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# The clock gene circuit in *Arabidopsis* includes a repressilator with additional feedback loops

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**Circadian clocks synchronise biological processes with the day/night cycle, using molecular mechanisms that include interlocked, transcriptional feedback loops. Recent experiments identified the evening complex (EC) as a repressor that can be essential for gene expression rhythms in plants. Integrating the EC components in this role significantly alters our mechanistic, mathematical model of the clock gene circuit. Negative autoregulation of the EC genes constitutes the clock's evening loop, replacing the hypothetical component *Y*. The EC explains our earlier conjecture that the morning gene *PSEUDO-RESPONSE REGULATOR 9* was repressed by an evening gene, previously identified with *TIMING OF CAB EXPRESSION1* (*TOC1*). Our computational analysis suggests that TOC1 is a repressor of the morning genes *LATE ELONGATED HYPOCOTYL* and *CIRCADIAN CLOCK ASSOCIATED1* rather than an activator as first conceived. This removes the necessity for the unknown component *X* (or TOC1mod) from previous clock models. As well as matching timeseries and phase-response data, the model provides a new conceptual framework for the plant clock that includes a three-component repressilator circuit in its complex structure.**
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## Introduction

Circadian clocks are found widely among organisms, ranging from cyanobacteria to mammals (Dong and Golden, 2008; Zhang and Kay, 2010). These internal time-keepers generate ~24 h rhythms of expression of multiple genes even in the absence of any environmental cues, allowing the organism to anticipate each new day. Circadian rhythms can enhance growth and survival (Dodd *et al*, 2005; Harmer, 2009; Zhang and Kay, 2010). In order to understand the behaviour, mechanisms and properties of the system, we previously built a mathematical model of the plant circadian clock (Pokhilko *et al*, 2010).

The clock was represented by a three-loop structure of interconnected morning and evening loops (Figure 1, upper right). The morning loop included MYB-related transcription factors *LHY* (*LATE ELONGATED HYPOCOTYL*) and *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*), which activate the expression of *PRR9*, *PRR7* and *PRR5/NI* (*PSEUDO-RESPONSE REGULATORs 9, 7, 5/night inhibitor*) (Farre *et al*, 2005; Nakamichi *et al*, 2010). Transcriptional co-regulators PRR9, PRR7 and PRR5 inhibit *LHY* and *CCA1* expression in the model, and in data that showed binding to their promoters (Nakamichi *et al*, 2010). The evening loop was represented by *TIMING OF CAB EXPRESSION1* (*TOC1*), which inhibited the expression of its unknown activator *Y*. The hypothetical gene *Y* was introduced in the model by Locke *et al* (2005) to describe the observed autonomous oscillations of *TOC1* expression in *lhy/cca1* double-mutant plants. GIGANTEA (GI), a large plant-specific protein, accelerated the degradation of TOC1 protein through stabilisation of the F box protein ZTL (ZEITLUPE) in the model, as in the data (Kim *et al*, 2007).

The connections between morning and evening loops were represented in the model by the inhibition of evening gene expression by LHY/CCA1 protein, which was well documented, and by activation of *LHY/CCA1* expression by TOC1. Previous models required unknown substances TOC1mod or *X* to match the observed ~12 h delay between *TOC1* expression and *LHY/CCA1* induction (Locke *et al*, 2005; Pokhilko *et al*, 2010). Pokhilko *et al* (2010) introduced an additional connection from the evening loop to the morning loop, based on timeseries data, through inhibition of *PRR9* expression by TOC1. This improved the model's description of plant rhythms but left open questions about core parts of the clock mechanism.

Loss-of-function mutants in each of the genes represented in previous clock models remained rhythmic, albeit with varying

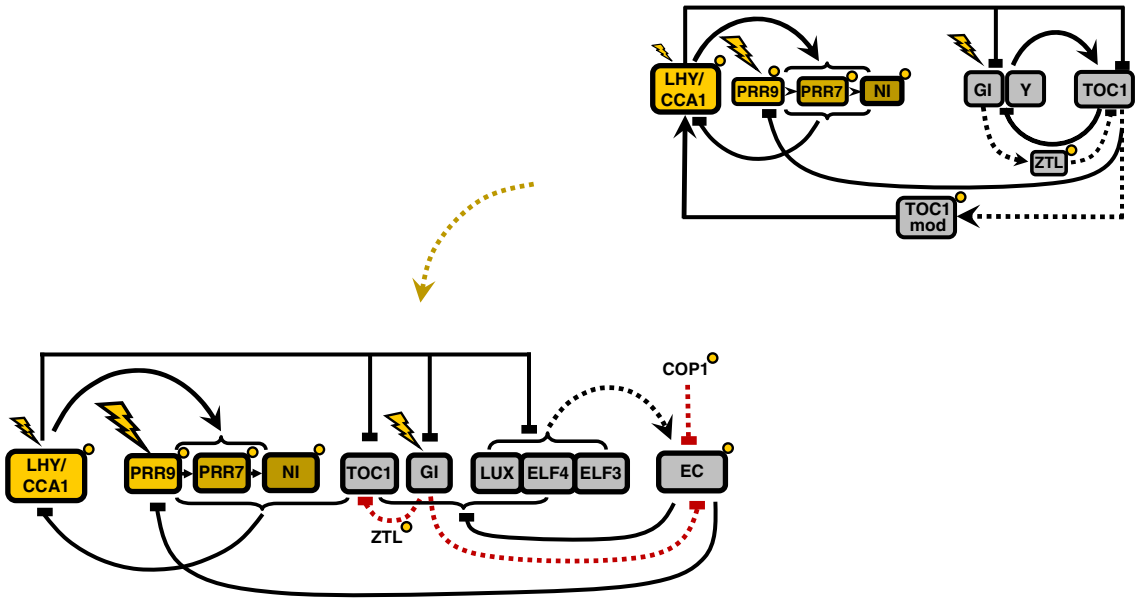


**Figure 1** The revised outline of the Arabidopsis circadian clock. Elements of the morning and evening loops are shown in yellow and grey, respectively. Proteins are shown only for EC, ZTL and COP1 for simplicity. Transcriptional regulation is shown by solid lines. EC protein complex formation is denoted by a dashed black line. Post-translational regulation of TOC1 and the EC by GI, ZTL and COP1 are shown by red dashed lines. Acute light responses in gene transcription are shown by flashes. Post-translational regulation by light is shown by small yellow circles. The previous outline circuit (Pokhilko *et al*, 2010) is shown on the upper right.

rhythmic properties. This was problematic, because the model required a hypothetical component *Y* to explain the rhythms observed in the *lhy/cca1* double mutant. GI, the first gene proposed as a candidate for *Y*, was known not to perform all of the required functions (Locke *et al*, 2005), so the biological identity of the missing components was unknown. Conversely, three mutants that did cause striking, arrhythmic phenotypes could not be integrated into the model, because the functions of the plant-specific proteins ELF3 (EARLY FLOWERING 3), ELF4 (EARLY FLOWERING 4) and the GARP transcription LUX (LUX ARRHYTHMO) (also known as PCL1) were unclear (Hicks *et al*, 1996; Covington *et al*, 2001; Doyle *et al*, 2002; Hazen *et al*, 2005).

Recent results demonstrate that *ELF3*, *ELF4* and *LUX* are the key regulators of clock gene expression at night (Onai and Ishiura, 2005; Kolmos *et al*, 2009; Dixon *et al*, 2011; Helfer *et al*, 2011). ELF3, ELF4 and LUX proteins were shown to form a complex, the EC (evening complex), which binds to the promoters of target genes (Nusinow *et al*, 2011). Although only LUX protein binds directly to promoters, both ELF3 and ELF4 proteins are important for EC function (Nusinow *et al*, 2011). The binding of the EC to the promoters of target genes, such as *PRR9* and *LUX* itself, suppresses their expression (Dixon *et al*, 2011; Helfer *et al*, 2011). The importance of the *ELF3/ELF4/LUX* complex for free-running rhythms in constant light, and for entrainment of both wild-type (WT) and the *lhy/cca1* double mutant (Hazen *et al*, 2005; Onai and Ishiura, 2005; Kolmos *et al*, 2009; Dixon *et al*, 2011), suggested that *ELF3*, *ELF4* and *LUX* (the EC genes) are the major elements of the evening loop of the clock. However, the evening loop's structure and integration with the rest of the clock circuit remained unclear.

To create the new clock structure, we first recast the evening loop to include the EC genes, together with post-translational regulation of ELF3 protein by the ubiquitin E3 ligase COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1) (Yu *et al*, 2008) (Figure 1, see Results for further detail). The oscillatory mechanism of the evening loop was analysed using data from the *lhy/cca1* double mutant, where only the evening loop sustains rhythmicity. We explored the function of GI in the new circuit, using data from the *lhy/cca1/gi* triple mutant. Second, we connected the evening loop to the rest of the clock and explored a new mechanism connecting the clock's evening components to the morning genes. In the context of the whole clock circuit, the observed repression of *PRR9* by the EC (Dixon *et al*, 2011; Helfer *et al*, 2011) creates a three-negative feedback ring structure, termed the repressilator. Another prediction relates to the regulation of *LHY* and *CCA1* expression by TOC1. Although the molecular details remain to be elucidated, our computational analysis revealed that timeseries data on the *ztl* and *prr7/prr9* mutants (Farre *et al*, 2005; Baudry *et al*, 2010) are more consistent with TOC1 being an inhibitor instead of an activator of *LHY* and *CCA1* expression. Besides, our new experiments with the *toc1* mutant and *TOC1*-overexpressing (*TOC1-ox*) plants further supported the negative role of TOC1 in regulation of *LHY* and *CCA1* genes inside the morning loop. The proposed clock circuit integrates both positive and negative connections, including the repressilator, into a complex, multi-loop structure. Our model of this circuit includes significantly more experimental data and explains the clock's responses to multiple genetic and environmental perturbations, now including the canonical response to short light pulses at various times (the phase-response curve (PRC)).

## Results

### Qualitative analysis leading to revision of the clock gene circuit in Arabidopsis

Figure 1 shows the principal scheme of the new clock model. We justify the new components and circuit structure in outline below, and examine its dynamic behaviour in the following sections. As in all previous models, *CCA1* and *LHY* were treated as a single component (*CCA1/LHY*). The model consists of 28 ordinary differential equations and 104 parameters. Values of 43 parameters were constrained based on the available data and 61 parameters were fitted to multiple timeseries data sets (see Supplementary Table S1). The value of the six Hill coefficients was set to two. A detailed description of the model is presented in the Supplementary information, together with a discussion of the model's limitations and its robustness to parameter variations (Supplementary Figures S3 and S4).

The evening loop of the clock was fundamentally revised in order to include the *ELF3*, *ELF4* and *LUX* genes (EC genes). The model includes the formation of the triple ELF3–ELF4–LUX protein complex, the EC (Figure 1), which was shown to be important for clock function (Nusinow *et al*, 2011). Multiple data show that the EC genes have repressive effects on clock gene expression, so that expression of *LUX*, *ELF4*, *GI*, *TOC1* and *PRR9* was derepressed in *elf3*, *lux* and *elf4* mutants (Fowler *et al*, 1999; Kikis *et al*, 2005; Kolmos *et al*, 2009; Dixon *et al*, 2011; Helfer *et al*, 2011). The model assumes that the EC suppresses the expression of these five target genes (Figure 1).

To define the minimal structure of the evening loop that remains in the *lhy/cca1* mutant, we analysed data on triple mutants *lhy/cca1/elf3*, *lhy/cca1/gi* and *lhy/cca1/toc1*. The data showed that clock entrainment is completely disrupted in the *lhy/cca1/elf3* mutant (Dixon *et al*, 2011), although the remnant circuit is still entrained in *lhy/cca1/toc1* (Ding *et al*, 2007) and *lhy/cca1/gi* (Locke *et al*, 2006). This suggested that the EC genes represent a core structure of the evening loop (Figure 1), which drives oscillations in the *lhy/cca1* mutant as described below, whereas *TOC1* and *GI* have different roles. Because the remnant circuit in *lhy/cca1* is entrained by light signals, we included light-dependent, post-translational regulation of the EC component ELF3 through its observed interactions with COP1 and GI, as detailed below. The new structure of the evening loop allowed us to describe our new data on the *lhy/cca1* and *lhy/cca1/gi* mutants without the hypothetical component *Y*.

Next, we connected the evening circuit to the rest of the clock. The connection from the morning to the evening loop was described through the suppression of *TOC1*, *LUX*, *ELF4*, *ELF3* and *GI* expression by CCA1 and LHY proteins (Harmer and Kay, 2005; Hazen *et al*, 2005; Kikis *et al*, 2005; Locke *et al*, 2005; Dixon *et al*, 2011; Li *et al*, 2011). The connections from the evening loop to the morning loop include the inhibition of *PRR9* expression by evening components. Based on indirect observations, we previously suggested the inhibition of *PRR9* by TOC1 (Pokhilko *et al*, 2010). Recent biochemical work demonstrated that *PRR9* expression is more likely to be inhibited by the EC (Dixon *et al*, 2011; Helfer *et al*, 2011) (Figure 1). Another important connection from the evening to the morning genes is related to the regulation of *LHY* and *CCA1* expression by TOC1 protein (Alabadi *et al*, 2001; Makino *et al*, 2002; Mas *et al*, 2003b; Baudry *et al*, 2010). Below we changed the sign of TOC1 function in the regulation of *LHY* and *CCA1* expression from an activator to an inhibitor, which improved the model's description of the existing data on the *ztl* and *prr7/prr9* mutants. This revision of TOC1 function removed the need for the hypothetical components *X* and TOC1mod of our previous models (Locke *et al*, 2005, 2006; Pokhilko *et al*, 2010), the most recent of which is hereafter referred to as the P2010 model. To further verify the proposed negative role of TOC1, we measured the level of *LHY* and *CCA1* expression in the *toc1* mutant and *TOC1-ox* plants. Our results showed that *LHY* and *CCA1* mRNA levels were reduced in the *TOC1-ox* plants and increased in the *toc1* mutant, which confirmed our model prediction about the negative regulation of *LHY* and *CCA1* genes by TOC1. Moreover, data published during revision of this manuscript further demonstrated the direct suppressive effect of TOC1 on *LHY* and *CCA1* expression (Gendron *et al*, 2012). Next, after connecting the loops, we tested the effects of the EC's repressive function on the dynamics of the whole system by comparing the simulated *elf3* mutant with WT, and also investigated the sensitivity of the new clock structure to light.

### New structure of the evening loop accounts for data on the lhy/cca1 and lhy/cca1/gi mutants

#### The EC as the main element of the evening loop

Based on the published data, we revised the structure of the evening loop, which supports oscillations in the *lhy/cca1* double mutant. It is represented by the formation of the EC by ELF3, ELF4 and LUX proteins (Nusinow *et al*, 2011). Inhibition of *ELF4* and *LUX* expression by the EC creates a negative feedback loop (Kikis *et al*, 2005; Helfer *et al*, 2011). To verify the new structure, we studied clock gene expression in the *lhy/cca1* double mutant computationally and experimentally under various light conditions. Expression peaks of the evening genes *TOC1* and *GI* were first shown to be advanced to a morning phase in RT–qPCR assays of the *lhy/cca1* mutant compared with WT plants grown under LD cycles (Figure 2A and B) (Alabadi *et al*, 2001; Mizoguchi *et al*, 2002; Locke *et al*, 2005). Transcriptome data from the *lhy/cca1* mutant identified *GI* as the most LHY/CCA1-responsive, evening-expressed gene (Supplementary Figure S2C). However, *ELF3*, *ELF4* and *LUX* were shown to be similarly affected (Supplementary Figure S2; Hazen *et al*, 2005; Kikis *et al*, 2005; Dixon *et al*, 2011; Li *et al*, 2011). We therefore extended the inhibitory action of LHY/CCA1 to all evening genes in our model, and the resulting simulations agreed with these data (Figure 2C): the early expression is caused by the loss of transcriptional inhibition by LHY/CCA1 in the morning. *LHY/CCA1* regulation in WT delays the rising phase of evening gene expression, as in previous models.

*TOC1* is also repressed by the EC in the model, which is based on the data on the high level of *TOC1* expression in the *elf3* and *elf4* mutants (Kolmos *et al*, 2009; Dixon *et al*, 2011) and the presence of two consensus *LUX*-binding sites, GAT(A/T)CG, in the *TOC1* promoter (Helfer *et al*, 2011). Simulated

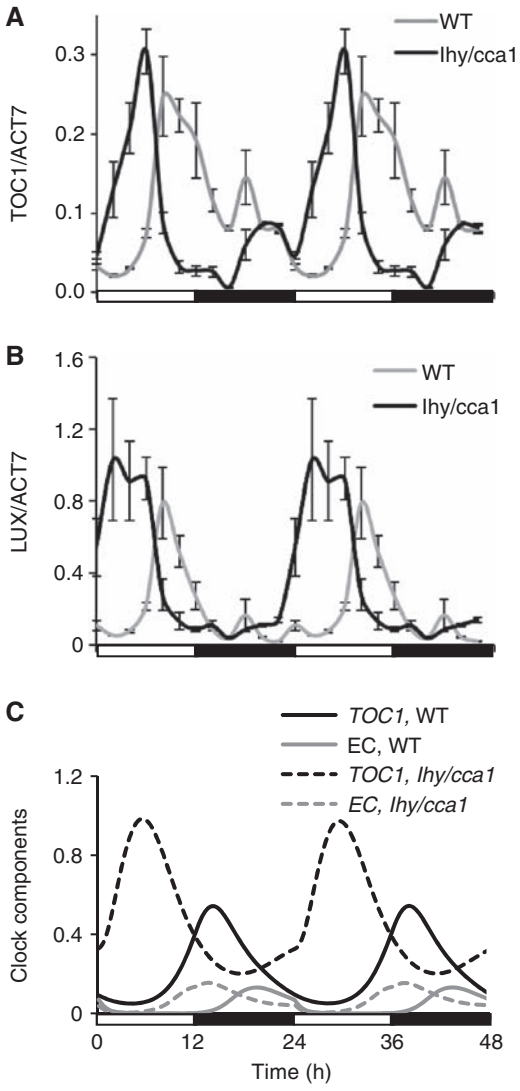


**Figure 2** Regulation of *TOC1* and *LUX* expression in the evening circuit of the clock. The phase advance of *TOC1* (**A**) and *LUX* (**B**) expression in the *lhy/cca1* double mutant (black line) compared with WT (grey line) was measured by qRT–PCR assays of plants grown under 12L:12D cycles, as described in Supplementary information. (**C**) Model simulations demonstrate that in both WT and *lhy/cca1* plants, the increase in EC (grey lines) coincides with the time of the fall in the expression of the EC's target genes (such as *TOC1*, black lines). Data are double-plotted to facilitate comparison to simulations. Light conditions are shown by open and filled bars below the figure.

*TOC1* RNA levels fall after dusk as EC levels rise, demonstrating that negative feedback from the EC is an important determinant of the falling phase of the evening genes' expression in the WT (Figure 2C). In the *lhy/cca1* mutant, this feedback is the only cause of oscillation in the model, so the profile of *TOC1* RNA level almost mirrors the EC profile (Figure 2C). The mutant's observed short period in constant conditions (17–18 h; Locke *et al*, 2005, 2006) reflects the lack of the additional delays from LHY and CCA1 inhibitor proteins (Supplementary Figure S5), which accelerates the expression of the EC genes. Formation of the EC then leads to autoinhibition of EC gene expression.

## Regulation of EC activity by COP1 and GI

The *lhy/cca1* mutant retained light entrainment (Alabadi *et al*, 2001; Mizoguchi *et al*, 2002; Locke *et al*, 2005), so light inputs must target at least one component of the evening loop. We therefore included the regulation of EC activity by light through targeted degradation of the EC component ELF3 by the COP1 ubiquitin E3 ligase, which was shown to be important for clock function (Millar *et al*, 1995b; Yu *et al*, 2008). To describe the kinetics of COP1 in diel cycles, we used the recent observation that COP1 protein exists in two different forms (Chen *et al*, 2010). Similarly to Pokhilko *et al* (2011), we assumed that light/dark transitions switch between the activities of these two, distinguishable E3 ligases, a night-active form (COP1n) and a day-active form (COP1d). Recent data suggested that COP1d might be related with a CULLIN 4 (CUL4) complex with COP1, where COP1 acts as a scaffold for a CUL4-based ubiquitin E3 ligase (Chen *et al*, 2006, 2010). Here, we assumed that COP1d is more active in the targeted degradation of the EC component ELF3, which thus alters the abundance profile of the EC (Supplementary Figure S6). ELF3 levels peak in the mid-night phase in the simulated WT, as observed (Liu *et al*, 2001; Dixon *et al*, 2011), so EC levels have already fallen substantially before dawn. Light regulation of COP1 activity (Pokhilko *et al*, 2011) then results in ELF3 degradation to a still lower level in the morning (Figure 2C). The further fall in EC levels is predicted to derepress the EC target genes such as *TOC1*. Its expression increases immediately after dawn in the *lhy/cca1* mutant (Figure 2; Supplementary Figures S2 and S6), though the LHY and CCA1 repressors mask this effect in WT plants. Thus, the model predicts that COP1 is important for the timing of evening gene expression in the *lhy/cca1* mutant. In the WT, this regulation would most affect genes that are more strongly regulated by LUX than by LHY and CCA1.

Additionally to COP1, GI protein also modulates the kinetics of the evening loop (Locke *et al*, 2005). Light-dependent stabilisation of ZTL by GI, and hence destabilisation of TOC1 protein (Kim *et al*, 2007), resulted in the simulated period lengthening in the *gi* mutant of the P2010 model. The short period of the most of *gi* mutants suggested another important function of GI in the clock (Martin-Tryon *et al*, 2007). Here, we added the binding of GI to ELF3 protein (Yu *et al*, 2008). The binding of GI to F box proteins in the presence of light suggested GI's ability to negatively regulate various protein targets (Kim *et al*, 2007; Sawa *et al*, 2007). Thus, we assumed that GI can accelerate the destruction of the EC by bringing F box proteins into its vicinity. Below we simulated computationally the possible outcomes of this role of GI and then tested the model predictions experimentally using the *lhy/cca1/gi* mutant.

The model predicted that the absence of GI should prevent EC levels from falling to their normal trough and thus reduce the peak levels of all EC-targeted evening genes (*LUX*, *TOC1*, *GI*, *ELF4*) in the *lhy/cca1/gi* triple mutant compared with *lhy/cca1* double mutant. Figure 3A and B show model simulations of *TOC1* and *LUX* expression, respectively. qRT–PCR measurements of *TOC1* and *LUX* expression confirmed this prediction (Figure 3C and D), demonstrating the indirect positive effect of *GI* on evening gene expression, consistent with previous reporter gene data (Locke *et al*, 2006). In our

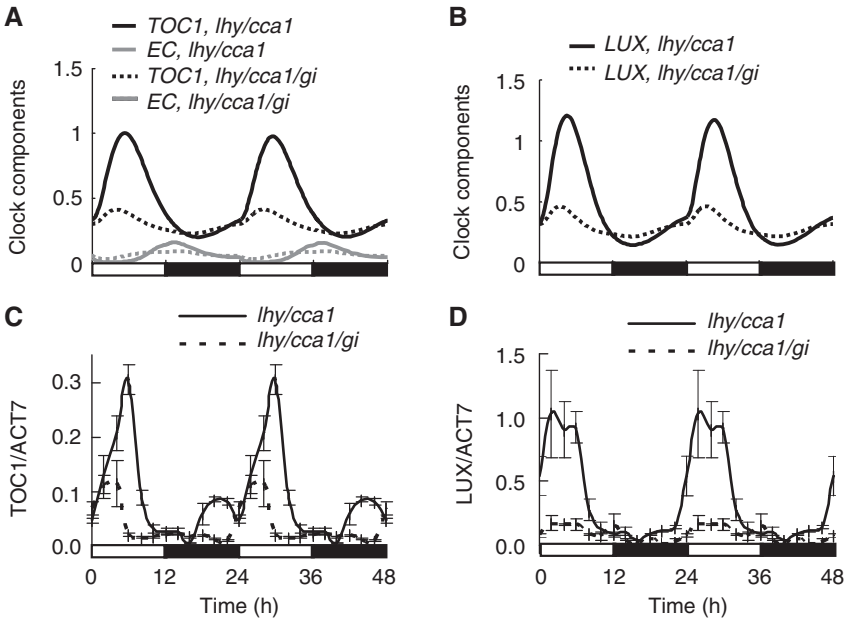


**Figure 3** The role of GI in the regulation of *TOC1* expression by the evening loop. Model simulations demonstrate lower peak levels of *TOC1* (**A**) and *LUX* (**B**) expression (black lines) in *lhy/cca1/gi* (dotted lines) compared with *lhy/cca1* mutants under 12L:12D cycles. This results from increased EC levels (grey lines) during the morning in the *lhy/cca1/gi* mutant. (**C**, **D**) qRT–PCR measurements of *TOC1* and *LUX* expression in *lhy/cca1/gi* and *lhy/cca1* mutants under 12L:12D. Data are double-plotted to facilitate comparison to simulations.

simulations of constant light conditions, this resulted in arrhythmia of the simulated *lhy/cca1/gi* mutants, in contrast to the short-period oscillations in *lhy/cca1* (Supplementary Figure S7), which both are consistent with experimental observations and with previous models (Locke *et al*, 2006).

The above simulations showed that COP1 and GI regulate the level of the ELF4–ELF3–LUX complex (EC) in both *lhy/cca1* mutants and WT. Despite this post-translational control of the complex, the temporal profiles of the bulk levels of ELF3, ELF4 and LUX proteins in our model mainly reflected the kinetics of the corresponding mRNAs, as shown in Supplementary Figure S8.

## Regulation of morning-expressed genes by the evening components of the clock

### TOC1—a repressor of the morning loop

The importance of the evening gene *TOC1* in the regulation of the morning components *LHY* and *CCA1* has long been assumed, based on multiple experimental observations (Alabadi *et al*, 2001; Mas *et al*, 2003b). However, the exact mechanisms of TOC1 action are still unknown. Based on gene expression analysis with mutant plants (Alabadi *et al*, 2001), TOC1 was previously suggested to play the role of an activator of *LHY/CCA1* expression (Alabadi *et al*, 2001; Locke *et al*, 2005; Baudry *et al*, 2010; Pokhilko *et al*, 2010). However, our analysis of the available data revealed inconsistency between the data and the activator role of TOC1. For example, the increase of TOC1 level in the *ztl* mutant, caused by the slowing of TOC1 protein degradation rate, leads to a substantial lengthening of the clock period (Mas *et al*, 2003b; Somers *et al*, 2004; Kevei *et al*, 2006). This lengthening was accompanied by a lower amplitude of *LHY* and *CCA1* expression in *ztl* plants (Baudry *et al*, 2010). This observation cannot easily be reconciled with the activation of *LHY* and *CCA1* expression by TOC1, which should result in a higher amplitude of *LHY* and *CCA1* in *ztl* mutants, as revealed by simulation of the P2010 model (Supplementary Figure S9). In addition, recent data showed that the expression of the *LHY* and *CCA1* inhibitors *PRR9* and *PRR7* is low in the *ztl* mutant (Baudry *et al*, 2010). It is hard to explain why *LHY* and *CCA1* mRNA does not rise in the *ztl* mutant, despite a low level of inhibitors and a higher level of the presumed activator TOC1.

On the contrary, our simulations below show that the results on *ztl* mutants can easily be described by assuming that TOC1 acts as a repressor of *LHY/CCA1* expression. The increase of TOC1 level in the *ztl* mutant results in a prolonged inhibition of *LHY/CCA1*, which lengthens the circadian period and reduces the amplitude of *LHY/CCA1* expression. As *TOC1 (PRR1)* belongs to the *PRR* gene family, the repressive function of TOC1 makes it consistent with the other PRR proteins, such as PRR9, PRR7 and PRR5 (Nakamichi *et al*, 2010; Pokhilko *et al*, 2010). Thus, we extended the wave of PRR inhibitors of *LHY/CCA1* in the model by including TOC1 (Figure 1), and explored the effect of TOC1 repression on the clock's dynamics.

Our simulation of the *ztl* mutant demonstrated that higher suppression of *LHY/CCA1* by TOC1 protein resulted in longer period and lower amplitude of *LHY/CCA1* in the *ztl* mutant compared with WT (Figure 4A and B). These results corresponded to the data (Baudry *et al*, 2010) and improved the description of *ztl* compared with the P2010 model (Supplementary Figure S9B). Figure 4A illustrates the participation of TOC1 in the wave of *LHY/CCA1* inhibitors in simulations of WT plants in constant light conditions. The absence of TOC1 resulted in a 2.5 h shortening of the period in

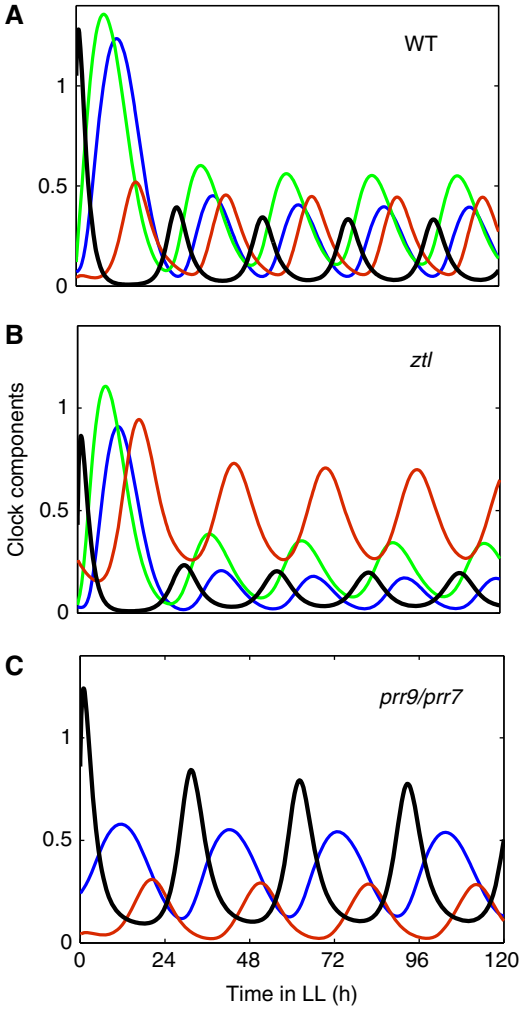


**Figure 4** The improved description of *ztl* and *prr7/prr9* mutants is related to the inhibition of *LHY/CCA1* expression by TOC1. The simulated level of *LHY/CCA1* mRNA (black) and the repressor proteins PRR7, NI and TOC1 (green, blue and red, respectively) are shown for WT (**A**), *ztl* mutant (**B**) and *prr7/prr9* mutant (**C**) plants. Simulations moved from 12L:12D cycles to constant light at time 0, corresponding to dawn in LD.

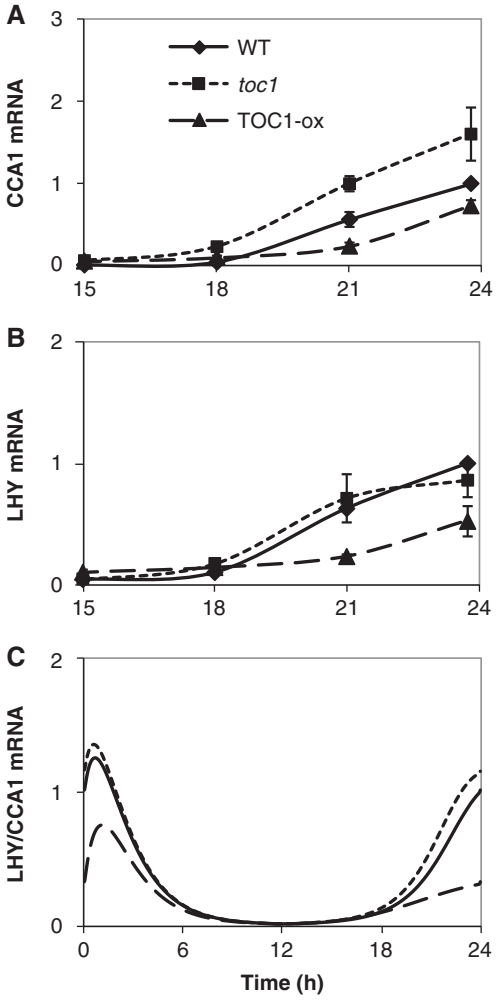


**Figure 5** The effect of TOC1 level on the kinetics of *LHY* and *CCA1* expression. qRT–PCR measurements of *CCA1* (**A**) and *LHY* (**B**) mRNA levels, and model simulations of *LHY/CCA1* (**C**) expression in *TOC1-ox*, *toc1* and WT plants under 12L:12D cycles were performed as described in the Supplementary information. *TOC1-ox* was simulated by adding a constant, unregulated activation of *TOC1* transcription with a rate constant equal to 0.3 per hour, which correspond the observed expression level of *TOC1* in TOC1-ox (Supplementary Figure S1).

the *toc1* mutant compared with WT (Supplementary Figure S10), which is close to the observed period shortening (Millar *et al*, 1995a; Mas *et al*, 2003a). *LHY/CCA1* levels were slightly reduced in the simulated *toc1* mutant under constant light, but this counter-intuitive result is also consistent with experimental data (Alabadi *et al*, 2001). The reduction was related to the higher trough level of the remaining *LHY/CCA1* inhibitors—the PRR9, PRR7 and NI proteins—in the *toc1* mutant (Supplementary Figure S10; please see Supplementary information for detail). Additionally to the correct description of *ztl* and *toc1* mutants, we greatly improved the description of the *prr7/prr9* double mutant compared with the P2010 model (Supplementary Figure S9C). The participation of TOC1 in *LHY/CCA1* inhibition resulted in robust oscillations in the *prr7/prr9* mutant under constant light with a period 30.6 h (Figure 4C), which corresponds to the experimental observations (Farre *et al*, 2005; Salome and McClung, 2005). In the P2010 model, the simulated period for *prr7/prr9* mutants was only 27.5 h, because the only remaining inhibitor (NI) could not provide a long enough delay in *LHY/CCA1* expression. Furthermore, the oscillations in the *prr7/prr9* mutant simulated by the P2010 model dampened faster than observed in the data (Supplementary Figure S9C). Thus, the introduction of TOC1 repressive function improved the description of multiple mutants in the new model of the clock.

To verify the repressive function of TOC1 further, we measured the expression levels of *LHY* and *CCA1* in the *toc1* mutant and *TOC1-ox* plants at the end of the night. TOC1 is predicted to have a larger role than the other, earlier-expressed PRR proteins at this time, when *LHY* and *CCA1* expression starts to rise as they are released from repression. Figure 5A and B show that *LHY* and *CCA1* mRNA levels rise more slowly in the *TOC1-ox* plants compared with WT, whereas the rise of *CCA1* is accelerated in the *toc1* mutant. The model simulations

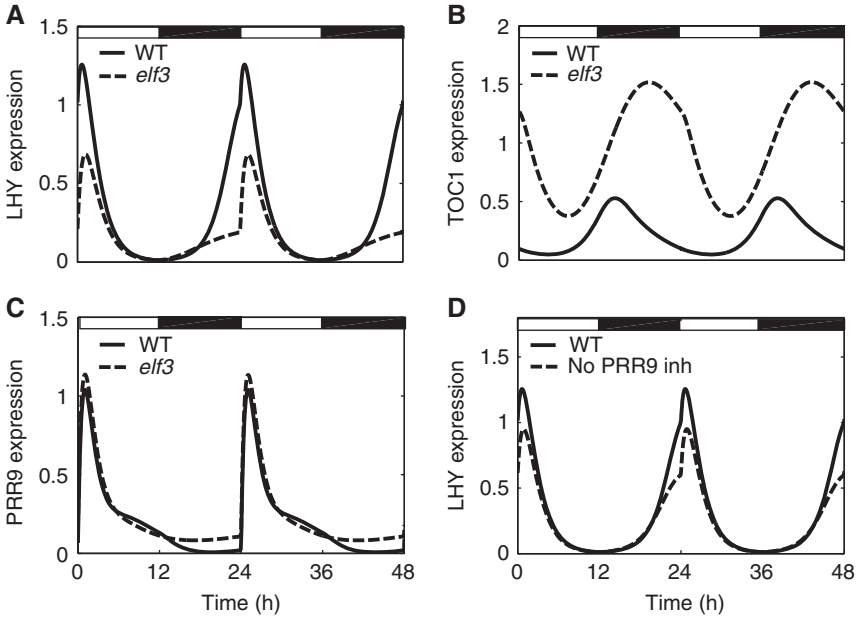


**Figure 6** Nighttime inhibition of *TOC1* and *PRR9* expression by the EC is important for the robust oscillation of *LHY/CCA1*. Model simulations (dashed lines) of the *elf3* mutant (**A**–**C**) and a hypothetical mutant without inhibition of *PRR9* by the EC (**D**) are shown together with WT simulations (solid lines). The simulations were run under 12L:12D conditions, which are indicated by open (light) and solid (dark) bars.

of *TOC1-ox* and *toc1* matched our experimental observations (Figure 5C), supporting the proposed repressive function of TOC1 towards *LHY* and *CCA1* expression. This change compared with earlier models affects only the sign of the interaction, not the level of abstraction in the model: the biochemical mechanism of TOC1 action remains to be determined.

### The EC controls *LHY* and *CCA1* expression through multiple PRRs

The EC components ELF3, ELF4 and LUX are known to be important for the high-amplitude oscillations of *LHY* and *CCA1* in diel cycles and for rhythmicity of the clock in constant light conditions (Doyle *et al*, 2002; Hazen *et al*, 2005; Kolmos *et al*, 2009; Dixon *et al*, 2011). We therefore explored the direct and indirect effects of EC action on the clock system, by simulating mutants in the EC genes. Figure 6A demonstrates the reduction of *LHY/CCA1* amplitude in the simulated *elf3* mutant compared with WT in a 12L:12D cycle, which agrees with experimental observations (Dixon *et al*, 2011; Helfer *et al*, 2011). The modelling also showed that this reduction of *LHY/CCA1* amplitude prevents rhythmicity in the remnant circuit of *elf3* mutants under constant light conditions (not shown), in line with observation (Hicks *et al*, 1996; Covington *et al*, 2001; Doyle *et al*, 2002; Hazen *et al*, 2005). The model suggested that the effect of the absence of EC on *LHY/CCA1* expression is related to the higher level of *LHY/CCA1* inhibitors TOC1 and PRR9 in the *elf3* mutant (Figure 6B and C), which corresponds to published data (Dixon *et al*, 2011). The effect of the *elf3* mutation on *PRR9* is quite subtle compared with its effect on *TOC1*. To separate the effects of derepressing *TOC1* and *PRR9* in *elf3* mutants, we simulated a hypothetical mutant that lacked only *PRR9* inhibition by the EC (Figure 6D). This simulation showed that the amplitude of *LHY/CCA1* oscillations falls by 46% in the *elf3* mutant and by 24% in the absence of inhibition of *PRR9* by EC (Figure 6A and D). Thus, the model predicted that the inhibition of both *PRR9* and *TOC1* expression by the EC at night is important for robust oscillations of *LHY/CCA1* and consequently for the anticipation of dawn by the clock.

## Improved light sensing by the new clock circuit

Various light input signals are used experimentally to study the mechanisms of light perception by the clock, which results in entrainment of the endogenous oscillator to the environmental day/night cycle. The most obvious manipulation changes the duration of the light interval or day length in an experimental light/dark cycle. Our simulations showed that, similarly to P2010, the new model retains the good match to *LHY/CCA1* mRNA data under various photoperiods (Supplementary Figure S11A). In the same time, the new structure of the evening loop provides some delay of evening gene expression in long days and thus provides a better match to the data compared with the P2010 model (Supplementary Figures S11B and S12).

Another way to investigate light sensing by the clock is the so-called PRC, which has a long history in the circadian field. The PRC represents the phase shift of the clock components, after light pulses given at different times to organisms that are kept in darkness (Figure 7A). It is characteristic that the clock sharply changes its response from phase delays to phase advances at a certain time of the subjective night (around 15 h after subjective dawn in Arabidopsis) (Covington *et al*, 2001). Here, we used our model to investigate the possible mechanisms of this phase shift. Our

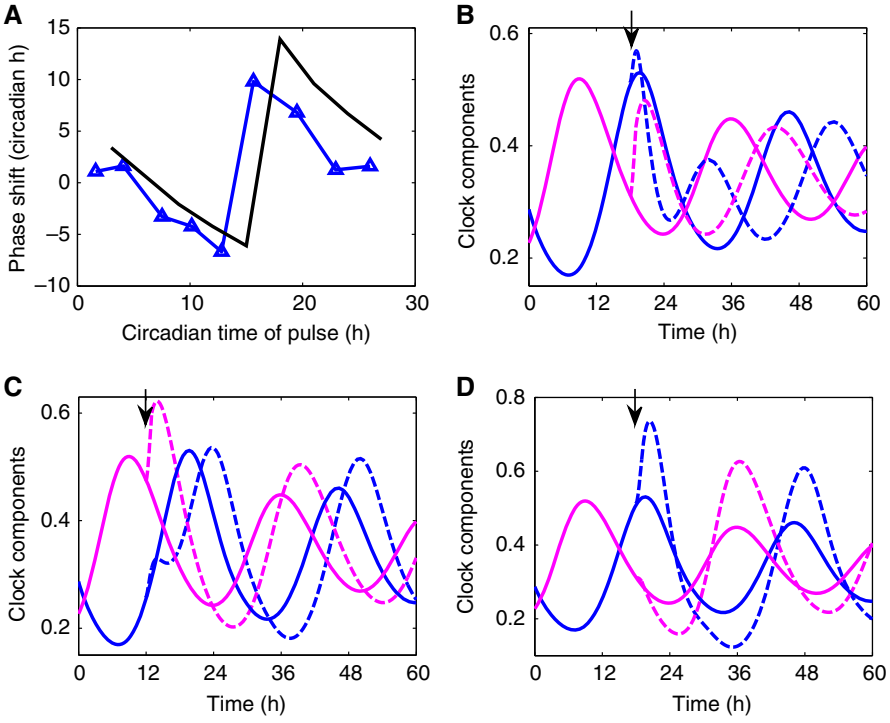


**Figure 7** The mechanism of the PRC in plants. (**A**) A PRC was simulated by monitoring the phase of peaks of *LUX* expression after light pulses of 1 h duration given on the second day in darkness after 12L:12D entrainment. Data points were taken from Covington *et al* (2001) for red light pulses. (**B**–**D**) The simulated profiles of *LUX* mRNA (blue) and LHY protein (magenta) with (dashed lines) or without (solid lines) light pulses given at indicated times (arrow)—at 18 h (B), 12 h (C) or at 18 h for a simulated mutant without an acute light response in *LHY* transcription (D; parameter $q1$=0). Time 0 refers to the beginning of the second day in darkness.

simulations matched the available data on the PRC of the evening components of the clock (Figure 7A). Although light affects the clock in several places (Figure 1), the PRC in our model is mostly determined by the acute light response in *LHY/CCA1* expression. This increase in *LHY/CCA1* expression immediately after 'lights-on' is caused by a fast transient activation of transcription. In this and all previous models, this is mediated by the yet-unidentified, dark-accumulating activator, protein P (Kim *et al*, 2003; Locke *et al*, 2005). A simulated mutant lacking only this response loses its phase response to light (compare Figure 7B and D), indicating that the response is necessary. The most closely related data show that transient, chemical induction of *CCA1* expression is sufficient to cause large phase shifts *in vivo* (Knowles *et al*, 2008). The increase in the level of LHY/CCA1 protein after the light pulse results in a fast decrease of the expression of LHY/CCA1 target genes, such as *LUX* (Figure 7B and C) in our model simulations. The resulting shift in *LUX* phase depends on the phase of *LUX* expression during the pulse. When the light pulse is given closer to or after the *LUX* mRNA peak (~18 h), the increase in LHY/CCA1 level accelerates the fall of *LUX* mRNA and advances the next peak (Figure 7B). However, earlier pulses delay the rise in *LUX* mRNA and the next peak (Figure 7C). The phase advance after a pulse at 18 h is lost in a simulated mutant that lacks an acute light response in *LHY/CCA1* (Figure 7C). Thus, the model predicted that the acute activation of *LHY/CCA1* expression by light is responsible for the observed transition from phase delay to phase advance in the PRC.

## Discussion

Based on very recent data, we updated the structure of the plant clock and used mathematical modelling to demonstrate a good correspondence of the new model to a wide spectrum of new and older data. The new model better described the clock's response to various genetic and environmental perturbations, and improved our understanding of the clock gene network.

### Comparison to earlier models

The most radical change in our model compared with the P2010 model is related to the introduction of the EC genes *ELF3*, *ELF4* and *LUX* into the clock scheme. The strong phenotypes of the single mutants of these genes suggested that they are very important for the clock. However, the structural relationships between EC genes and the rest of the clock were unknown. Our recent data suggested that the EC is absolutely necessary for the rhythmicity and entrainment of the *lhy/cca1* mutant (Dixon *et al*, 2011). We therefore started building the new structure of the clock from the evening loop, which represents the minimal, EC-containing rhythmic element.

The previous structure of the evening loop was based on the observed, rhythmic *TOC1* expression in the *lhy/cca1* double mutant (Locke *et al*, 2005; Pokhilko *et al*, 2010). In the P2010 model, the evening loop consisted of the hypothetical activator *Y* of *TOC1* expression, which was transcriptionally suppressed by TOC1 protein. In the new model, we replaced *Y* with the EC

genes, which feedback negatively to their promoters, providing oscillations in the *lhy/cca1* mutant. The expression profiles of the EC target genes, such as *TOC1*, are described through the repression from the EC instead of the earlier model's activation by *Y*. Light regulation is important for the observed entrainment of the evening loop. In previous models, light directly regulated *Y* expression (Locke *et al*, 2005). In the new model, light input is provided by the light-dependent degradation of the EC component ELF3, in which COP1 and a related ubiquitin E3 ligase may participate (Yu *et al*, 2008; Chen *et al*, 2010). This does not preclude other contributions to light regulation, such as the recently described transcriptional induction of *ELF4* (Li *et al*, 2011).

The next changes in the scheme of the evening loop were related to GI, which was previously proposed as a candidate that accounted for some but not all of *Y*'s functions, on the basis of RNA data and genetic evidence (Locke *et al*, 2005, 2006). Indeed, *GI* retains functions in the present model that are consistent with *Y* but *GI* appears to be a modulator rather than the major effector. GI still increases *TOC1* expression in the model, for example, as observed in data (Figure 3B) (Locke *et al*, 2006) but the mechanism is by a double inhibition rather than direct activation: GI protein is a negative regulator of the EC, which inhibits *TOC1* expression. The introduction of the negative effect of GI on the EC improved the description of the *gi* mutant compared with the P2010 model: the 2.6 h shortening of circadian period in the simulated *gi* mutant provides a better match to the data (Park *et al*, 1999; Gould *et al*, 2006; Martin-Tryon *et al*, 2007) than the 2 h lengthening of the *gi* period simulated in the P2010 model. Thus, we removed the hypothetical gene *Y* and redrew the structure of the evening circuit by including the important clock components ELF3, ELF4, LUX and COP1, re-connecting them to GI to provide a more realistic structure for the evening loop.

After rebuilding the evening loop, we connected it to the rest of the clock circuit and re-examined the connections from the evening genes to the morning loop. Based on genetic data, it was previously assumed that TOC1 activates *LHY/CCA1* expression (Alabadi *et al*, 2001; Locke *et al*, 2005; Pokhilko *et al*, 2010). The ~12 h time delay of peak *LHY* compared with *TOC1* expression required a hypothetical, intermediate clock component, *X* or TOC1mod, in previous clock models to ensure the required, long-lasting positive effect of TOC1 on *LHY/CCA1* expression (Locke *et al*, 2005; Pokhilko *et al*, 2010). However, our model analysis demonstrated that the data on *ztl* and *prr7/prr9* mutant plants agreed better with a negative role for TOC1 in *LHY* and *CCA1* expression. In addition to Baudry *et al* (2010), other published data also show lower levels of *LHY* and *CCA1* mRNA in multiple mutants with increased amounts of TOC1 protein (Makino *et al*, 2002; Somers *et al*, 2004; Kim *et al*, 2011). Our data on *LHY* and *CCA1* expression in the *TOC1-ox* and *toc1* mutants further supported the negative role of TOC1 (Figure 5). This repressive function was consistent with TOC1 protein acting immediately after *TOC1* RNA expression, allowing us to further improve the model by removing the hypothetical delaying component. Together, the EC-based evening loop and the change in the sign of *TOC1* function suggest that the *toc1* mutation merely removes the last component in the wave of PRR repressors, leaving the EC-based evening loop intact. The biochemical mechanisms of TOC1's suppressive action remain the object of further studies: our data inform only the sign of the interaction between genes and cannot exclude greater complexity in the molecular interactions involved.

Additionally to removing *X* and *Y*, we further simplified the model by greatly reduced number of transcriptional regulators with Hill kinetics, which imply a complex or multimeric regulation. The remaining Hill coefficients are set to 2, which corresponds to the well-justified dimerisation of plant clock components (Fujiwara *et al*, 2008; Wang *et al*, 2010; O'Neill *et al*, 2011). This provided a more realistic description of the clock compared with the P2010 model.

Mutation of an EC gene removes the evening loop in the model but leaves the potential for oscillatory feedback(s) among LHY, CCA1 and the PRRs. Oscillations and related behaviour have been observed in EC gene mutants in some conditions (Hicks *et al*, 1996; McWatters *et al*, 2000; Covington *et al*, 2001; Hall *et al*, 2003; Wenden *et al*, 2011). The new model recapitulates the more severe circadian phenotypes of EC gene mutants under constant light conditions (Reed *et al*, 2000; Doyle *et al*, 2002; Hazen *et al*, 2005), which suggest that the morning loop cannot support self-sustaining oscillations, in contrast to the P2010 model. The new model matches the data for EC gene mutants in diel cycles (Figure 6), indicating that the EC also contributes to high-amplitude oscillations of *LHY* and *CCA1* under entrained conditions. Thus, the new model describes a complex, integrated clock structure, with interdependent dynamics. Weak, damping oscillations from the evening loop alone are stabilised by coupling to the morning loop in the intact system.

## Abstraction of regulatory circuits from observed pairwise interactions

The model of the plant circadian clock was modified, based entirely on known components and their interactions. Interestingly, the emerging structure of the clock includes a ring of three sequential negative steps, each representing the inhibition of earlier-expressed clock components by the later ones: inhibition of EC genes by the rise of LHY/CCA1 in the late night, of *PRR* genes by EC in the early night, and of *LHY/CCA1* by PRRs in the day. This new structure allows us to re-interpret several previous observations. First, EC genes were previously suggested to be activators of *LHY* and *CCA1* expression based on genetic studies (Doyle *et al*, 2002; Hazen *et al*, 2005; Kikis *et al*, 2005; Onai and Ishiura, 2005). The new data demonstrated that EC proteins repress the expression of *PRR* genes (Kolmos *et al*, 2009; Dixon *et al*, 2011; Helfer *et al*, 2011). This, together with the previously known, negative regulation of *LHY* and *CCA1* expression by PRR proteins (Farre *et al*, 2005; Mizuno and Nakamichi, 2005; Nakamichi *et al*, 2010) allowed the re-interpretation of the positive genetic interaction from the EC genes to *LHY* and *CCA1* as a double-negative interaction (Dixon *et al*, 2011; Helfer *et al*, 2011) (Figure 8A). We demonstrated both computationally and experimentally that mutation of the EC components resulted in the decrease of the *LHY/CCA1* amplitude (Figure 6A–C), in agreement with the experimental findings (Doyle *et al*, 2002; Hazen *et al*, 2005; Kolmos *et al*, 2009). Thus, the double-negative

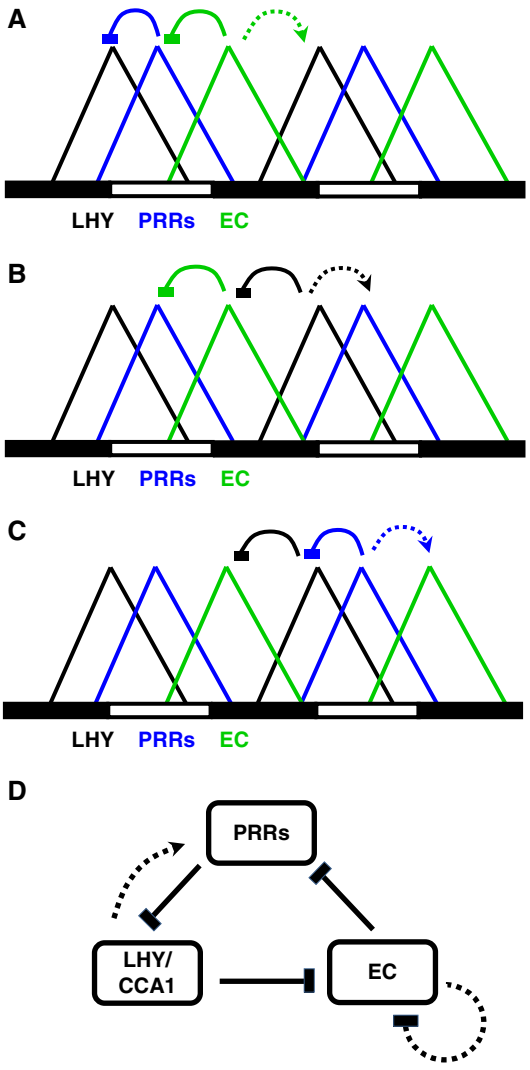


**Figure 8** Core interactions in the clock model form a repressilator circuit. (**A**–**C**) The sequential expression of *LHY/CCA1* (black), *PRR* genes (blue) and EC genes (green) are sketched relative to a 12L:12D diel cycle. Their regulatory interactions can be explained by double-negative (solid, blunt arrows) or single-positive (dashed arrow) connections, for (A) *LHY/CCA1* activation by the EC genes; (B) *PRR* gene activation by LHY/CCA1; (C) activation of EC genes by PRRs. (**D**) The core structure of LHY–PRR–EC interactions in the model is shown to include a repressilator, a three-inhibitor ring oscillator (solid lines). Other interactions between LHY, PRRs and the EC (dotted lines) include the activation of *PRR*s by LHY/CCA1, which was identified as the morning loop, and the autoinhibition of the EC, which represents the evening loop. For clarity, the light inputs, GI and the post-translational regulators are omitted.

feedback from EC to *LHY/CCA1* is sufficient to describe the experimental data.

Similarly, the model has a double-negative feedback from LHY/CCA1 to *PRR* genes, which is also based on experimental data. Indeed, LHY and CCA1 proteins inhibit the expression of the EC genes (Hazen *et al*, 2005; Kikis *et al*, 2005; Portoles and Mas, 2010; Dixon *et al*, 2011; Li *et al*, 2011). The repression of *PRR* genes by the EC forms a double-negative connection from LHY and CCA1 to the *PRR*s. This double-negative connection can also be represented by a positive regulation of *PRR* expression by LHY/CCA1 (Figure 8B), which was previously suggested based on genetic studies (Farre *et al*, 2005; Ding *et al*, 2007). Based on the present data, it is not possible to distinguish between double-negative and direct positive connections from LHY to the *PRR*s, because either or both of them could lead to the observed decrease of *PRR* expression in the *lhy/cca1* double mutant. However, the observed increase of *LUX* and *ELF4* expression in the *lhy/cca1* mutant (Hazen *et al*, 2005; Kikis *et al*, 2005) suggests that the double-negative feedback mechanism might underlie the decrease of *PRR* gene expression in the *lhy/cca1* mutant. Additionally to the double-negative connection, our current model retains the direct positive connections from LHY/CCA1 to the *PRR*s, which is supported by the existence of LHY/CCA1-binding sites in the promoter of *PRR9* and *PRR7* genes (Farre *et al*, 2005; Harmer and Kay, 2005) and the observed binding of CCA1 to *PRR9* and *PRR7* promoters (Portoles and Mas, 2010), which can have high affinity (O'Neill *et al*, 2011). The positive connections were also found in other circadian systems, such as mamallian and fly clocks (Zhang and Kay, 2010). The functional role of the positive connections might be related with increased robustness of oscillations, as was shown for the various negative feedback networks with an additional positive connection (Tsai *et al*, 2008). Further biochemical studies are required to dissect the relative impact of the positive and double-negative connections from LHY and CCA1 to the *PRR*s.

Finally, the double-negative feedback from PRRs to the EC genes via LHY and CCA1 is also presented in the model (Figure 8C). Analogously, this follows from the data showing that PRR proteins inhibit *LHY* and *CCA1* expression, which in turn inhibit expression of the evening genes. The double-negative connection could lead to the indirect activation of evening gene expression by *PRR* genes, which was previously suggested from genetic studies (Mizuno and Nakamichi, 2005; Nakamichi *et al*, 2005).

A counter-intuitive aspect in each of these connections is that the target gene is expressed before its immediate regulator within the day–night cycle. It was therefore natural to propose that LHY and CCA1 activated *PRR* gene expression, and the EC activated *LHY* and *CCA1* expression, in line with the genetic results from stable mutant plants or mis-expression lines (dashed arrows in Figure 8A–C). Dynamic manipulations of the circuit and direct biochemical studies were required to demonstrate the double-negative mechanisms (Nakamichi *et al*, 2010; Portoles and Mas, 2010; Dixon *et al*, 2011; Helfer *et al*, 2011; this paper). Our mathematical model suggests that the double-negative connections are consistent with the data. Currently, we have left only one positive connection from LHY/CCA1 to the *PRR* genes, because it is supported by data on the direct binding of CCA1 protein to *PRR* promoters. Future experiments are necessary to investigate the functional consequence of this binding on *PRR* gene expression.

A repressilator, the three-inhibitor ring oscillator, was first constructed as a synthetic circuit in *Escherichia coli* (Elowitz and Leibler, 2000) and is one of a class of well-studied ring systems (reviewed in Purcell *et al*, 2010). Here, we show that the repressilator structure is present as an integrated element of the more complex circuit in our current model (Figure 8D). Interestingly, a similar repressilator structure was recently found in the mammalian clock, where it also represents only part of the system (Hogenesch and Ueda, 2011; Ukai-Tadenuma

*et al*, 2011). Importantly, the repressilator structures were discovered in both plant and mammalian networks based directly on experimental data. This suggests that, although the real biological systems are more complicated than the simplified structure of the repressilator, some features of repressilator behaviour might be important for clock function. However, as described in the Results, the whole structure of the plant clock includes such important additional elements as the autoregulation of the EC genes; post-translational regulation of the EC by COP1 and GI; and the wave of multiple PRR inhibitors of *LHY/CCA1* expression. Additionally, multiple light inputs affect the kinetics of the plant system. In summary, we propose that the plant clock functions as an integrated multi-feedback system, which maintains robust oscillations and entrainment under multiple perturbations.

## 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 (www.nature.com/msb). The model is available in SBML format 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) and the authors' web site (http://www.amillar.org).

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*Author contributions:* AP, APF, KJH, MMS and AJM designed the experiments; APF, MMS and KDE performed PCR and transcriptome experiments, respectively; AP and AJM designed the computational analysis; AP performed the computational analysis; AP and AJM wrote the paper with comments from all the authors.

## Conflict of interest

The authors declare that they have no conflict of interest.

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