Supplemental methods

1 Experimental Methods

gi-11 was isolated in a screen of T-DNA insertion lines described in (Richardson *et al.*, 1998; Fowler *et al.*, 1999). The *CAB:LUC+* and *CCR2:LUC* transgenes in the WS background were as described in (Hall *et al.*, 2002) and (Doyle *et al.*, 2002), respectively. The *toc1-9* allele introduces a termination codon at W138 of TOC1, as described (Kevei *et al.*, 2006). *toc1-10* was isolated from a T-DNA mutagenised population (E.K., B.F. and F.N. unpublished data). The mutation is caused by a deletion that removes the coding region of TOC1 (At5g61380) after S255 and the adjacent gene (At5g61390,encoding an exonuclease-like protein). *toc1-9* and *toc1-10* were generated in the same WS *CAB:LUC* background (Hall *et al.*, 2002) and both alleles show indistinguishable photomorphogenic and circadian phenotypes. To create *TOC1:LUC*, a 2068 bp region upstream of the *TOC1* coding region was amplified (forward primer: tctagacttctctgaggaatttcatc, reverse primer: ggatccgatcagattaacaactaaac) and inserted into pZPΩLUC (Schultz *et al.*, 2001). The construct was transformed into wild type Ws plants. Transgenic lines carrying single insertion of the transgene were selected and characterised. The *cca1-11* and *lhy-21* mutants were isolated from the Arabidopsis Functional Genomics Consortium population (Krysan *et al.*, 1999) and these were used to produce a *lhy;cca1* double mutant. Both the double and single mutants have been described in (Hall *et al.*, 2003). The triple mutant was produced by crossing the *cca1-11;lhy-21* double mutant with *gi-11*. Late flowering plants were selected in the F2 generation and genotyped with 3 allele-specific mutant and WT primer sets. *CAB:LUC+* was transformed into both the *lhy;cca1* double and the *gi;lhy;cca1* triple mutants. At least 4 independently transformed lines expressing the luciferase construct were analysed for each genotype. The data in figure 2 is of one representative line. A *CCR2:LUC+* or *TOC1:LUC+* transgene was introgressed into both the *lhy;cca1* double and the *gi;lhy;cca1* triple mutants by genetic crossing.

2 Rhythm Analysis

The seedlings were then sown on Murashige-Skoog media contain 3% sucrose and 1.5% agar. Seeds were kept at 4°C for 2 days and then grown in 12L:12D cycles of 80 μmol m^{-2}s^{-1} in a Sanyo MLR350 (Sanyo Gallenkamp PLC, UK). Temperatures both during entrainment and during experiments

were logged using Hobo temperature loggers (Onset computer corporation, USA). Luminescence levels were analysed using an ORCA-II-BT 1024, 16bit camera cooled to $-80°C$ (Hamamatsu photonics, UK). The camera was housed on top of a Sanyo MIR-553 cooled incubator maintaining a uniform temperature $\pm 0.5°C$ (Sanyo Gallenkamp PLC, UK). Illumination was provided by 4 red/blue LED arrays (MD electronics, UK). Image acquisition and light control was driven by WASABI imaging software (Hamamatsu photonics, UK). The images were processed using Metamorph 6.0 image analysis software (Molecular Devices corporation, USA). Alternatively, luminescence was recorded by an automated luminometer equipped by red and blue LED arrays, essentially as described (Hall *et al.*, 2002). Individual period estimates were generated by importing data into BRASS (available from www.amillar.org) and using BRASS to run fast fourier transform-nonlinear least squares (FFT NLLS) analysis programs (Plautz *et al.*, 1997) on each data trace to generate period estimates and relative amplitude errors (Rel. amp. Error). The data is representative of at least 2 independent experiments.

3 Computational Methods

We have built upon our network equations for the proposed interlocked feedback loop model for the Arabidopsis circadian clock (Locke *et al.*, 2005a), as a recent report suggests there is an additional feedback loop involving *LHY/CCA1* and the genes *PRR7* and *PRR9* (Farre *et al.*, 2005). The interlocked loop model consists of a feedback loop between *LHY*, which represents the function of both *CCA1* and *LHY* and is acutely light activated, and *TOC1*, and an additional loop between *TOC1* and a proposed gene *Y*, which is also light activated. An additional gene *X* is also proposed to be activated by TOC1 and then go on to activate *LHY* transcription, as TOC1 levels are low at dawn when *LHY* transcription is activated. We have added to this network an additional loop; *PRR7* and *PRR9* transcription is proposed to be activated by LHY/CCA1, and then PRR7 and PRR9 go on to repress *LHY* and *CCA1* transcription (Farre *et al.*, 2005). This gives us a three loop model for the clock (Figure 1).

We incorporated the *PRR7/9 - LHY/CCA1* feedback loop into the clock as follows. *PRR9* (Ito *et al.*, 2003) and *PRR7* (Yamamoto *et al.*, 2003) peak at the beginning and middle of the day respectively, with *PRR9* transcription acutely light activated. The functions of PRR7 and PRR9 in the clock are individually modest and hard to distinguish, notwithstanding their differing

light regulation, whereas the double *prr7;prr9* mutant gives a strong period phenotype (Farre *et al.*, 2005), so we combined their functions into a single gene in the model, termed *PRR7/9* (Eqns 14-16). *PRR7/9* transcription was given both an acute light activation term and a constant light activation term, as we previously used for Y, and is activated by nuclear LHY protein. However, our optimisation scheme minimised the parameters associated with the constant light activation of *PRR7/9*, so this term was removed from our equations for *PRR7/9* mRNA (Eqn 14).

We modified our terms for *LHY* mRNA levels to include the role of PRR7/9 (Eqn 1). PRR7/9 represses both *LHY*'s light activation and the activation by *TOC1*. In addition to the acute light reponse, we gave *LHY* mRNA levels a constant light activation term $\Theta_{\text{light}}(t)\, n_0$ as *LHY* transcription appears to be light activated throughout the day in an *prr7;prr9* plant (Farre *et al.*, 2005). $\Theta_{\text{light}} = 1$ when light is present, 0 otherwise.

We took the following as our mathematical model for the central circadian network, which involves the cellular concentrations $c_i^{(j)}(t)$ of the products of the i^{th} gene ($i = L$ labels LHY, $i = T$ labels TOC1, $i = X$ labels X, $i = Y$ label Y, $i = A$ labels PPR7/9) where $j = m, c, n$ denotes that it is the corresponding mRNA, or protein in the cytoplasm or nucleus respectively.

$$\frac{dc_L^{(m)}}{dt} = \left(\frac{g_0^\alpha}{(g_0^\alpha + c_A^{(n)\alpha})}\right)\left(\Theta_{\text{light}}(t)\left(q_1 c_P^{(n)} + n_0\right) + \frac{n_1 c_X^{(n)^a}}{g_1^a + c_X^{(n)^a}}\right) \times -\frac{m_1 c_L^{(m)}}{k_1 + c_L^{(m)}} \quad (1)$$

$$\frac{dc_L^{(c)}}{dt} = p_1 c_L^{(m)} - r_1 c_L^{(c)} + r_2 c_L^{(n)} - \frac{m_2 c_L^{(c)}}{k_2 + c_L^{(c)}} \quad (2)$$

$$\frac{dc_L^{(n)}}{dt} = r_1 c_L^{(c)} - r_2 c_L^{(n)} - \frac{m_3 c_L^{(n)}}{k_3 + c_L^{(n)}} \quad (3)$$

$$\frac{dc_T^{(m)}}{dt} = \left(\frac{n_2 c_Y^{(n)^b}}{g_2^b + c_Y^{(n)^b}}\right)\left(\frac{g_3^c}{g_3^c + c_L^{(n)^c}}\right) - \frac{m_4 c_T^{(m)}}{k_4 + c_T^{(m)}} \quad (4)$$

$$\frac{dc_T^{(c)}}{dt} = p_2 c_T^{(m)} - r_3 c_T^{(c)} + r_4 c_T^{(n)} - ((1 - \Theta_{\text{light}}(t))m_5 + m_6)\frac{c_T^{(c)}}{k_5 + c_T^{(c)}} \quad (5)$$

$$\frac{dc_T^{(n)}}{dt} = r_3 c_T^{(c)} - r_4 c_T^{(n)} - ((1 - \Theta_{\text{light}}(t))m_7 + m_8)\frac{c_T^{(n)}}{k_6 + c_T^{(n)}} \quad (6)$$

$$\frac{dc_X^{(m)}}{dt} = \frac{n_3 c_T^{(n)^d}}{g_4^d + c_T^{(n)^d}} - \frac{m_9 c_X^{(m)}}{k_7 + c_X^{(m)}} \quad (7)$$

$$\frac{dc_X^{(c)}}{dt} = p_3 c_X^{(m)} - r_5 c_X^{(c)} + r_6 c_X^{(n)} - \frac{m_{10} c_X^{(c)}}{k_8 + c_X^{(c)}} \quad (8)$$

$$\frac{dc_X^{(n)}}{dt} = r_5 c_X^{(c)} - r_6 c_X^{(n)} - \frac{m_{11} c_X^{(n)}}{k_9 + c_X^{(n)}} \quad (9)$$

$$\frac{dc_Y^{(m)}}{dt} = \left(\Theta_{\text{light}}(t)\, q_2 c_P^{(n)} + \frac{(\Theta_{\text{light}}(t)\, n_4 + n_5) g_5^e}{g_5^e + c_T^{(n)^e}}\right) \times \left(\frac{g_6^f}{g_6^f + c_L^{(n)^f}}\right) - \frac{m_{12} c_Y^{(m)}}{k_{10} + c_Y^{(m)}} \quad (10)$$

$$\frac{dc_Y^{(c)}}{dt} = p_4 c_Y^{(m)} - r_7 c_Y^{(c)} + r_8 c_Y^{(n)} - \frac{m_{13} c_Y^{(c)}}{k_{11} + c_Y^{(c)}} \quad (11)$$

$$\frac{dc_Y^{(n)}}{dt} = r_7 c_Y^{(c)} - r_8 c_Y^{(n)} - \frac{m_{14} c_Y^{(n)}}{k_{12} + c_Y^{(n)}} \quad (12)$$

$$\frac{dc_P^{(n)}}{dt} = \left(1 - \Theta_{\text{light}}\left(t\right)\right) p_5 - \frac{m_{15} c_P^{(n)}}{k_{13} + c_P^{(n)}} - q_3 \Theta_{\text{light}}\left(t\right) c_P^{(n)} \quad (13)$$

$$\frac{dc_A^{(m)}}{dt} = \Theta_{\text{light}}\left(t\right) \left(q_4 c_P^{(n)}\right) + \frac{n_6 c_L^{(n)g}}{g_7^g + c_L^{(n)g}} - \frac{m_{16} c_A^{(m)}}{k_{14} + c_A^{(m)}} \quad (14)$$

$$\frac{dc_A^{(c)}}{dt} = p_6 c_A^{(m)} - r_9 c_A^{(c)} + r_{10} c_A^{(n)} - \frac{m_{17} c_A^{(c)}}{k_{15} + c_A^{(c)}} \quad (15)$$

$$\frac{dc_A^{(n)}}{dt} = r_9 c_A^{(c)} - r_{10} c_A^{(n)} - \frac{m_{18} c_A^{(n)}}{k_{16} + c_A^{(n)}} \quad (16)$$

Here the various rate constants n_j, g_j etc parameterise transcription (n_j, g_j), degradation (m_j, k_j), translation (p_j), and the nuclear $\leftrightarrow$ cytoplasmic protein transport (r_j). The Hill coefficients are represented by α, a, b, c, d, e, f, g. Light is known to give an acute, transient activation response for expression of *LHY* and *CCA1* (Kim *et al.*, 2003; Kaczorowski & Quail, 2003; Doyle *et al.*, 2002). This was modelled as in (Locke *et al.*, 2005a,b), using a simple mechanism involving an interaction of a light sensitive protein P, with concentration $c_P^{(n)}$ with the *LHY* gene promoter. $\Theta_{\text{light}} = 1$ when light is present, 0 otherwise. The values of the four parameters that appear in the equation for $c_P^{(n)}$ are chosen so as to give an acute light activation profile which is close to that observed in experiment. The essential features of Eq 13 are that P is produced only when light is absent and is degraded strongly when light is present.

3.1 Parameter Optimisation

The parameter values for the optimum solution for the interlocked feedback loop model (Locke *et al.*, 2005a) were taken as our starting point. In order to reduce parameter space, the acute light activation term for *PRR7/9* q_4 was set to the same value as the acute light response for the LHY promoter q_1, g, the Hill coefficient of *PPR7/9* activation by LHY was set to the same value as c, the Hill coefficient of *TOC1* repression by LHY, and g_0, the constant of repression of LHY by APPR7/9 was set to 1. The value of the Hill coefficients were constrained through optimisation to take biological reasonable values of between 1 and 4, and the minimum value of the constant light activation term to *LHY*, n_0, was set to 0.5, in order to ensure the possibility of light activation through out the day.

The parameters in Eqn 1 were reoptimised to take into account that in a *prr7;prr9* plant the period of the clock is approx 30 hours in LL (Farre

et al., 2005). In order to model the *prr7;prr9* mutation the translation rate of PRR7/9, p_6, was set to 0, and then the equations were solved for 100000 simulated annealing points in order to minimise a qualitative cost function as defined in (Locke *et al.*, 2005a) which quantifies the goodness of fit of the solutions to several key pieces of experimental data. We briefly outline the terms of the cost function below, but for a full description of the method please see (Locke *et al.*, 2005a,b).

The equations were solved using MATLAB, integrated using the inbuilt stiff equation solver ODE15s (Shampine & Reichelt, 1997). The optimisation process described in the following sections was carried out by compiling the MATLAB code into C and running the code on a task farm super computer consisting of 31 x 2.6 GHz Pentium4 Xeon 2-way SMP nodes (62 CPUs in total). In order to evaluate the terms of the cost function, we solved numerically Eqns 1-16 over 600h, 300h in 12:12 LD cycles, and then 300h in LL conditions (the first 200h of each solution are discarded as transitory). In what follows we identify 1nM and 1h as the typical concentration and time scales, and measure all concentrations and rate constants in units where these are unity. We initialised our simulation at $c_i^{(j)} = 1$.

We made modifications to the WT cost function as defined for the interlocked loop model (Locke *et al.*, 2005a). We repeat a description of these terms here for completeness. The WT cost function is defined as:

$$\Delta = \delta_{\tau_{ld}} + \delta_{\tau_d} + \delta_{\phi} + \delta_{\text{size}} + \delta_{c_L} + \delta_{\phi d} \tag{17}$$

we now describe each term of the cost function, Eqn.17, in turn.

First, $\delta_{\tau_{ld}}$ measures the difference between the experimental target period and the mean period of the oscillation in mRNA levels of *LHY* and *TOC1* in light:dark (LD) cycles as exhibited by the model;

$$\delta_{\tau_{ld}} = \sum_{i=L,T} \langle (24 - \tau_i^{(m)})^2 / 0.15 \rangle_{ld} \tag{18}$$

This is the summed error in the period, τ, for *LHY* (L) and *TOC1* (T) mRNA levels (m) in light:dark cycles (LD), where $\langle\rangle_{ld}$ gives the average over the cycles between $200 < t < 300$, and a marginally acceptable period difference of $\approx$ 25mins contributes O(1) to the cost function for each term.

Second, the term δ_{τ_d} gives a similar measure in constant darkness (DD). These two terms ensure that the entrained and free running clocks are near limit cycles with the experimentally observed period (stably entrained in LD cycles and with a free running period greater than 24h (Millar *et al.*, 1995)),

$$\delta_{\tau_d} = \sum_{i=L,T} \langle (25 - \tau_i^{(m)})^2 / f \rangle_d \tag{19}$$

where the average of $\langle\rangle_d$ is now over $300 < t < 600$ (DD). The biological evidence strongly indicates that the free running period of the clock is greater than 24 (Millar *et al.*, 1995), probably about 25, but we have less confidence in assigning a precise value hence we adopt values of $f = 0.05$ if $\tau_i^{(m)} \leq 25$ and $f = 2$ if $\tau_i^{(m)} > 25$.

Thirdly δ_ϕ measures the difference between the target phase and the average phase of the peaks of *LHY* and *TOC1* mRNA expression in LD. It also ensures that the oscillations are entrained to the LD cycles,

$$\delta_\phi = \sum_{i=L,T} \left[\langle \Delta\Phi_i^2 \rangle_{ld} + \left(\frac{\sigma[c_i^{(m)}(t_{\rm p})]_{ld}}{0.05 \langle c_i^{(m)}(t_{\rm p}) \rangle_{ld}} \right)^2 + \left(\frac{\sigma[\Delta\Phi_i]}{5/60} \right)^2 \right] + \delta_{ent} \qquad (20)$$

The first term compares the mean difference in phase over the LD cycles, where $\Delta\Phi_i = \bar{\phi}_i - \phi_i$, ϕ_i is the phase (from dawn) of the RNA peak in the model and $\bar{\phi}_L = 1hr$, $\bar{\phi}_T = 11hr$ are the target phases of the peaks in $c_L^{(m)}$ and $c_T^{(m)}$ respectively. We assume a cost that is O(1) for solutions that differ by an hour. The next two terms ascribe a cost of O(1) for limit cycle solutions in LD cycles whose peak heights vary only within 5 percent of one another, and whose variations in peak phases are 5 minutes. $\sigma[]_{ld}$ is the standard deviation for the cycles in LD. The term δ_{ent} checks that the solution is truly entrained to the light/dark cycle, i.e is not oscillating with the correct phase simply because of the initial conditions chosen. This is achieved as follows: the solution is rerun for 75h, taking the solution at 202h and shifting it back 3h, i.e initialising the $t = 202$ solution as the t = 199 solution. The new phase of the second peak is compared to the original phase of the second peak. If the phase discrepancy is still near 3 h, then the solution is too weakly entrained, and the solution is pathological. The LD cycles have failed to phase shift the response. We assume that the rate of adjustment of the phase is linear in the discrepancy of the phase. This gives us a phase discrepancy that goes to 0 exponentially in time (like the radioactive decay equation). The characteristic time is then trivially related to the log of the phase discrepancy. It is this logarithmic variation that is reflected in our choice of δ_{ent}. Hence δ_{ent} takes the form of $\log(0.5)/\log(\delta\phi/3)$, where $\delta\phi$ is the phase discrepancy in hours between the shifted and original solution, and $\delta\phi/3$ is therefore the fraction of the imposed 3h phase shift remaining after 2 periods. The term log(0.5) gives the acceptable remaining phase difference of 1.5h for the second cycle, which results in an O(1) contribution to the cost function.

Next $\delta_{\rm size}$ checks that the oscillation sizes are large enough to be detectable experimentally, and quantifies the degree to which the clock in the model is

damped in constant conditions: we require that it is not strongly damped,

$$\delta_{\text{size}} = \sum_{i=L,T} \left[\left(\frac{1}{\langle \Delta c_i^{(m)} \rangle_{ld}} \right)^2 + \left(\frac{\tau_o}{\tau_e} \right)^2 \right]. \tag{21}$$

The first term introduces a > 1 cost for solutions in LD cycle with oscillation sizes, $(\Delta c_i^{(m)} = {c_i^{(m)}}_{max} - {c_i^{(m)}}_{min})$, less than 1nm, and the second term penalises oscillations that decay too quickly when entering DD as follows: τ_o is a time characterising the decay in the oscillations over the 300h in DD, $\tau_o = -300/\log(({\Delta c_T^{(m)}}_{ld} - {\Delta c_T^{(m)}}_{d})/{\Delta c_T^{(m)}}_{ld})$, and τ_e gives the marginally acceptable decay time, $-300/\log(0.75)$.

The term δ_{c_L} contains a measure of how broad the peak of *LHY* mRNA expression is in the proposed solution in LD cycles and is small only if the trace peaks sharply, as observed experimentally. This term is also only small if the peak heights of *LHY* mRNA expression drop when going from LD to DD,

$$\begin{aligned} \delta_{c_L} &= \sum_{i=2,-2} \langle \left(\frac{2/3 c_L^{(m)}(t_{\text{p}})}{c_L^{(m)}(t_{\text{p}}) - c_L^{(m)}(t_{\text{p}} + i)} \right)^2 \rangle_{ld} + ... \\ &\langle \left(\frac{0.05(c_L^{(m)}(t_{\text{p}} - 2) - c_L^{(m)}(t_{\text{m}}))}{c_L^{(m)}(t_{\text{m}}) - c_L^{(m)}(t_{\text{m}} + i)} \right)^2 \rangle_{ld} + 10 \left(\frac{\langle c_L^{(m)}(t_p) \rangle_d}{\langle c_L^{(m)}(t_p) \rangle_{ld}} \right)^4 \end{aligned} \tag{22}$$

The first term penalises *LHY* mRNA expression profiles that do not have a sharp peak in LD cycles, with an O(1) contribution if *LHY*'s expression level has dropped by 2/3 of its oscillation size within 2h before and after its peak of expression (at time t_p). The second term checks that *LHY* mRNA expression has a broad minimum, with an O(1) contribution if 2h before and after the minimum point (at time t_m) *LHY*'s expression has only increased to 5 percent of the level 2 h before *LHY*'s peak. The last term checks that the peak of *LHY* mRNA expression drops from LD into DD, as it loses its acute light activation.

Finally, $\delta_{\phi d}$ constrains an appropriate phase difference between the peak times of *LHY* (ϕ_L) and *TOC1* mRNA (ϕ_T), $\Delta\Phi_d = \phi_T - \phi_L$ (modulo half the period), with a characteristic prefactor of 10h.

$$\delta_{\phi d} = (10/\Delta\Phi_d)^2 \tag{23}$$

In order to model the *prr7;prr9* mutant the cost function error term for the WT period in DD, δ_{τ_d} was replaced with an error term for the period in LL,

$\delta_{\tau_{ll}}$ in order to find a solution in LL with a period of 30h, as opposed to a DD solution with a period of 25h. (Supplementary Table One, Supplementary figure 1).

$\delta_{\tau_{ll}}$ is given by:

$$\delta_{\tau_{ll}} = \sum_{i=L,T} \langle (30 - \tau_i^{(m)})^2 / f \rangle_{ll}. \tag{24}$$

This represents the summed error in the period, τ, for *LHY* (L) and *TOC1* (T) mRNA levels in constant light conditions, where $\langle\rangle_{ll}$ gives the average over the cycles between $300 < t < 600$. The biological evidence strongly indicates that the free running period of the clock in an *prr7;prr9* mutant plant is not less than 30h (Farre *et al.*, 2005), but we have less confidence in assigning a precise value hence we adopt values of $f = 0.05$ if $\tau_i^{(m)} \leq 30$ and $f = 2$ if $\tau_i^{(m)} > 30$. Also the error terms for the oscillation under constant conditions in δ_{size} and δ_{c_L} were calculated for LL, rather than DD.

The parameters for PPR7/9 were then optimised (Eqn 14-16) in order to model a WT plant. As in (Locke *et al.*, 2005a,b) the equations were solved for 1 million quasi random points in parameter space, and $\delta_{\tau_{ll}}$ was altered in order to search for a period in LL of 24h rather than 30h $\delta_{\tau_{ll}} = \sum_{i=L,T} \langle (24 - \tau_i^{(m)})^2 / 0.1 \rangle_{ll}$. The costfunction was also altered to find a short period oscillation in the *toc1* background (Mas *et al.*, 2003), as opposed to a short period oscillation in a *lhy;cca1* background (Locke *et al.*, 2005a). This gives a cost function:

$$\Delta = \delta_{\tau_{ld}} + \delta_{\tau_{ll}} + \delta_{\phi} + \delta_{\text{size}} + \delta_{c_L} + \delta_{\phi d} + \delta^{toc1}_{\tau_{ld}} + \delta^{toc1}_{\tau_{ll}} + \delta^{toc1}_{\phi} + \delta^{toc1}_{\text{size}} + \delta^{toc1}_{c_Y} \tag{25}$$

where the first 6 WT terms are as defined as above, and the label (toc1) denotes the new cost function for the *toc1* mutant plant. We define below the terms for the new *toc1* mutant terms of the cost function:

$$\delta^{toc1}_{\tau_{ld}} = \sum_{i=A,L} \langle (24 - \tau_i^{(m)})^2 / 0.15 \rangle_{ld} \tag{26}$$

is the summed error in the period, τ, for A (PRR7/9) and T (*LHY*) mRNA (m) levels in LD cycles. We penalise solutions with a period of *PRR7/9* greater than 20 hours under constant light conditions. $\delta^{(m)}_{\tau_{ll}} = 0$ if the period is less than 20 hours, otherwise:

$$\delta^{toc1}_{\tau_{ll}} = \langle (20 - \tau_A^{(m)})^2 / 0.1 \rangle_{ll} \tag{27}$$

The next term δ_{ϕ}^{toc1} is defined as:

$$\delta_{\phi}^{toc1} = \left[\langle \Delta\Phi_L^2 \rangle_{ld} + (\sigma[\Delta\Phi_L])^2\right] \tag{28}$$

Here the first term compares the mean difference in phase over the LD cycles, where $\Delta\Phi_i = \bar{\phi}_L - \phi_L$, ϕ_L is the phase (from dawn) of the *LHY* mRNA peak in the model and $\bar{\phi}_L = 1h$ is the target phase of the peak in $c_L^{(m)}$. The second term describes a cost of $O(1)$ for solutions whose variations in peak phase are 1h. Next,

$$\delta_{\text{size}}^{toc1} = \sum_{i=A,L} \left(\frac{1}{\langle \Delta c_i^{(m)} \rangle_{ld}} \right)^2 \tag{29}$$

This term costs for solutions in LD cycle with oscillation sizes, ($\Delta c_i^{(m)} = {c_i^{(m)}}_{max} - {c_i^{(m)}}_{min}$), less than 1nm. Finally,

$$\delta_{c_Y}^{toc1} = \sum_{i=2,-2} \langle \left(\frac{2/3 c_L^{(m)}(t_{\text{p}})}{c_L^{(m)}(t_{\text{p}}) - c_L^{(m)}(t_{\text{p}} + i)} \right)^2 \rangle_{ld} \tag{30}$$

The first term checks that the *LHY* mRNA expression profile has a sharp peak in LD cycles, with an O(1) contribution if *LHY*'s expression level has dropped by 2/3 of its oscillation size within 2 hours before and after its peak of expression. As for previous optimisations, throughout the implementation the cost function was "capped" at $\Delta_{\max} = 10^4$, such that $\Delta \rightarrow Min(10^4, \Delta)$. The sum of the *toc1* cost function terms was also capped at 10^3.

The output of the model is the same as for the interlocked loop model when simulating a *lhy;cca1* plant, as PRR7/9 and LHY are no longer part of the functional clock in this case. A further 100000 simulated annealing points was carried out on the 10 best solutions found from the search of parameter space, to find the optimal parameter set (Supplementary Table One).

3.2 Parameter Stability Analysis

We examined the robustness of the optimised 3 loop model to parameter changes by calculating the period and amplitude of LHY mRNA oscillations over 300h in LL after a 5% increase or decrease of each parameter value in turn (Supplemental figure 2). The resulting change in period varied from 0 to 3%, similar to that seen for the interlocking loop model (Locke *et al.*, 2005a), and an improvement over the robustness properties for the one loop model (Locke *et al.*, 2005b). The model was most sensitive to alterations

in *PRR7/9* transcription and degradation (e.g see 4 points with mean *LHY* mRNA levels less than 0.5 in Supplemental figure 2). Longer transients after the transition from LD to LL are also seen using parameters with a 5% reduction in *PRR7/9* transcription compared to WT, although not in the transition from LD to DD (Data not shown). This further points to the need to investigate the role of light in the feedback loops.

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