonant T input to the oscillator.

Fig. 3 A circadian oscillator that resonates with the environment is required for anticipation of dawn. (a) Mean time after dawn of maximum CAB2::LUC bioluminescence in C24 wild-type (red), toc1-1 (grey) and ztl-1 (blue) under 20 h (T20), 24 h (T24) and 28 h (T28); (b) mean time before dawn of minimum CAB2::LUC bioluminescence; (c) mean time after dawn of peak (CCA1::LUC) or minimum (TOC1::LUC, CCR2::LUC) bioluminescence; (d) mean time before dawn of peak (TOC1::LUC, CCR2::LUC) or minimum (CCA1::LUC) bioluminescence. Data are mean (n = 6) values derived from bioluminescence timecourse imaging of six clusters of 15–20 Arabidopsis thaliana seedlings ± SEM. Because TOC1::LUC was arrhythmic under T20 light: dark (LD), peak times were estimated from the bioluminescence peak that was closest to dawn. Dashed and dotted lines indicate gradients of –1 and 0, respectively, for comparison with slope of data. (a, b) Statistical significance of time of peak or trough of each genotype, determined with one-way ANOVA, indicated above compared data points.

(Fig. 3b; Table 1). This could be because the long FRP of ztl-1 resulted in no circadian anticipation of dawn when T cycle < period, which may have been accentuated by the relatively long period of ztl-1 in our experiments (c. 30 h after entrainment to T24). This may alternatively relate to the ZTL-mediated light input to the oscillator.

Simulated oscillator behaviour after forcing to dissonant T cycles

We wished to determine whether existing mathematical models for the circadian oscillator incorporate properties that explain the behaviour of the circadian oscillator after entrainment to LD cycles that differ from the FRP. To provide a perspective based on current understanding, we used mathematical models of the circadian oscillator to simulate the transition of the oscillator from entrained LD T cycles to free run under simulated LL. We performed this for a simulated wild-type oscillator for several T cycles lengths, using three published models for the oscillator, to understand whether different network architectures could give different predictions about the behaviour of the oscillator during transition from entrained cycles to free running conditions (Fig. 4). All three models predict a graded transition of phase from the forced period to the FRP that takes approximately twice the duration of the T cycle period. We termed this unstable state between the forced and steady state the ‘transitional period’. For example, the ‘repressilator’ model (Pokhilkho et al., 2012) predicts that it takes c. 40 h for the oscillator to adopt a stable FRP following transition from T20 to LL, and 54 h to adopt a stable FRP following transition from T28 to FRP (Fig. 4c,i). Similarly, the model of Pokhilkho et al. (2010) predicts that stable oscillations will be reached c. 56 h (Fig. S2c,i). The model of Locke et al. (2006) was unable to resolve its endogenous near-24 h period with forcing to

### Table 1 Relationship between T cycle duration and waveform under light: dark cycles, for CAB2::LUCIFERASE activity in period mutants and wild-type Arabidopsis thaliana seedlings

<table>
<thead>
<tr>
<th>Gradient for each genotype</th>
<th>toc1-1</th>
<th>C24 wild-type</th>
<th>ztl-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time after dawn of peak bioluminescence</td>
<td>Gradient T20–T24</td>
<td>–0.2</td>
<td>–0.1</td>
</tr>
<tr>
<td>Mean gradient T20–T28</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Time before dawn of minimum bioluminescence</td>
<td>Gradient T20–T28</td>
<td>–0.1</td>
<td>–0.2</td>
</tr>
<tr>
<td>Mean gradient T20–T28</td>
<td>–1.5</td>
<td>–0.9</td>
<td>–0.0</td>
</tr>
</tbody>
</table>

### Table 2 Relationship between T cycle duration and waveform under light: dark cycles, for reporters of circadian clock function in Arabidopsis thaliana seedlings

<table>
<thead>
<tr>
<th>Gradient for each reporter</th>
<th>CCA1::LUC (peak)</th>
<th>TOC1::LUC (peak)</th>
<th>CCR2::LUC (peak)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradient T20–T24</td>
<td>0.4</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Gradient T24–T28</td>
<td>–0.2</td>
<td>–0.5</td>
<td>–0.5</td>
</tr>
<tr>
<td>Mean gradient T20–T28</td>
<td>0.1</td>
<td>–0.1</td>
<td>–0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gradient for each reporter</th>
<th>CCA1::LUC (trough)</th>
<th>TOC1::LUC (trough)</th>
<th>CCR2::LUC (trough)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradient T20–T24</td>
<td>–0.3</td>
<td>–0.1</td>
<td>–0.1</td>
</tr>
<tr>
<td>Gradient T24–T28</td>
<td>–1.8</td>
<td>–1.4</td>
<td>–1.5</td>
</tr>
<tr>
<td>Mean gradient T20–T28</td>
<td>–1.1</td>
<td>–0.8</td>
<td>–0.8</td>
</tr>
</tbody>
</table>
light: dark cycles of 20 or 28 h. Simulations in T20 and T28 produced irregular oscillations, in which the amplitude of successive cycles had low frequency oscillations (with a period of \(c. 10\) d). Therefore, the model was unable to maintain stable entrainment to T20 and T28. However, a rapid recovery was observed after LD entrainment to T23 and T26 (28 and 52 h respectively; Fig. S2c,i). A transitory excursion in the abundance of \(LHY/CCA1\) and \(TOC1\) transcripts was predicted by two models (Pokhilko et al., 2010, 2012) during the transition from LD to LL (Figs 4, S1). This was not specific to non-24 h T cycles because simulated transcript abundance excursions also occurred during the transition from driven T24 LD to LL (Figs 4f, S1f).

In summary, models for the circadian oscillator predict that following a transition from a forced non-24 h LD cycle, the oscillator will return gradually to the steady-state FRP rather than adjust instantly to its steady state.

Oscillator dynamics in plants after forcing to dissonant T cycles

We used the structure of our simulated timecourses to design experiments to test whether the Arabidopsis circadian network...
incorporates plasticity of period. For seedlings entrained to T20 and T28, there was a gradual transition from the forced period to the steady-state FRP (Fig. 5c,i). After forcing to T20 and T28, it took c. 55 h (T20) and 40 h (T28) of constant conditions for the oscillator to assume steady state (Fig. 5c,i). There was variation in time to steady state depending on the reporter; CCA1::LUC tended to reach steady state before TOC1::LUC and CCR2::LUC. Although this could reflect the earlier phase of CCA1 promoter activity within the 24 h cycle, this did not occur within the simulations so different parts of the network might reach steady state at different rates.

Therefore, after forcing to dissonant LD T cycles, the oscillator returns gradually from the forced period to steady-state period. This was predicted by three mathematical models, and the time taken for the oscillator to reach steady state is comparable between plants and simulations (40–55 h). The simulated excursions in period that were predicted by two models (Figs 4c,f,i, S1c,f,i), including brief period overshoots, did not occur in seedlings (Fig. 5c,f,i).

Steady state oscillator period is robust to entrainment T cycle

We found that the oscillator returns to a steady state 40–55 h after forcing to T cycles that differed from FRP (Fig. 5). This demonstrates that there is plasticity in the period that can be adopted by the oscillator. The plasticity is not sufficient to adapt the oscillator completely to T cycle variations as demonstrated by the altered timing of anticipation of dawn when the T cycle is mismatched to the FRP (Fig. 3). We therefore investigated whether entrainment to a range of T cycle regimes alters period or phase under steady-state free run. C24 wild-type, short (toc1-1) and long (ztl-1, tej) period mutants expressing C2B::LUC (Panda et al., 2002), and seedlings expressing CCA1::LUC, TOC1::LUC and CCR2::LUC were grown under LD cycles (T20, T24 and T28) before bioluminescence imaging under LL. We used tej to determine whether T cycle-induced variation in period of ztl-1 was specific to the ztl-1 mutation, or whether the phenotypes are general features of long period mutants. In all lines and reporters, after entrainment to photocycles ranging from 20 to 28 h the FRP was stable and generally dependent on genotype rather than entrainment (Fig. 6a,b). The exception was ztl-1. After entrainment to T20 all replicates of ztl-1, apart from one, were arrhythmic. The rhythmic replicate had low-amplitude oscillations of C2B::LUC following entrainment to T20 (Fig. S3a), and a longer period following entrainment to both T20 (31.5 h) and T28 (33.7 ± 0.3 h) compared with T24 (29.8 ± 0.2 h; Fig. 6a). There was also some FRP variation between entrainment regimes for the long period mutant tej (Fig. 6a).

We assessed the rhythmic robustness of circadian oscillations of C2B::LUC, CCA1::LUC, TOC1::LUC and CCR2::LUC following entrainment to T cycles far from and similar to FRP. Relative amplitude error (RAE) provides a measure of the rhythmic robustness of an oscillation, where RAE = 0 is a perfect sine wave and RAE ≥ 0.6 is considered arrhythmic (Alabadi et al., 2002).

For all T cycles and entrainment regimes, RAE was < 0.4 and in most cases RAE ≤ 0.2 (Fig. 6a,b). Therefore, entrainment to T cycles far from the FRP did not reduce the rhythmic robustness of the circadian oscillator.

We investigated whether there were T cycle-dependent alterations in phase during free run. For reporters of circadian clock associated genes (CCA1, TOC1, CCR2), longer T cycles caused later phases (Fig. 6b). The later phase may be an adaptation of the oscillator to changes in the photoperiod of the T cycle, as has been described for oscillator components and circadian oscillations of cytosolic free Ca2+ after entrainment to T24 cycles with long and short photoperiods (Love et al., 2004; Sawa et al., 2007). There was no consistent phase hierarchy for C2B::LUC (Fig. 6a).

Taken together, we conclude that oscillator period can be altered by the entrainment regime, but this is temporary and it is not possible to alter the steady-state free running period.

Discussion

A circadian oscillator resonating with the environment is important during the night and for anticipation of dawn

Because many rhythmic events in plants are controlled by both the circadian clock and light signals (Dalchau et al., 2010), we wished to determine the contribution of the circadian clock to daily timing of cellular activity in light dark cycles. We used promoters of genes associated closely with the circadian network (CCA1, TOC1, CCR2), and a circadian-regulated photosynthesis gene promoter (C2B) that is an output from the circadian clock, to investigate the role of circadian regulation in anticipation of daily changes in the light environment. Under LD, circadian regulation was of greatest importance for controlling the phase of pre-dawn regulation of reporters in the wild-type and circadian period mutants. If scaled to incorporate all circadian-regulated genes, this may be why resonance between the circadian oscillator and the external T cycle can increase the fitness of higher plants and cyanobacteria (Ouyang et al., 1998; Woelfle et al., 2004; Dodd et al., 2005). The consequence of dissonance between circadian period and T cycle was less pronounced during the light period. For example, the timing relative to dawn of the day peaks of CCA1::LUC and C2B::LUC, and the troughs of TOC1::LUC and CCR2::LUC, was not dependent on T cycle duration (Fig. 3). This is because the oscillator is reset by dawn and so little time passed between oscillator resetting and daytime events, so less time existed for the oscillator to fall out of phase with the environment. This suggests that circadian regulation is especially important for anticipation of dawn and also the timing of events during the dark period, such as the degradation rate of transitory starch (Graf et al., 2010). Therefore, whilst light signalling adjusts circadian timing in the light (Dalchau et al., 2010), in darkness circadian timing predominates and assumes a more important role.

The relative importance of circadian regulation to the daytime and nocturnal timing of transcription appears relatively independent from gene function. We found across all reporters that an
Fig. 5 Poincaré analysis and measurements of oscillator recovery following T cycle entrainment in Arabidopsis thaliana seedlings. Rhythms of CCA1::LUC, TOC1::LUC and CCR2::LUC bioluminescence analysed for transitions to constant light conditions from entrained T cycles of (a–c) 20 h, (d–f) 24 h, and (g–i) 28 h. (a, d, g) Data are mean values derived from bioluminescence imaging (n = 6; black lines), with SEM indicated by grey areas. Intersections between the phase plot and the Poincaré sections are indicated by coloured markers. White and black bars on x-axes indicate light and dark periods, respectively, and grey bars indicate light in the subjective night. (b, e, h) Phase plots for simulations in (a, d, g), showing the relationship between the concentrations at a fixed interval corresponding to approximately one quarter of the entrainment period (solid black lines). Poincaré sections are indicated by dashed black lines, and intersections with the phase plots by coloured markers. (c, f, i) Time between successive Poincaré intersection points, representing instantaneous period estimates. In (a, b, d, e, g, h), open circles indicate undesirable ‘extra’ Poincaré intersections that arise as a consequence of variability in the measurement signal, which were removed from the period estimate analysis in (c, f, i).
oscillator that resonates with the environment is important for timing during the dark period. CCA1 and CAB2 are light induced, and the integration of light and circadian signals is essential for adjustment of phase to match different photoperiod lengths (Hicks et al., 1996; Roden et al., 2002; Dalchau et al., 2010). Therefore, the oscillator may be sufficiently stable that additional information must be combined with timing signals to confer a degree of plasticity to gene expression that is absent from circadian control alone.

Circadian oscillators have transient plasticity of period

We investigated the properties of the oscillator during the transition from a forced cycle to free run. We found that there is a gradual rather than instantaneous waveform transition from the forced period to the steady-state FRP (Fig. 5). This suggests that driving the oscillator to assume dissonant T cycles alters the abundance or activity of oscillator components in a way that temporarily alters the period. Upon transfer to free running conditions, the oscillator readjusted to its genetically encoded FRP. Because the transition begins immediately following the onset of LL, the oscillator phase cannot remain synchronized with LD T cycles that differ from the steady-state FRP.

The period of animal circadian oscillators can also be altered by forcing the oscillator to nonresonant T cycles. For example, entrainment of the hamster to light : dark cycles of T23 or T25 alters the FRP of its activity rhythm (Pittendrigh & Daan, 1976). After entrainment to T23, the FRP returns over c. 40 d to 24 h, whereas after entrainment to T25, the FRP remains longer than 24 h for up to 100 cycles (Pittendrigh & Daan, 1976). Similarly, cockroaches entrained to T22 have a short FRP (Barrett & Page, 1989), mice entrained to T20 have FRP up to 1.5 h shorter than mice entrained to T28 (Aton et al., 2004), and short T cycles cause a slightly shorter (0.15 h) FRP in humans (Scheer et al., 2007). In these studies, the oscillators did not adopt the period of the T cycle and remained within 1.5–2 h of 24 h. In comparison with this very slow transition in mammals, the Arabidopsis oscillator returned rapidly in constant conditions to its steady state following an imposed T cycle that differed substantially from the FRP (Fig. 5). The short-lived transitional period that occurred in Arabidopsis is therefore analogous to a short-lived ‘transient’ rather than a long-term ‘after effect’ (Pittendrigh & Daan, 1976). One interpretation of this is that the Arabidopsis circadian network has greater robustness to perturbations than the mammalian circadian system, because it returns much more rapidly to its steady-state FRP following perturbation.

Oscillator response to perturbation may also depend on cell and tissue type. The oscillator within the suprachiasmatic nucleus (SCN) has rigid period and phase that cannot be shifted by 5 d exposure to non-24 h thermocycles (Abraham et al., 2010). Intercellular coupling of individual oscillators is thought to confer this rigidity (Abraham et al., 2010). In comparison, the period and phase of uncoupled cells can be altered by non-24 h thermocycles. T28 thermocycles alters phase, whilst T20 thermocycles shorten the FRP transiently (Abraham et al., 2010). Because intercellular oscillator coupling in plants is thought to be weak or
absent (Wenden et al., 2012), the gradual transition from forced to steady-state FRP that occurred in our experiments may parallel oscillator plasticity in uncoupled animal cells such as peripheral tissues, rather than the SCN. Despite this, the oscillator in Arabidopsis shifted to the steady-state FRP more rapidly (c. 55 h) than the uncoupled peripheral oscillator in mouse, which took at least 3 d (Abraham et al., 2010).

Unlike mammals, a longer term alteration of the steady-state FRP of the Arabidopsis oscillator was not detected after exposure to dissonant LD cycles (Pittendrigh & Daan, 1976; Fig. 6). This indicates that in Arabidopsis, FRP is an emergent property of the oscillator rather than an environmental response. The only environmental effect on steady-state FRP that we detected was variation in FRP in ztl-1 entrained to T20, T24 and T28 photocycles (Fig. 6a). In ztl-1, the oscillator did not entrain well to T20; the oscillation amplitude of CAb2::LUC was low compared with other experiments and reporters, and 83% of replicates were arrhythmic (Fig. S3). In our experiments, the FRP of ztl-1 was c. 30 h following entrainment to T24, so under T20 the 10 h difference between endogenous FRP and T cycle duration might have prevented stable entrainment. Alternatively, low-amplitude oscillations of ztl-1 after T20 might have arisen from poorly synchronized oscillations between cells, tissues or seedlings, so the bioluminescence signal incorporated overlapping oscillations with different phases. In ztl-1 under T20 LD cycles, the peaks of CAb2::LUC bioluminescence had variation in amplitude and peak shape between each cycle (Fig. 1a). This suggests the ztl-1 oscillator also does not function well under T20 LD. Simulations predict the mammalian circadian oscillator will entrain more easily when T cycle > FRP than when T cycle < FRP (Abraham et al., 2010). Our finding that ztl-1 entrains poorly to a short T cycle, combined with slightly greater FRP variability in the long-period mutant tej, suggests that this might also be the case in plants. A caveat for this interpretation is that ztl-1 modulates oscillator function depending on light intensity (Somers et al., 2000). Therefore, whilst our data are consistent with the finding that natural variation in period has a genetic basis (Boikoglou et al., 2011), our experiments show that the entrainment regime also induces some variation in FRP in certain genotypes.

Mathematical models of the Arabidopsis oscillator describe the progressive transition from T cycle period to FRP, such as the gradual nature of the transition and its duration (Figs 4, 5). Three existing simulated networks therefore incorporate properties that allow temporary plasticity of period. There were simulated excursions in transient period that did not occur in plants during the transition from LD to LL. As the Poincaré analysis approximates transitional period via transient differences in the timing of equivalent points on the oscillations, the excursions in transient period result from differences in phase across an LD to LL transition. This could arise from sensitive component(s) of the model during transition to free run, such as a particularly sensitive light input parameter. Alternatively, a component that is not captured within current models could buffer light input to the oscillator. Because the models incorporate most but not all aspects of the transitional period, future iterations of Arabidopsis circadian oscillator models could uncover factor(s) that underlie transitional changes in period. This might be conferred by specific biochemical components or by network topology that is already incorporated into recent simulations of the oscillator. We have generated experimental observations and analysis approaches that provide a platform for future improvements in the accuracy of model dynamics during transient transitional changes in period. Future refinements to the model may also provide mechanistic explanations for the more rapid transition of the Arabidopsis oscillator to FRP, following forcing to nonresonant T cycles, compared with animal systems. This has the potential to provide novel information about oscillator properties, particularly entrainment of the oscillator (Johnson et al., 2003), and about the physiological plasticity of organisms within unpredictable environments.

If periodic plasticity was required within the Arabidopsis circadian oscillator, considerable complexity would be required to confer memory of the entraining period and an oscillator is unlikely to be the most appropriate system to provide this plasticity. Our experiments support the notion that the oscillator has evolved to provide a fixed cycle that accommodates small seasonal adjustments and has robustness to environmental perturbations in order to anticipate accurately the changes in the light environment essential to the functioning of photosynthetic organisms.

Acknowledgements

A.N.D. is grateful to The Royal Society for awarding a University Research Fellowship. This work was supported by a Royal Society Research Grant and BBSRC research grant BB/I005811/1. We thank Dr M. Hanaoka (Chiba University, Japan) for constructive criticism.

References


**Fig. S2** Poincaré analysis and simulations of oscillator recovery following T cycle entrainment, using the Pokhilko 2010 model of the Arabidopsis circadian network.

**Fig. S3** Rhythms of *CAB2::LUCIFERASE* activity in C24 wild-type, toc1-1 and ztl-1 after entrainment to T20, T24 and T28 in Arabidopsis seedlings.

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.