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Data assimilation constrains new connections and components in a complex, eukaryotic circadian clock model

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Circadian clocks generate 24-h rhythms that are entrained by the day/night cycle. Clock circuits include several light inputs and interlocked feedback loops, with complex dynamics. Multiple biological components can contribute to each part of the circuit in higher organisms. Mechanistic models with morning, evening and central feedback loops have provided a heuristic framework for the clock in plants, but were based on transcriptional control. Here, we model observed, post-transcriptional and post-translational regulation and constrain many parameter values based on experimental data. The model's feedback circuit is revised and now includes *PSEUDO-RESPONSE REGULATOR 7* (*PRR7*) and *ZEITLUPE*. The revised model matches data in varying environments and mutants, and gains robustness to parameter variation. Our results suggest that the activation of important morning-expressed genes follows their release from a night inhibitor (NI). Experiments inspired by the new model support the predicted NI function and show that the *PRR5* gene contributes to the NI. The multiple *PRR* genes of Arabidopsis uncouple events in the late night from light-driven responses in the day, increasing the flexibility of rhythmic regulation.
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Introduction

Circadian rhythms are widespread in the biology of almost all eukaryotic organisms, including plants. The self-sustained 24 h oscillations of the core clock genes drive rhythmic expression of >30% of the Arabidopsis genome (Michael and McClung, 2003; Edwards *et al*, 2006; Covington *et al*, 2008; Michael *et al*, 2008) and thus provide plants with an adaptive advantage to anticipate the daily changes in environmental conditions, such as light/dark cycles (Dodd *et al*, 2005). The current concept of the Arabidopsis circadian clock is of a genetic network consisting of morning and evening feedback loops interconnected by a central loop (Locke *et al*, 2006; McClung, 2006). The complexity of the mechanism, consisting of two coupled oscillators, makes mathematical modelling a useful tool for the analysis of functional properties of the system.

Our previous model (Locke *et al*, 2006), hereafter referred to as L2006, was based on the three-loop circuit (Figure 1). The dawn-expressed genes *LATE ELONGATED HYPOCOTYL (LHY)* and *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* form a morning negative feedback loop through activation of their inhibitors, *PSEUDO-RESPONSE REGULATOR 9* (*PRR9*) and *PRR7*. *LHY* and *CCA1* also control the phase of evening genes by inhibiting their expression in the morning. The important evening gene *TIMING OF CAB EXPRESSION 1* (*TOC1*), which is expressed at dusk, regulates itself in the evening loop of the clock through inhibition of its hypothetical activator, gene *Y*. The evening-expressed gene *GIGANTEA* (*GI*) was previously suggested to be a component of *Y* (Locke *et al*, 2006). The evening loop feeds back to the morning loop through the hypothetical gene *X*, which activates *LHY/CCA1* expression in the late night. Light stimulates the expression of *LHY/CCA1*,

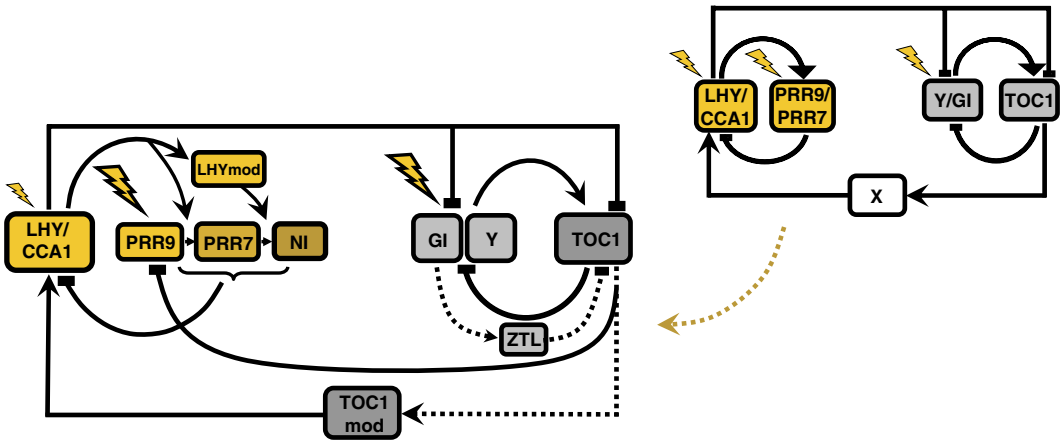

Figure 1 The main elements of the extended Arabidopsis circadian clock model. The previous circuit (Locke *et al*, 2006) is shown, upper right. Elements of the morning and evening oscillators are shown in yellow and grey, respectively. For clarity, proteins are shown only for ZTL, LHY modified (LHY_{mod}) and TOC1 modified ($TOC1_{mod}$). Genetic interactions, solid arrows; post-translational regulation, dashed arrows. Light inputs to gene transcription are marked by flashes. The full scheme of the model is presented in SBGN format in Supplementary Figure 1.

PRR9 and *Y* and thus provides daily resetting or entrainment of the clock.

Although it remains a foundation for understanding the Arabidopsis circadian clock, the L2006 model did not include recent experimental findings, particularly on post-translational regulation. GI protein regulates the clock at the post-translational level by stabilization of the F-box protein ZEITLUPE (ZTL) in the presence of light (Kim *et al*, 2007). ZTL, in turn, is necessary for the targeting of TOC1 protein to degradation by the proteasome (Mas *et al*, 2003b; Kim *et al*, 2007). The results from *gi* mutants have questioned the implication that GI directly regulates *TOC1* (Martin-Tryon *et al*, 2007), which was proposed for Y in the L2006 model. The evening-expressed gene *PRR5* was not explicitly included in the model, though together with the morning genes *PRR9* and *PRR7*, *PRR5* is important for the regulation of *LHY* and *CCA1* expression (Farre *et al*, 2005; Nakamichi *et al*, 2005, 2010). Several results (Farre and Kay, 2007; Ito *et al*, 2007; Kiba *et al*, 2007) also suggest that PRRs are regulated by light at the protein level. Finally, the model could not describe the low level of *PRR9* mRNA in transgenic plants that overexpress *TOC1* (*TOC1-ox*) (Makino *et al*, 2002).

Here, we have integrated the new data into the formal description of the clock gene network. First, we explicitly modelled GI and ZTL function, separating GI from Y and including the stabilization of ZTL by GI. The updated ZTL regulation retained a good match to previous data on the control of *TOC1* transcription by GI, but by an unexpected mechanism. Second, we introduced a new element, the night inhibitor (NI) of *LHY/CCA1* expression and suggested *PRR5* as a candidate component of the NI, with an important function in controlling the phase of morning gene expression. After showing that the model could match multiple sets of molecular time-series data, we analysed the clock responses to perturbations of the light conditions combined with mutations of the clock genes. New data on *prr5/prr7* double mutant plants showed a good match to the model, supporting the idea that PRR5 is an essential part of the NI. In summary, the model integrates new and existing experimental data and allows us to understand (describe, explain and predict) the complex responses of the clock to environmental and genetic perturbations.

Results

Modification of the circadian clock circuit

An extended three-loop circuit for the circadian clock in Arabidopsis is proposed. Figure 1 shows the principal scheme of the model. A more detailed description of the full reaction scheme, model assumptions and network equations are presented in Supplementary information. The proposed scheme incorporates several new features compared with the L2006 model, based on experimental data as detailed below. The *LHY* and *CCA1* genes are represented by a single *LHY/CCA1* component, as before. In contrast to the previous models (Locke *et al*, 2005, 2006), we identified data that constrained 35 of the 90 parameters in the model (see Supplementary information). The remaining parameters were fitted to two types of data: the quantitative profiles of molecular components of the clock and the values of free-running periods of circadian rhythms, in wild-type (wt) plants and *lhy/cca1*, *toc1*, *ztl* and *prr7/prr9* mutants, under varying environmental conditions (see Supplementary information). The parameter values used for all calculations are shown in Supplementary Table 1. We then applied the model to analyse the mechanisms of circadian regulation under additional genetic and environmental perturbations.

Revised evening loop

We started the modification of the clock scheme from the evening loop, in which we included the post-translational regulation of TOC1 protein by GI (Kim *et al*, 2007). The model described the stabilization of the ZTL protein in complex with GI protein and the acceleration of TOC1 protein degradation by ZTL (Mas *et al*, 2003b; Kim *et al*, 2007). Thus, GI inhibits *TOC1* function in the model at the level of TOC1 protein, through positive regulation of ZTL. Together with the inhibition of *Y*

expression by TOC1 protein, this results in indirect activation of *TOC1* mRNA expression by GI in the model, as described in a later section. Previously, GI was also considered as the main candidate for *Y*, the direct activator of *TOC1* expression, accounting for 70% of *Y* activity (Locke *et al*, 2006). The high *TOC1* mRNA levels in *gi* mutants (Martin-Tryon *et al*, 2007) suggested that this aspect of *Y* function was not related to *GI*. We, therefore, removed the direct activation of *TOC1* expression by GI in the model, and discovered that the revised structure of the evening loop retained the main features of the L2006 model.

Regulation of Y and TOC1 expression under different photoperiods

The structure of the evening loop of the clock, with activation of *TOC1* transcription by Y, was originally based on the data on autonomous oscillations of the evening genes in *lhy/cca1* double mutant plants (Locke *et al*, 2005). The entrainment of rhythmic *TOC1* expression by light in the *lhy/cca1* mutant (Mizoguchi *et al*, 2002), together with the absence of direct activation of *TOC1* expression by light (Matsushika *et al*, 2000), implied that *Y* was the light-responsive component of the evening loop. In addition, the evening expression of *TOC1* in wt plants suggested that the expression of *Y* was delayed through inhibition by LHY/CCA1 protein, analogous to *GI* (Locke *et al*, 2005).

We used the revised model to predict the properties of Y and its regulation of *TOC1* expression. As the structure of the evening loop is inherited from our L2006 model, its components showed qualitatively similar behaviour. The predicted profiles of *Y* and *TOC1* mRNA expression in wt plants under light–dark cycles with various photoperiods are shown in Figure 2A. The first peak of *Y* after dawn is caused by the acute light activation of *Y* expression, whereas a second, major peak 10 h after dawn is related to the release of *Y* expression from its inhibition by LHY/CCA1 protein. The broad expression of Y during the light period allows a measure of 'dusk sensing' by the evening loop of the clock. Interestingly, the model predicts a later phase of peak *TOC1* mRNA in short photoperiods compared with 9L:15D cycles, which corresponds to our experimental data (Figure 2C). The model explains this effect by the lower activation of *Y* expression in darkness, which slows down *TOC1* accumulation in cycles with <9 h of light.

The simulated double-peaked shape of *Y* mRNA profiles (Figure 2A) and the delayed phase of *TOC1* in short days (Figure 2C) were also observed in the simulations of wt plants using the L2006 model (Supplementary Figures 5 and 6). However, the current model predicted a stronger response to photoperiod in the *lhy/cca1* double mutant (Figure 2B and Figure 7 of Supplementary information). Figure 2B shows the simulated profiles of *Y* and *TOC1* mRNA in *lhy/cca1* plants under various photoperiods. *Y* mRNA is abundant in *lhy/cca1* during the light period, which results in a broader profile of *TOC1* mRNA in long days compared with short days. Our results on *TOC1:LUC* expression in *lhy/cca1* mutant plants under 6L:18D and 18L:6D photoperiods confirm this prediction (Supplementary Figure 19). The characteristic profiles predicted for *Y* expression in *lhy/cca1* suggest that candidate genes for *Y* could be identified by testing genome-wide expression profiles in *lhy/cca1* mutant plants under various photoperiods.

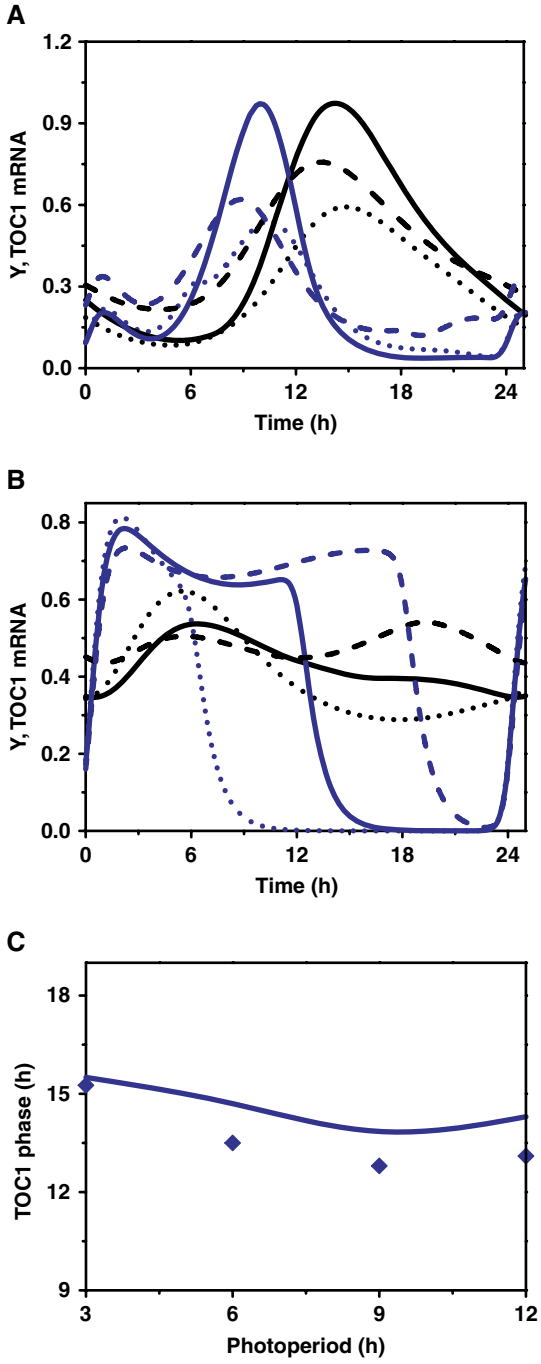

Figure 2 Regulation of the evening components *Y* and *TOC1* by photoperiods. Simulated expression profiles of *Y* (blue lines) and *TOC1* (black lines) mRNA in wt (**A**) and *lhy/cca1* mutant (**B**) plants are shown for 6L:18D (dotted lines); 12L:12D (solid lines) and 18L:6D (dashed lines). (**C**) Dependence of peak *TOC1* phase on the day length in wt plants. Simulations are shown by a line and the experimental data points for the peak phase of *TOC1:LUC* reporter expression are taken from Edwards *et al* (2010). The level of each clock component in the model is normalized to its maximum level in 12L:12D (see Supplementary information).

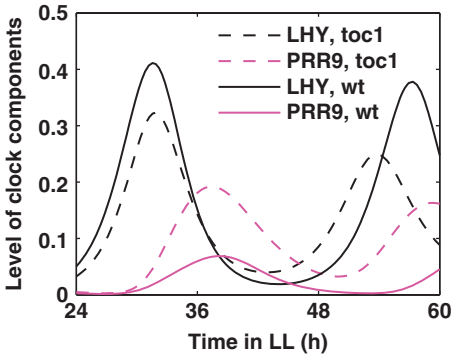

Figure 3 Importance of *PRR9* inhibition by TOC1 for high-amplitude oscillations of *LHY/CCA1* in constant light. Simulated profiles of *LHY/CCA1* mRNA (black lines) and PRR9 protein (magenta lines) are shown for wild-type plants (solid lines) and for a simulated partial *toc1* mutant that specifically lacks *PRR9* inhibition by TOC1 (dashed lines).

Feed-forward regulation of LHY/CCA1 amplitude by TOC1 and PRR9

In addition to the revision of the structure of the evening loop, we modified the connection between morning and evening oscillators. First, the unknown activator of *LHY/CCA1* expression (gene *X*) was replaced by a post-translational modification or complex dependent on TOC1 protein ($TOC1_{mod}$), based on the data on TOC1 binding to protein complexes at the *CCA1* promoter (Pruneda-Paz *et al*, 2009). Second, an inhibition of *PRR9* expression by TOC1 was introduced, because overexpression of *TOC1* was shown to reduce *PRR9* mRNA to a negligible level (Makino *et al*, 2002; Ito *et al*, 2005).

Next, the model was used to analyse the regulation of *LHY/CCA1* by TOC1 in more detail. The scheme of Figure 1 suggests that there are three steps in *TOC1* action in the clock: (1) autoregulation of *TOC1* expression through the inhibition of *Y*, (2) activation of *LHY/CCA1* expression and (3) inhibition of *PRR9* expression. Model simulations showed that the third mechanism enhances the second, because the inhibition of *PRR9* by TOC1 delays *PRR9* expression relative to *LHY/CCA1*. *LHY/CCA1* expression can, therefore, rise further under the influence of $TOC1_{mod}$ before the onset of inhibition by PRR9 protein. The effect of TOC1's inhibition of *PRR9* on the expression profile of *LHY/CCA1* is especially pronounced in constant light (LL) conditions. The expression level of *PRR9* is low in LL, because the acute induction of *PRR9* at lights-on does not occur in these conditions. Figure 3 shows the simulated profiles of *LHY/CCA1* mRNA and PRR9 protein for wt plants and a hypothetical *toc1* mutant that is unable to inhibit *PRR9* expression. The release of *PRR9* from inhibition by TOC1 results in a higher level of PRR9 protein, which in turn reduces the amplitude of *LHY/CCA1* expression and shortens the period in this partial *toc1* mutant.

The model predicts that the phenotype of *toc1* null mutants is similar to the partial *toc1* mutant described above (Supplementary Figure 9). Thus, the experimentally observed 4 h shortening of the period and decrease of *LHY/CCA1* amplitude in the *toc1* mutant compared with wt (Mas *et al*, 2003a) can be mostly ascribed to the above effect of the absence of *PRR9* inhibition by TOC1, rather than direct activation of *LHY/CCA1*. The proposed mechanism of *LHY/CCA1* regulation by TOC1 is known in the biology of transcriptional networks as a coherent feed-forward motif, when one transcription factor affects another and both of them jointly regulate a target gene (Mangan and Alon, 2003). In this case, TOC1 regulates *LHY/CCA1* through a double negative connection through *PRR9*, in parallel with activation through $TOC1_{mod}$.

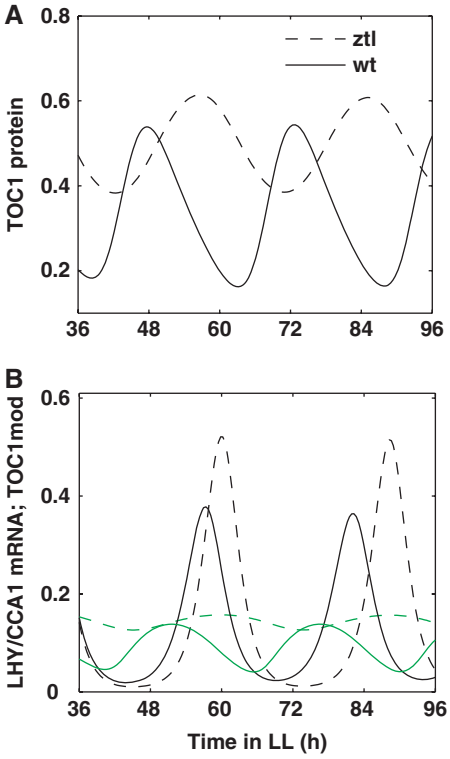

Figure 4 Mechanism of ZTL function under LL conditions. Simulated profiles for wild-type and *ztl* mutant plants are shown by solid and dashed lines, respectively. (**A**) TOC1 protein; (**B**) *LHY/CCA1* mRNA (black) and $TOC1_{mod}$ protein (green).

Updating of ZTL effects on the clock

The L2006 model included ZTL-dependent degradation of TOC1 protein, but it did not include the regulation of ZTL by the clock and light (Locke *et al*, 2006). Updating the mechanism of ZTL regulation by including its stabilization by GI in the presence of light allowed us to explore ZTL action in the clock. We found that in the absence of ZTL, the level of TOC1 protein is higher and its rhythm has lower amplitude in *ztl* mutants compared with wt plants, which corresponds to experimental observations (Mas *et al*, 2003b). Figure 4 shows TOC1 protein (panel A) and $TOC1_{mod}$ protein (panel B) profiles in wt and *ztl* mutant plants in LL conditions. The change of TOC1 profile, in turn, results in the delay of the *LHY/CCA1* mRNA peak (Figure 4B) and is in agreement with the experimentally observed, long period of the *ztl* mutant (Mas *et al*, 2003b).

To understand the influence of ZTL on the evening loop, we revisited our previous data on the kinetics of *TOC1* expression in the *lhy/cca1* double and *lhy/cca1/gi* triple mutant plants in LL conditions (Locke *et al*, 2006). Our simulations showed that the ZTL level would be low in *lhy/cca1/gi* (Figure 5A), because of the rapid degradation of ZTL in the absence of GI protein (Kim *et al*, 2007). The lower ZTL increased TOC1 protein levels in *lhy/cca1/gi* compared with *lhy/cca1*. Next, the negative feedback from TOC1 to *Y* resulted in decreased *Y* mRNA in *lhy/cca1/gi*. As Y activates *TOC1* expression, this resulted in a lower level of *TOC1* mRNA and reduction of oscillation amplitude in the *lhy/cca1/gi* mutant plants (Figure 5A and B). This prediction of the model suggested that GI stabilization of ZTL causes an unexpected, indirect activation of *TOC1* expression, consistent with the 2.5-fold increase of mean *TOC1:LUC* expression observed in *lhy/cca1* compared with *lhy/cca1/gi* (Locke *et al*, 2006). In summary, the above simulations of the wt, *ztl* and *lhy/cca1/gi* mutants showed good agreement of the updated ZTL mechanism with existing experimental data.

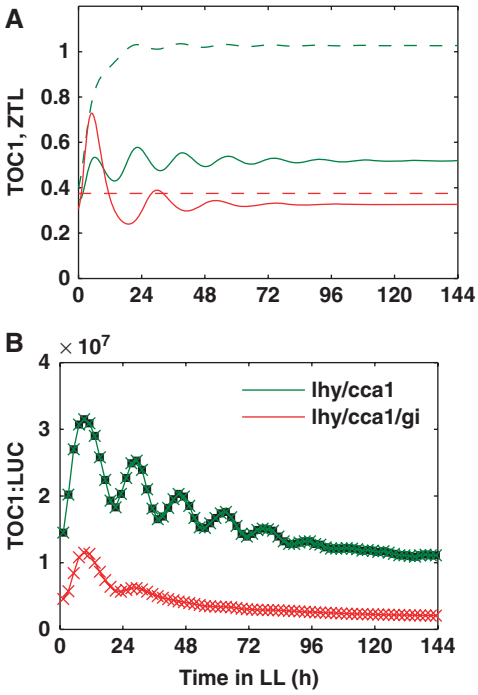

Figure 5 Mechanism of *TOC1* regulation by GI. The predicted (**A**) and experimental (**B**) profiles of *TOC1* expression in *lhy/cca1* double and *lhy/cca1/gi* triple mutants. Green lines correspond to *lhy/cca1* and red lines to *lhy/cca1/gi*. (A) *TOC1* mRNA expression is shown by solid lines and the total content of ZTL protein is shown by dashed lines. (B) Bioluminescence of transgenic mutant plants carrying the *TOC1:LUC* reporter, in the *lhy/cca1* double and *lhy/cca1/gi* triple mutant backgrounds (taken from our previous data (Locke *et al*, 2006)).

Regulation of LHY/CCA1 expression by a wave of inhibitors allows dawn and dusk sensitivity

The morning loop was extended by introducing a new element, the NI of *LHY/CCA1* (NI). Introducing NI was originally motivated by the levels of *LHY* and *CCA1* mRNA, 100- to 1000-fold lower than their dawn peak, that were observed in the middle of the night (Figure 6; Supplementary Figure 1). The identity of NI is discussed in the next section. Its effect, together with the day-phased inhibitors PRR9 and PRR7 (Farre *et al*, 2005), guarantees that *LHY/CCA1* mRNA quickly falls after dawn and stays at a very low level until the middle of the night. Figure 6 shows the simulated profile of *LHY/CCA1* mRNA together with experimental data and the wave of inhibitor proteins. The high level of activator $TOC1_{mod}$ at night (Supplementary Figure 8) ensures a steep rise of *LHY/CCA1* mRNA before dawn, when the inhibitor proteins degrade (Figure 6).

The model provides sequential expression of *PRR9*, *PRR7* and *NI* using a simple, effective formulation, because the molecular details underlying the striking 'PRR wave' of mRNA profiles (Matsushika *et al*, 2000) are unclear. *PRR9* expression in the model is activated by light at dawn and by LHY/CCA1 protein, consistent with data (Ito *et al*, 2005). PRR9 protein activates *PRR7*, and PRR7 activates *NI*. A modified form of LHY/CCA1 protein, LHY_{mod}, is introduced to activate *PRR7* and *NI*. The PRR9/PRR7/NI activation cascade and LHY_{mod} have no other function in the model. Phosphorylation of CCA1 protein, which is known to affect timing *in vivo* (Daniel *et al*, 2004), provides one example of the type of post-translational modification envisaged for LHY_{mod}. Alternative formulations are discussed in Supplementary information.

Modelling the PRR/NI wave allowed us to explore the functional impact of multiple genes with divergent timing and light regulation in the morning loop. In addition to light-activated *PRR9* transcription, the model includes the light-dependent stabilization observed for PRR9 and PRR7 proteins and assumed for NI (Farre and Kay, 2007; Ito *et al*, 2007; Kiba *et al*, 2007). The PRR/NI wave has the potential to respond both to dawn, when *PRR9* is activated, and to dusk, when the PRR/NI proteins are degraded, and thus to alter circadian timing in response to changing photoperiods. Figure 7A shows simulated *LHY/CCA1* mRNA profiles under short 6L:18D photoperiods, standard 12L:12D photoperiods and long

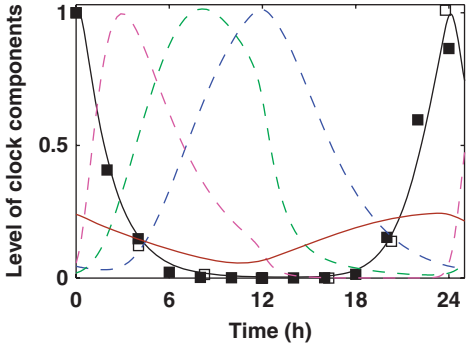

Figure 6 Regulation of *LHY/CCA1* expression by the wave of inhibitors and the activator $TOC1_{mod}$. Simulations are shown during 12L:12D entrainment of wild-type plants. *LHY/CCA1* mRNA is shown by the black line. Inhibitor proteins PRR9, PRR7 and NI are shown by dashed magenta, green and blue lines, respectively. $TOC1_{mod}$ is shown by the brown line. Experimental data on *LHY/CCA1* expression are shown by filled squares (Edwards *et al*, 2010) and open squares (Farre *et al*, 2005).

18L:6D photoperiods. PRR9 was activated strongly after dawn in all photoperiods, matching published data (Matsushika *et al*, 2000; Supplementary Figure 3). The model predicts that the PRR7 and NI proteins will fall to low levels during the long dark interval in 6L:18D, owing to their higher degradation rate in darkness (Figure 7A; Supplementary Figure 3). Consequently, *LHY/CCA1* expression is expected to be de-repressed earlier in short photoperiods compared with long photoperiods. This corresponds closely to our experimental data, in which *CCA1* mRNA levels rise 3 h earlier in short photoperiods compared with long photoperiods (Figure 7B).

Thus, the inclusion of the PRR/NI wave improved the entrainment of the model to various photoperiods compared with L2006 (Supplementary Figure 4). In addition, the PRR/NI wave allowed the model to entrain in a broad range of T cycles (light/dark cycles of total duration T), again in contrast with L2006 (Supplementary Figure 6).

Validation of the inhibitor wave and the identity of NI

We used a classical protocol of circadian biology, the skeleton photoperiod (Pittendrigh and Daan, 1976), to test the predicted regulation of *LHY/CCA1* by the inhibitor wave. The skeleton photoperiod uses two short light treatments per daily cycle to entrain the clock, separating the contributions of light at dawn and dusk. Previous work had shown that the Arabidopsis clock could be entrained by skeleton photoperiods as expected (Millar, 2003), but that >3 h light per cycle was required to maintain normal plant development (Millar, unpublished results). Simulations were, therefore, conducted to match the experiments: initial entrainment in 12L:12D was followed by simulated skeleton photoperiods with 3 h light treatments starting at dawn and ending at dusk (3L:6D:3L:12D). The *LHY/CCA1* mRNA profile showed correct entrainment, with a strong peak at the first ('dawn') pulse and only a small shoulder of expression at the second pulse (Figure 8A). The L2006 model failed to match this entrained pattern (see Supplementary information). *PRR9*, in contrast, was predicted to show strong expression in response to both light pulses (Figure 8C).

The responses of wt plants to skeleton photoperiods were tested experimentally by measuring the bioluminescence of the *CCA1:LUC* and *PRR9:LUC* reporter genes. Our data confirmed the prevalence of the 'dawn' response in *CCA1* expression (Figure 8B), contrasting with major light induction of *PRR9:LUC* expression after both light pulses (Figure 8D). The slower decrease and delay in the peak of *CCA1:LUC* and *PRR9:LUC* activity (Figure 8B and D) compared with the simulated mRNA in the model (Figure 8A and C) may result from a slower degradation rate of LUC mRNA and protein (Finkenstadt *et al*, 2008) compared with rapid degradation of *CCA1* and *PRR9* mRNA (Ito *et al*, 2007; Yakir *et al*, 2007, 2009). Supplementary Figure 17 shows the faster decrease of *CCA1* and *PRR9* mRNA in qPCR measurements compared with *CCA1:LUC* and *PRR9:LUC*, supporting this interpretation. We conclude that light input pathways are functional during both light pulses in the skeleton photoperiod, yet *LHY/CCA1* was only weakly induced at dusk.

The model explains the mechanism of *LHY/CCA1* expression at 'dawn', because the inhibitory PRR7 and NI proteins

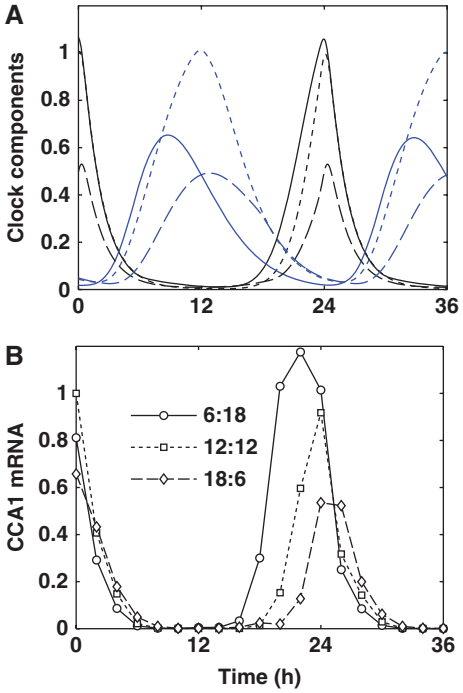

Figure 7 Regulation of *LHY/CCA1* phase. Simulations are shown under entrainment of wild-type plants to various photoperiods. (**A**) Simulated profiles of *LHY/CCA1* mRNA (black) and NI protein (blue) are shown for 6L:18D (solid lines), 12L:12D (dotted lines) and 18L:6D (dashed lines). (**B**) Experimental data on *CCA1* mRNA for 6L:18D, 12L:12D and 18L:6D entrainment were taken from Edwards *et al* (2010).

are degraded during the longer period of darkness that represents the night phase (Figure 8A). In contrast, the PRR/NI inhibitors are strongly expressed during the day phase, which results in a low *LHY/CCA1* response to the 'dusk' pulse of light.

The model predicted that the absence of both PRR7 and NI would result in the loss of the 'dawn preference' of *LHY/CCA1* expression in the skeleton protocol (Figure 8E). The existing data suggested that PRR5 is a good candidate for NI, because PRR5 together with PRR7 inhibits *LHY* and *CCA1* RNA accumulation in darkness (Nakamichi *et al*, 2005) and data published during revision of this manuscript revealed physical association of PRR5 to *LHY* and *CCA1* promoters (Nakamichi *et al*, 2010). *CCA1:LUC* expression in the *prr7/prr5* double mutant responded at nearly equal levels to both light pulses (Figure 8F). This result indicates that PRR5 is a good candidate for NI, because it participates in the inhibition of *LHY/CCA1* expression at night together with PRR7.

To test the relative importance of PRR7 and PRR5 *in vivo*, we analysed the behaviour of the *prr7* and *prr5* single mutant plants under skeleton conditions. Our experiments showed some impairment of *CCA1* inhibition in the 'dusk' light pulse in both single mutants (Figure 8G and H) compared with wt plants (Figure 8B). The difference between the *prr7/prr5* double mutant and the single mutants showed that both PRR7 and PRR5 normally contribute to repress *CCA1* expression in the late day.

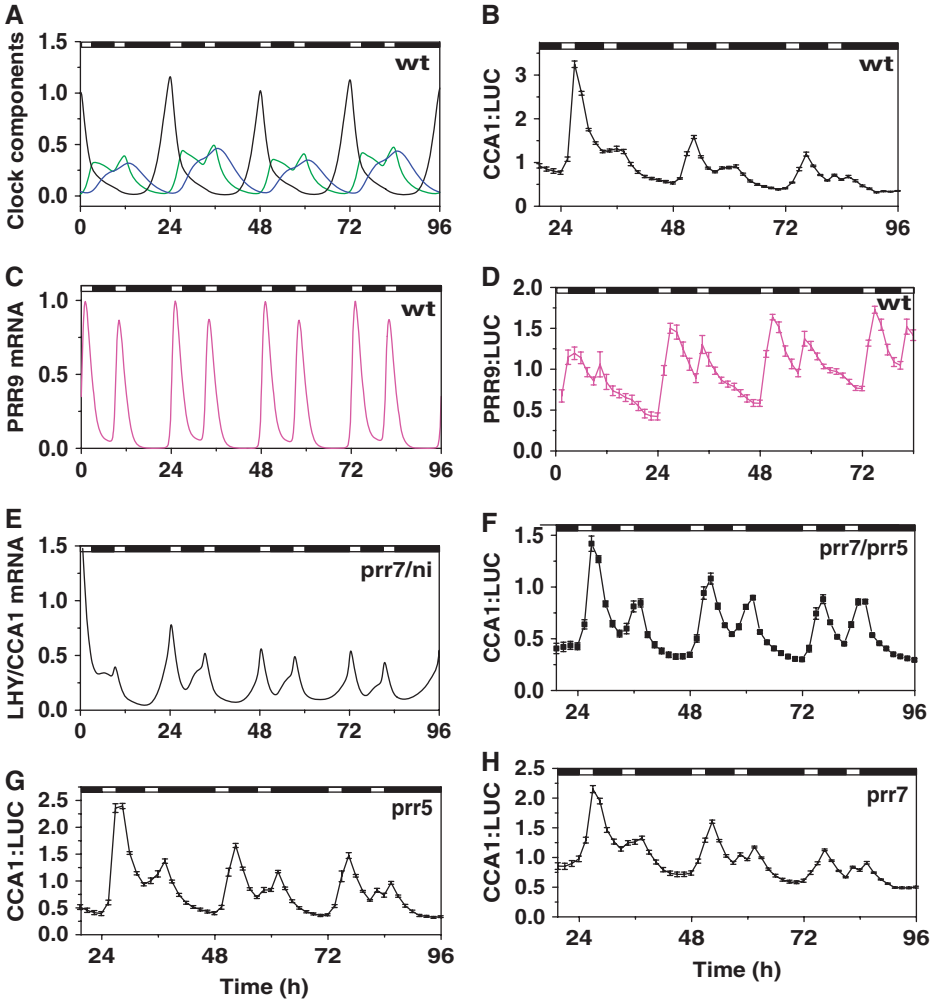

Figure 8 Theoretical and experimental profiles of clock components during skeleton entrainment. Plants were transferred from 12L:12D to skeleton entrainment with 'dawn' and 'dusk' pulses of light, each of 3 h duration (3L:6D:3L:12D), at time 0 h in simulations (**A**, **C**, **E**) and experiments (**B**, **D**, **F–H**). Black lines correspond to simulated *LHY/CCA1* mRNA in wild-type (A) or *prr7/ni* double mutants (E), or normalized luminescence from transgenic *CCA1:LUC* plants in wild-type (B) or *prr7/prr5* (F), *prr7* (H) or *prr5* (G) mutant backgrounds. (A) Simulated levels of inhibitor proteins PRR7 and NI in wild type are shown by green and blue lines. (C) Simulated levels of *PRR9* mRNA are shown by the magenta line. Normalized luminescence from wild-type *PRR9:LUC* plants is shown in panel D. Luminescence data represent the mean of data from 9 to 18 individual plants, in which each plant's trace was normalized to the mean luminescence level for the plant. Data shown are representative of two independent experiments.

Discussion

The main aim of this study was to understand better the structure of the circadian clock circuit based on existing and new experimental data. To achieve this, we improved our previous modelling approach (Locke *et al*, 2006) by including more kinetic data to build and constrain the model. Quantitative time-series data from dynamic perturbation experiments are common in circadian biology and are essential to this approach. Experiments to measure specific parameter values are also helpful, because the sensitive parameters in complex biological models cannot generally be located *a priori*. Each measured parameter value, even with associated experimental uncertainty, progressively constrains the high-dimensional search space of biologically realistic values. The final model's behaviour may be robust (insensitive) to variation in many parameter values, but this does not justify a *post hoc* deprecation of laborious parameter measurements (Gutenkunst *et al*, 2007). Measuring the degradation rates of the *LHY/CCA1* and *TOC1* mRNA, for example, was a relatively tractable experiment (Supplementary Figure 18), and these were unexpectedly among the most sensitive parameters in our model (Supplementary Figures 15 and 16).

We were able to introduce several new components and interactions into the clock circuit (Figure 1) using these constraints, starting from the revision of the evening loop. The effects of the *GI* gene on the clock are not completely understood. The L2006 model suggested that *GI* was part of the hypothetical component *Y*—an activator of *TOC1* transcription (Locke *et al*, 2005). However, recent data (Kim *et al*, 2007; Martin-Tryon *et al*, 2007) show that *GI* influences *TOC1* not on the transcriptional, but on the protein level. We, therefore, modified the structure of the evening loop by separating GI and Y functions in the model. Y increases *TOC1* mRNA level, whereas GI decreases TOC1 protein level through stabilization of ZTL protein. The decreased TOC1 protein tends

to relieve repression of *Y* by TOC1, acting in the evening negative feedback loop. Increased *Y* expression in turn activates *TOC1* expression. Both Y (directly) and GI (indirectly) activate *TOC1* expression in the model. This allows us to give a new explanation of the previous experimental data from the *gi/lhy/cca1* triple mutant (Locke *et al*, 2006; Figure 5), which show that the *gi* mutation reduces *TOC1* expression.

However, we included only one function of GI in the model. In addition to the interaction with ZTL, GI can function as a scaffold cofactor in the formation of multi-protein activator complexes at gene promoters (Sawa *et al*, 2007). This raises the possibility that GI can also accelerate the expression of some clock genes, similarly to the hypothetical activation of *TOC1* by Y. Experimental data show a variation of the periods of *gi* mutant alleles from short to long compared with wt plants (Park *et al*, 1999; Gould *et al*, 2006). The shortening of the period of some *gi* mutants was less pronounced in blue light (Martin-Tryon *et al*, 2007), which is necessary for GI–ZTL interaction, suggesting that a perturbation of the GI–ZTL interaction could be responsible for the longer period of *gi* alleles that cause amino-acid substitutions, such as *gi-596* (Gould *et al*, 2006). This observation is consistent with the 2 h period lengthening of the simulated *gi* mutant relative to wt in our model (data not shown), which incorporated only the effect of the GI–ZTL interaction upon the degradation of TOC1 protein. The short periods observed in other *gi* mutant alleles are possibly related with the loss of other functions of GI as a scaffold, which might affect protein targets other than TOC1. We do not yet have data to include this function of GI explicitly in the model, so only Y retained the function in transcriptional activation, and simulated *y* mutants had short periods in our model (data not shown), as in Locke *et al* (2005, 2006).

We also used the model to study the ZTL effects on the clock. We showed that the long period of *ztl* mutant is related to changes in TOC1 protein levels. This is consistent with experimental data, which show that the main effect of ZTL is related with *TOC1*: the *ztl/toc1* double mutant has a short period close to the *toc1* single mutant (Mas *et al*, 2003b). In addition to its effect on TOC1, ZTL is known to participate in the degradation of PRR5 (Kiba *et al*, 2007), which is probably responsible for some part of NI activity. The potential for ZTL-like control of NI protein degradation was taken into consideration by increasing the rate of NI protein degradation in darkness, analogous to PRR9 and PRR7.

Our current understanding of the Arabidopsis clock leaves some open questions about the structure of the evening loop and its connection to the morning loop. Mostly, they are related with Y, the unknown *TOC1* transcriptional activator. The model predicts that the characteristic property of *Y* is its broad expression in *lhy/cca1* double mutants in the daytime, which may allow identification of this gene in future. This profile is most obvious in long days, in which *Y* mRNA is predicted to rise to a second peak before dusk (Figure 2B). Another question is related with the multiple functions ascribed to TOC1. The data on *TOC1* co-expression with the transcription factor *LUX* in constant light, and short- and long-photoperiod conditions, as well as in multiple mutant backgrounds (Hazen *et al*, 2004, 2005), suggest that some TOC1 functions in the model might be realized through LUX *in vivo*. New experimental data on *LUX* expression and LUX functions will be required to understand how LUX contributes to the evening loop and its connection to the morning loop of the clock (Hazen *et al*, 2005).

An important aspect of this study is related to regulation of the important morning components *LHY* and *CCA1*, which in turn control the expression of multiple genes with evening and morning elements in their promoters (Harmer and Kay, 2005). The central function of *LHY* and *CCA1* in the clock may explain the precise regulation of their expression by multiple inhibitors (Locke *et al*, 2006; McClung, 2006). When the *LHY* and *CCA1* profiles are altered, the phase of all other clock components is changed. Here, we introduced NI, an inhibitor of *LHY/CCA1* expression in the model, based on existing data (Nakamichi *et al*, 2005, 2010; Pruneda-Paz *et al*, 2009).

Our model predicts that regulation of the wave of inhibitor proteins PRR9, PRR7 and NI by light is important for correct phasing of the clock. The simulated profiles of *LHY/CCA1* expression match our experimental data under multiple light conditions including various photoperiods, constant light conditions and in skeleton photoperiods, in which the clock is reset by 'dawn' and 'dusk' pulses of light. The model shows that the wave of inhibitors may contribute to the observed response of morning genes to the time of dusk (Figure 7B), whereas dusk sensitivity was previously ascribed only to the evening loop (Locke *et al*, 2006). The combined, *PRR9/7* gene of the L2006 model did not match either this dusk-responsive behaviour or the strong induction of *PRR9* observed at dawn, because the regulation of that single component represented a difficult compromise between the two patterns. Consequently, the morning loop in L2006 showed no response to changing photoperiods (Edwards *et al*, 2010; Supplementary Figure 4), and parameters of the *PRR7/9* component were the most sensitive in the model (Locke *et al*, 2006; Treenut Saithong and Millar, unpublished results). The L2006 model was also relatively slow to reach stable entrainment, because it lacked strong light activation of *PRR9* (see Supplementary information). The introduction of three inhibitor components (*PRR9*, *PRR7* and *NI*) instead of one (combined *PRR7/9*) greatly increased the flexibility of entrainment and the robustness of the present model to parameter changes, compared with the L2006 model. These advantages arise generically from the duplication and divergent regulation of clock components. Organisms with large genomes show this effect in clock gene families, such as the plant *PRR* genes and the mammalian *Period* genes, but other biochemical mechanisms could evolve to provide the same properties. The differential regulation of a single clock gene in distinct, though coupled, cells offers one alternative, as in Drosophila.

The model predicted that the initial phase of *LHY/CCA1* transcription in the late night depends on PRR7 and NI proteins, which are present at night. Such regulation of *LHY/CCA1* would not result simply from the PRR9 protein profile, which falls at the start of the night (Nakamichi *et al*, 2010; Supplementary Figure 3). Considering PRR5 as a candidate for NI based on previous data (Nakamichi *et al*, 2005), we investigated in more detail the effects of PRR7 and PRR5 on *LHY/CCA1* expression. Our experimental data on the *prr7* and *prr5* single and *prr7/prr5* double mutant plants showed that both PRR7 and PRR5 are important for the higher induction of *LHY/CCA1* at dawn compared with dusk under skeleton

photoperiods, in agreement with the model prediction (Figure 8). The higher level of *LHY* and *CCA1* expression at dusk in the *prr7* and *prr7/prr5* mutants was expected from published data (Farre *et al*, 2005; Nakamichi *et al*, 2005). The increase of *CCA1* mRNA levels at dusk in *prr5* was not expected from previous data under normal photoperiods (Eriksson *et al*, 2003), emphasizing the usefulness of the skeleton photoperiod protocol in understanding the clock mechanisms. Thus, our data on *prr7/prr5* and *prr5* mutants confirm that PRR5 is a good candidate for NI. During revision of this manuscript, experimental results showing that PRR5 associates with and inhibits expression of *LHY* and *CCA1* further supported this hypothesis (Nakamichi *et al*, 2010).

Previous data showed that the *prr7/prr5* double mutant alters the entrained phase in normal photoperiods (Yamashino *et al*, 2008). Our model also shows a phase shift (data not shown), but the extent of the shift is dependent on the quantitative contributions and mutual regulation of the PRR genes, which cannot yet be defined from data. In addition, recent experimental data (Pruneda-Paz *et al*, 2009) show the inhibition of *CCA1* by another dusk-expressed gene, *CHE* (*TCP21*), the product of which interacts with TOC1 at the *CCA1* promoter. On the basis of these data we expect that PRR7 and PRR5 may work together with CHE protein at night in the inhibition specifically of *CCA1*. Thus, the NI component of our model may represent a protein complex with elements additional to PRR5.

The model simulations showed that, in addition to inhibitors, the activator $TOC1_{mod}$ has an impact on *LHY/CCA1* expression by determining the amplitude of the *LHY/CCA1* mRNA rhythm. The model predicts that TOC1 has two, coherent effects on *LHY/CCA1* expression. As well as direct activation of *LHY/CCA1*, TOC1 inhibits activation of *PRR9* expression by LHY/CCA1, which further increases the amplitude of *LHY/CCA1* expression (Figure 3). Thus, inhibition of *PRR9* by TOC1 at night increases robustness of oscillations. In the morning, however, *PRR9* is quickly induced by light. The molecular mechanism of the switch from inhibition to activation at the *PRR9* promoter is unknown. In our model, we assumed that the acute induction of *PRR9* expression by light through protein P does not depend on TOC1, allowing a robust acute induction of *PRR9* by both pulses of light in the skeleton photoperiods (Figure 8C). However, the observed *PRR9* response to the 'dusk' pulse of light is smaller than the response to the 'dawn' pulse (Figure 8D). This would be consistent with some minor inhibition of *PRR9* light induction by TOC1. Future studies of the protein complexes at the *PRR9* promoter will allow a more detailed description of *PRR9* inhibition by TOC1 and the competition between TOC1 and light activation for the *PRR9* promoter.

The model emphasises that *LHY/CCA1* regulation must switch from inhibition by PRR9, PRR7 and PRR5 in the early night to activation by $TOC1_{mod}$ in the late night, but the molecular mechanism of this switch is unknown. One possible scenario is that the function of CHE, and perhaps other similar DNA-binding proteins, is modulated through binding of different effectors, such as PRR proteins other than TOC1 (PRR1). In that case, competition between PRR proteins that inhibit or activate *CCA1* might also be important in the modulation of the morning loop by evening components. Thus, our approach both highlights general principles of circadian circuits and also focuses experimental work, from introducing an inhibitory function for PRR5 to considering how this function must end in the night.

Materials and methods

Computational and experimental methods are described in detail in Supplementary information.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (http://www.nature.com/msb). The model will be available from the Biomodels database (Le Novere *et al*, 2006) and the Plant Systems Modelling portal (http://www.plasmo.ed.ac.uk). Experimental data used in the study will be available in a standard format from the Centre for Systems Biology at Edinburgh (http://www.csbe.ed.ac.uk).

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Author contributions: AP, SKH, KK, KDE and AJM designed experiments; SKH, KK, KDE and AWT performed experiments; TM supplied biological materials; AP, KS and AJM designed computational analysis; AP and KS performed computational analysis; AP and AJM wrote the paper with comments from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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