

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

Alexandra Pokhilko, Aurora Piñas-Fernández, Kieron D. Edwards, Megan M Southern, Karen J. Halliday, Andrew J. Millar

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### Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

09 September 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, substantial concerns that preclude its publication in its present form.

There was a clear divide between the first two reviewers, who were generally positive, and the last reviewer whose opinion was clearly negative. Nonetheless, it is clear that the reviewers, especially the second and the third actually raised some very similar concerns. Molecular Systems Biology now encourages reviewers to comment on each other's reports, and during this process the second reviewer wrote, "I can understand the points [of] the 3rd reviewer - for any physicist the parameter fitting is not satisfying. In fact, assuming, e.g., a certain topology, Hill coefficients of 2 and linear degradation kinetics makes the model somewhat arbitrary. However, there are no more comprehensive quantitative data available and thus the model cannot be perfect, but it can be useful. The proposed model seems to me close [to] the upper limit of what data and experiences allow. Thus deficiencies in parameter estimations could be tolerated if the biological insight is inspiring." With these comments in mind, we highlight some important concerns that would need to be addressed in any revised manuscript.

-- Model parameters. As outlined by the first two reviewers it will be important to acknowledge the limitations of the current model and current parameter estimates, and provide information on the relative confidence of various model aspects. In addition, some attempt should be made to demonstrate that the key conclusions arising from this analysis (e.g. a repressive role for TOC1) are robust to reasonable variation in the parameter values.

-- The Repressilator. The third reviewer found the functional relevance of the repressilator concept less than convincing given that this structure represents a selectively emphasized portion of a more complex network. Similarly, the second reviewer wonders whether there is evidence that the entire network actually contains subnetworks capable of independent oscillatory behavior. Additional conceptual clarification is needed here, and it may be necessary to reduce your claims or use more cautious language.

The second reviewer also felt that a substantial effort should be made to make sure that this work is clear and accessible to scientists who are not familiar with the Arabidopsis circadian clock, a point we would like to emphasize given the broad readership of Molecular Systems Biology.

In addition, the editor notes the recent work by Lau et al. (2011, Mol Cell). While this work does not appear to impinge on the novelty of the findings reported here, this work and its implications for the current model should be discussed.

We also ask that you deposit your circadian clock model in a public repository like BioModels before submitting your revised work, and incorporate the accession number (or a confidential reviewer login) into the methods section of the manuscript.

\*\*\* PLEASE NOTE \*\*\* As part of the EMBO Publications transparent editorial process initiative (see our Editorial at <http://www.nature.com/msb/journal/v6/n1/full/msb201072.html>), Molecular Systems Biology will publish online a Review Process File to accompany accepted manuscripts. When preparing your letter of response, please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this File, which will be available to the scientific community. More information about this initiative is available in our Instructions to Authors. If you have any questions about this initiative, please contact the editorial office [msb@embo.org](mailto:msb@embo.org).

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

Yours sincerely,

Editor - Molecular Systems Biology  
[msb@embo.org](mailto:msb@embo.org)

### Referee reports

Reviewer #1 (Remarks to the Author):

In this study, Pokhilko and co-workers present an improved model for the central circadian clock in Arabidopsis. The new model is largely based on a model previously published by the same group in 2010, which has been significantly extended with the inclusion of the EC (evening complex) genes ELF3, ELF4, and LUX. Figure 1 is particularly helpful in illustrating the similarities and differences between the two models (especially for non-Arabidopsis-experts like myself) and the authors should consider directing the reader's attention to this figure near the beginning of the description on pages 2-4, rather than at the end. The paper is extremely well-written and proceeds in a logical fashion from the inadequacies of the previous model (especially its reliance on the unknown factors "X" and "Y"), through the modifications used in the new model, to presenting a simplified abstraction of the new model to the well-known repressilator topology. The creation of a model this complex involves a large number of choices that can seem arbitrary, but the authors took pains to explain why they made the choices they did and where other choices could have been made (such as the choice between double-repression or direct-activation interactions between LHY and the PRRs).

Minor Concerns:

1. One thing that might improve the conclusion of the paper would be a brief paragraph assessing

the authors' confidence in various aspects of the model. The choices made were clearly motivated by experimental evidence and by improving the model's agreement with observed behavior, but some of those choices must have been made more confidently than others. Providing readers with a sense of which areas are less-than-completely established will likely aid in future refinements and improvements. On a related note, Supplementary Table 1 contains values that had been fit to multiple datasets, but doesn't convey a sense of the uncertainty in these fitted values, or of which parameters were better-constrained by the data than others. A little more description of the parameter-fitting procedure would be helpful, along with something to convey a sense of the uncertainty in the fitted parameters and (more importantly) the simulated model output.

2. One thing that might improve the generality of the paper would be a brief paragraph discussing the repressilator structure found in circadian clocks of other organisms. A similar repressilator structure has been also found in mammalian circadian clocks (Ukai-Tadenuma M et al, Cell 144(2):268-81 (2011), Hogenesch JB et al, Nature Reviews Genetics Jun;12(6):407-16 (2011)). Some discussion of the generality and biological relevance of this structure in circadian clock might be useful for the readers.

A few (more minor) comments on the text itself:

On page 14: "We demonstrated computationally, that mutation of the EC components resulted in the decrease of the LHY/CCA1 amplitude (Figure 5A), in agreement with the experimental findings." The first comma is unnecessary: "We demonstrated computationally that mutation of the EC components..."

On page 15: "This double negative connection can also be represented by a positive regulation of PRR expression by LHY/CCA1 (Figure 9B)," I think the authors mean Figure 8B.

Supplementary materials, page 7: "In total, parameters 43 were constrained and 61 parameters were fitted." -- should be "43 parameters were constrained..."

Supplementary materials, page 11: "(?add refs: Gould et al, 2006; Mizoguchi et al, 2002; Yakir et al, 2009)" Looks like Gould and Mizoguchi are there; Yakir is missing.

Supplementary materials, page 14: "The detailed kinetic measurements of the activity and abundance of the protein complexes in the plant clock, such as COP1 and GI protein complexes are awaiting for the future elucidation." -- should be "...await future elucidation."

Reviewer #2 (Remarks to the Author):

The authors address an exciting question - the feedback loop design of the circadian clock in *A. thaliana*. Old and recent published data together with new own data combined with a deep understanding of the core clock leads to a significantly updated model of the Arabidopsis clock. The novel model has a new loop design (including repressilator structure) and a well-done mathematical description in terms of ODEs. In this way a better conceptual understanding of this oscillator is achieved. The paper is an excellent combination of data, biological insight and mathematical modelling and should be published in a good journal.

However, the current version of the manuscript is not easy to read by a scientist not familiar with the Arabidopsis clock and previous papers such as P2010, L2005 and L2006. The authors should reduce the amount of specialized information and should focus on fewer messages. In particular, I suggest removal of many of the gene names from the text. The link between abbreviations and genes names might be provided in footnotes, in a separate table or in the supplement.

At the end of the introduction 4 aims are listed (EC, GI, PRR9, TOC1). In the results and the discussion these topics are indeed studied in detail but the non-expert has problems to follow all the lines of thought. I suggest to reduce the aims as much as possible (perhaps 2-3), to enumerate them, and to construct Results and Discussion according to these aims. Some redundancy is helpful, side remarks should be reduced as much as possible.

The complete model is conceptually a combination of 3 oscillators: morning, evening and

repressilator. Thus in principal independent oscillations with different periods, tori and chaos might be expected. Is there any evidence of bifurcations of coupled oscillators? Otherwise all the loops constitute just one complex oscillator and the terms morning oscillator, evening oscillator and repressilator should be used with care.

I am aware that the available data do not allow the construction of a perfect quantitative model. For example, details of the transcriptional regulations or of enzymatic degradations are not known and thus Hill coefficients of 2 and in most cases linear degradation kinetics are assumed. Thus even after careful parameter fitting the model is plausible, explains many data, but it cannot be regarded as a precise quantitative model. Consequently, it would be appropriate to stress at some point the limitations of the model.

Specific comments:

1. Interesting title! Are there also repressilator structures in other model organisms (*Neurospora*, *Drosophila*, mammals)?
2. The discussion of flexibility and robustness is vague. Without quantifying these concepts a comparison of simple feedbacks and multiple loops is difficult. I can also imagine that a simple delayed negative feedback is somehow robust.
3. Some of the motivations in the introduction are technical (removal of X and Y, role for ELF3, ELF4, LUX). What are the open biological issues requesting a new model? What are the major discrepancies to the data justifying a novel design? I am aware that these questions are implicitly addressed. Thus no extension of the text is necessary but focussing on biological insight instead of technical improvement.
4. I miss in the main text a short description of the core properties of the model (number of equations, parameters, nonlinearities). I appreciate that few and mild nonlinearities were sufficient. Is the saturated degradation kinetics in 2 cases experimentally justified or is it just used to avoid larger Hill coefficients?
5. Fig.2 (data and interpretation) is very clear. For Fig.3 and its message (GI) a new subsection might be justified to structure the lengthy text.
6. There is no Figure 9B.
7. If reduction is necessary - Fig. 5 is not urgent. There is no direct comparison with data and the effect might be described in words (and the figure might be shown in the supplement).
8. Figure addresses an interesting issue (phases for different photoperiods) but it is a bit at the periphery of the manuscript. Moreover, the differences between models and data are comparable to the yearly variation and thus difficult to interpret.
9. Figure 8 is a good summary. However, the meaning of A-C is not really clear to me. I see the same information in Figure 8 D.

Reviewer #3 (Remarks to the Author):

As I am not a biologist, I cannot comment much on the biology (I am sure other reviewers will). I will mainly focus on the modeling aspects of the paper.

I believe that the work from these authors is very important for the community of plant circadian clocks as it provides a starting point for many researchers. With such models, one can make hypothesis and quickly test them by simulating the system. Hence, these results should be published and available to others. However, this paper feels more like an updated version of some software and so the question is where should it be published.

My major concern with this paper is that this is simply an update of existing models. As with other updates, this new model concludes that there were missing species and wrong links in previous models. For example, previous models claimed that TOC1 was an activator of CCA1/LHY and now it is proposed that it is a repressor. This is valuable information, but the question is: if previous models got it wrong, what guarantees do we have that this model is correct? How do we know that the next version will not change everything again?

There are several problems with finding detailed models and drawing conclusions from them.

- 1) Lack of knowledge of hidden variables. At any given time, only part of a network components are

known and/or measured. Models are typically build with those variables and perhaps a few more to better explain the data. When new components are discovered they are added to the model, which means that existing parameters may change substantially. Conclusions drawn from properties of current models (such as repression, activation, network topology, etc) may be incorrect.

2) Large number of parameters. In most cases, data are not rich enough to correctly identify parameters for large dimensional systems, such as the one in the paper. What this means is that there are probably several parameter sets that fit the data. If they are enough far apart, then different parameters may lead to different models. Even finding suitable parameters is hard, since it typically involves solving a nonlinear optimization problem. Global optima parameters are almost impossible to find as the optimization tends to get stuck in local minimums. The set of parameters is very large and as the models get more complex the search for "good" parameters gets harder and harder. In this paper, several parameters are fixed from previous models. This facilitates finding other parameters. However, there is no guarantee that the fixed parameters are accurate. They were a good fit for a previous model, which does not mean it is a good fit for this model. Also, regarding the mutant data, unless they correspond to gene knockouts, it is not clear at what level parameters should be changed. Perhaps the corresponding parameters should also be variables. For *cop1*, *n5* was reduced? Why by 10%?

3) Data comes mostly from oscillations. When modeling oscillatory systems, measured signals tend to be oscillations. The problems is that, in the frequency domain, such signals have most of their energy centered at around  $1/T$ ,  $T=24h$ . Thus, there is little information at high and especially at low frequencies. Thus, models can only explain this particular range of frequencies and not the whole spectrum.

Along these lines, it may be true that *TOC1* is a repressor of *CCA1/LHY*. However, just because the existing model seems to favor this particular property, it is also possible that, by including other still unknown species, the model may change to be an activator. Or, it is also possible that there are other sets of parameters with a goof fit to the data where we have an activator. I find it very hard to believe one can decide activation versus repression solely based on oscillating data. To be sure, ideally we would need data where feedback loops were disrupted to remove oscillations in *TOC1* and *CCA1* and then *TOC1* expression would either be increased or decreased. The effect in *CCA1* would then be clear.

Finally, I think the word repressilator in the title is misleading. Repressilators are much simpler circuits which offer very little robustness. As stated in the main text, the network here is much more complex with additional feedback loops. Probably other networks that do not resemble the repressilator could be constructed by picking other components of the system. I suggest that the authors remove this analogy from the paper.

We are very grateful for the Editor's and reviewers' comments, which we address below, quoting both from the comments and from the revised paper.

### Editor's comments:

1. -- Model parameters. As outlined by the first two reviewers is will be important to acknowledge the limitations of the current model and current parameter estimates, and provide information on the relative confidence of various model aspects. In addition, some attempt should be made to demonstrate that the key conclusions arising from this analysis (e.g. a repressive role for TOC1) are robust to reasonable variation in the parameter values.

To address these points we have included two new sections "3f. Limitations of the model" and "3g. Parameter stability analysis" in the Supplementary information. The main text at the beginning of p. 5 notes: "*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.*". Section 3f. details the assumptions and other issues that affect our confidence in various aspects of the model. Section 3g. presents new analytical results for parameter sensitivity, showing that the present model is more robust than previous versions.

Also, we included a new paragraph about the estimation of parameter values and their confidence in the first paragraph on p.8 of the Supplementary information: "*The constrained parameters were estimated from timeseries data of the clock mRNAs or proteins: LHY/CCA1 ... Parameters, that were directly measured or estimated from the experimental data, namely  $n_4$ ,  $m_1$ ,  $m_2$ ,  $m_3$ ,  $m_4$ ,  $m_5$ ,  $m_{12}$ ,  $m_{14}$ ,  $m_{16}$ ,  $m_{20}$ ,  $m_{21}$ ,  $m_{25}$ ,  $m_{28}$ ,  $m_{34}$ ,  $m_{38}$ ,  $p_1$ ,  $p_2$ ,  $p_{22}$ ,  $p_{30}$ ,  $g_5$ ,  $g_6$ ,  $g_9$ ,  $g_{15}$ ,  $g_{16}$ ,  $a$ ,  $b$ ,  $e$ ,  $f$  have higher confidence than the rest of the parameters.*".

2. -- The Repressilator. The third reviewer found the functional relevance of the repressilator concept less than convincing given that this structure represents a selectively emphasized portion of a more complex network. Similarly, the second reviewer wonders whether there is evidence that the entire network actually contains subnetworks capable of independent oscillatory behavior. Additional conceptual clarification is needed here, and it may be necessary to reduce your claims or use more cautious language.

As we note in the reply to the reviewer 2, we agree that the whole network might represent one complex oscillator, and have modified the text, title and abstract to address this point. All references to morning and evening oscillators were removed, and we now comment on the complex, integrated structure in several places.

At the same time, there are two heuristic reasons to emphasize the presence of the repressilator structure. First, it provides a framework to re-interpret some long-standing molecular data on the plant clock, as we note in the Discussion. This helps to clarify results that were previously confusing, so we argue that it will benefit our understanding of this clock. Second, a similar repressilator structure was recently found in the mammalian clock circuit. This suggests that the repressilator might be a functionally relevant motif for clocks. The revised text therefore retains the repressilator concept, presented in measured terms.

3. The second reviewer also felt that a substantial effort should be made to make sure that this work is clear and accessible to scientists who are not familiar with the Arabidopsis circadian clock, a point we would like to emphasize given the broad readership of Molecular Systems Biology.

Following the suggestions of reviewer 2, we have re-written several parts of the paper to make the text easier to follow, especially in the introductory sections.

4. In addition, the editor notes the recent work by Lau et al. (2011, Mol Cell). While this work does not appear to impinge on the novelty of the findings reported here, this work and its implications for the current model should be discussed.

We now comment on this interesting paper in point 6 of the section “Limitations of the model” on p. 11 of the Supplementary information:

*“Very recent data suggest the involvement of additional proteins, such as DET1, in the regulation of gene expression by LHY and CCA1 (Lau et al, 2011). Further biochemical characterization of the kinetics of DET1 activity, its co-regulators, and their modulation by light and the clock will be necessary in order to include them in future clock models.”*

Reviewer #1 (Remarks to the Author):

Figure 1 is particularly helpful in illustrating the similarities and differences between the two models (especially for non-Arabidopsis-experts like myself) and the authors should consider directing the reader's attention to this figure near the beginning of the description on pages 2-4, rather than at the end.

We appreciate the suggestion, and have done so by including the following sentences at the beginning of p. 3:

*“The clock was represented by a three-loop structure of interconnected morning and evening loops (Figure 1 upper right).”*

and on p.4 (second paragraph):

*“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 (Yu et al, 2008) (Figure 1, see Results for further detail).”*

Minor Concerns:

1. One thing that might improve the conclusion of the paper would be a brief paragraph assessing the authors' confidence in various aspects of the model. The choices made were clearly motivated by experimental evidence and by improving the model's agreement with observed behaviour, but some of those choices must have been made more confidently than others. Providing readers with a sense of which areas are less-than-completely established will likely aid in future refinements and improvements. On a related note, Supplementary Table 1 contains values that had been fit to multiple datasets, but doesn't convey a sense of the uncertainty in these fitted values, or of which parameters were better-constrained by the data than others. A little more description of the parameter-fitting procedure would be helpful,

along with something to convey a sense of the uncertainty in the fitted parameters and (more importantly) the simulated model output.

We addressed these points in full, by including two new sections in the Supplementary information, “3f. Limitations of the model” and “3g. Parameter stability analysis”, as described above in response to Editor’s comment 1.

2. One thing that might improve the generality of the paper would be a brief paragraph discussing the repressilator structure found in circadian clocks of other organisms. A similar repressilator structure has been also found in mammalian circadian clocks (Ukai-Tadenuma M et al, Cell 144(2):268-81 (2011), Hogenesch JB et al, Nature Reviews Genetics Jun;12(6):407-16 (2011)). Some discussion of the generality and biological relevance of this structure in circadian clock might be useful for the readers.

We agree that these are interesting and relevant results, which we now discuss on p.16:

*“A repressilator, the three-inhibitor ring oscillator, was first constructed as a synthetic circuit in E. coli (Elowitz & 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 7D). Interestingly, a similar repressilator structure was recently found in the mammalian clock, where it also represents only part of the system (Hogenesch & 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.”*

A few (more minor) comments on the text itself:

On page 14: "We demonstrated computationally, that mutation of the EC components resulted in the decrease of the LHY/CCA1 amplitude (Figure 5A), in agreement with the experimental findings." The first comma is unnecessary: "We demonstrated computationally that mutation of the EC components..."

On page 15: "This double negative connection can also be represented by a positive regulation of PRR expression by LHY/CCA1 (Figure 9B)," I think the authors mean Figure 8B.

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Looks like Gould and Mizoguchi are there; Yakir is missing.

Supplementary materials, page 14: "The detailed kinetic measurements of the activity and abundance of the protein complexes in the plant clock, such as COP1 and GI protein complexes are awaiting for the future elucidation." - should be "...await future elucidation."

All these corrections were done as suggested.



## Reviewer #2 (Remarks to the Author):

However, the current version of the manuscript is not easy to read by a scientist not familiar with the Arabidopsis clock and previous papers such as P2010, L2005 and L2006. The authors should reduce the amount of specialized information and should focus on fewer messages. In particular, I suggest removal of many of the gene names from the text. The link between abbreviations and genes names might be provided in footnotes, in a separate table or in the supplement.

In response to this reviewer's suggestion, which was stressed in Editor's comment 3, we re-wrote the paper by simplifying and re-structuring the text to make it easier to follow. All abbreviations are now included in a new Glossary on p.2.

At the end of the introduction 4 aims are listed (EC, GI, PRR9, TOC1). In the results and the discussion these topics are indeed studied in detail but the non-expert has problems to follow all the lines of thought. I suggest to reduce the aims as much as possible (perhaps 2-3), to enumerate them, and to construct Results and Discussion according to these aims. Some redundancy is helpful, side remarks should be reduced as much as possible.

We revised the text according to these comments, leaving two aims: the structure of the evening loop and its connection to the rest of the clock. We also simplified the logic and removed some of the unnecessary detail for clarity.

The complete model is conceptually a combination of 3 oscillators: morning, evening and repressilator. Thus in principal independent oscillations with different periods, tori and chaos might be expected. Is there any evidence of bifurcations of coupled oscillators? Otherwise all the loops constitute just one complex oscillator and the terms morning oscillator, evening oscillator and repressilator should be used with care.

We agree that the whole clock structure might represent one complex oscillator, and we say so in the text, as described above (it is particularly stressed in the discussion as cited below). Indeed, the intact structure provides robust free-running oscillations. The evening loop demonstrates only damping oscillations (in the *lhy-cca1*- double mutant). The morning loop does not provide autonomous oscillations (the single mutants of EC genes are arrhythmic, as discussed in the new introduction and discussion). And we have never observed tori or chaos in this model with realistic parameters. Accordingly, we removed references to the morning and evening oscillators, throughout the paper.

We commented on the complex clock structure in several places, including p.14: *"The new model recapitulates the more severe circadian phenotypes of EC gene mutants under constant light conditions (Doyle et al, 2002; Hazen et al, 2005; Reed et al, 2000) 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 5), 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 stabilized by coupling to the morning loop in the intact system."*

However, as we note in response to comment 2 of reviewer 1, the presence of the repressilator structure in the mammalian clock also suggests that this might have functional relevance, as described above.

I am aware that the available data do not allow the construction of a perfect quantitative model. For example, details of the transcriptional regulations or of enzymatic degradations are not known and thus Hill coefficients of 2 and in most cases linear degradation kinetics are assumed. Thus even after careful parameter fitting the model is plausible, explains many data, but it cannot be regarded as a precise quantitative model. Consequently, it would be appropriate to stress at some point the limitations of the model.

We agree that the model does not describe in detail the precise molecular mechanism of reactions because current knowledge does not allow this. Moreover, the mechanisms of some connections are not clear yet. We discussed this in more detail in the new section “3f. Limitations of the model” of the Supplementary information.

Specific comments:

1. Interesting title! Are there also repressilator structures in other model organisms (Neurospora, Drosophila, mammals)?

As outlined above, we note in the Discussion that a repressilator structure was recently identified in the mammalian clock system.

2. The discussion of flexibility and robustness is vague. Without quantifying these concepts a comparison of simple feedbacks and multiple loops is difficult. I can also imagine that a simple delayed negative feedback is somehow robust.

We removed the references to flexibility and robustness from the introduction and also included new analysis of the robustness of the model to parameter variations in a new section “3g. Parameter stability analysis” of the Supplementary information.

3. Some of the motivations in the introduction are technical (removal of X and Y, role for ELF3, ELF4, LUX). What are the open biological issues requesting a new model? What are the major discrepancies to the data justifying a novel design? I am aware that these questions are implicitly addressed. Thus no extension of the text is necessary but focussing on biological insight instead of technical improvement.

The Editor’s comment 3 also stresses this point and we re-wrote the paper as described above, emphasizing the biological questions and minimizing technical details.

4. I miss in the main text a short description of the core properties of the model (number of equations, parameters, nonlinearities). I appreciate that few and mild nonlinearities were sufficient. Is the saturated degradation kinetics in 2 cases experimentally justified or is it just used to avoid larger Hill coefficients?

We appreciate the suggestion and have extended the description of the model in the main text at the beginning of the Results:

*“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 1). 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 discussion of the model’s limitations and its robustness to parameter variations”*

All degradation rates in the model are linearly dependent on their substrate concentrations. The reviewer likely refers to the modification of LHY/CCA1 protein (eq.2-3) and the modulation of EC degradation by GI (eq. 24). The Hill coefficient of 2 for modification of LHY/CCA1 protein (parameter  $c$ ) is based on the dimerization data for this protein (Yakir et al. 2009; O’Neill et al. 2011). It was already present in our earlier model (as noted in Table1). The regulation of EC by GI is much less studied. It is mentioned in point 10 of Supplementary Information section “3f. Limitations of the model”. We now comment on this Hill coefficient on p 8 (second paragraph) of the Supplementary information:

*“The Hill coefficient for the modulation of the EC degradation by GI ( $d$ ) was set to 2 for simplicity.”*

5. Fig.2 (data and interpretation) is very clear. For Fig.3 and its message (GI) a new subsection might be justified to structure the lengthy text.

To address this point, we restructured the text. GI is described in a new section “2b. Regulation of the EC activity by COP1 and GI” on p.8.

6. There is no Figure 9B.

We corrected this reference.

7. If reduction is necessary - Fig. 5 is not urgent. There is no direct comparison with data and the effect might be described in words (and the figure might be shown in the supplement).

We would agree, but Fig.5 corresponds to published data and provides a new interpretation, which will be interesting for the circadian field.

8. Figure addresses an interesting issue (phases for different photoperiods) but it is a bit at the periphery of the manuscript. Moreover, the differences between models and data are comparable to the yearly variation and thus difficult to interpret.

Indeed, we have moved former Figure 7 and the associated text into the Supplementary information.

9. Figure 8 is a good summary. However, the meaning of A-C is not really clear to me. I see the same information in Figure 8 D.

The schematic timeseries in panels A-C on this figure are intended for experimental biologists, to illustrate our new interpretation of the clock circuit. Such readers may

be less expert than the reviewer in inferring expression profiles from the circuit diagram in panel D, so we retain these panels.

### Reply to Reviewer #3

I believe that the work from these authors is very important for the community of plant circadian clocks as it provides a starting point for many researchers. With such models, one can make hypothesis and quickly test them by simulating the system. Hence, these results should be published and available to others. However, this paper feels more like an updated version of some software and so the question is where should it be published.

The Editor's comment 3 stresses this point. We have rewritten substantial sections of the paper to address it, as described above. Notably, the previous models lacked the EC genes (ELF3/ELF4/LUX), because there had been no experimental evidence to identify their targets. Experimental work from our own and other laboratories now allows us to incorporate these genes for the first time. It is clear that they are essential for clock function, because mutation of any of them results in arrhythmicity in the *elf3*, *elf4* or *lux* single mutants. All other single mutants of the clock genes are rhythmic, as we discuss in the introduction. Our combination of computational and experimental approaches to integrate these recent advances brings significant new understanding, which reconciles the new data with the previous concepts of the clock. The new clock structure is then able to describe both new and published data, resolving previous paradoxes by identifying TOC1 as a repressor.

My major concern with this paper is that this is simply an update of existing models. As with other updates, this new model concludes that there were missing species and wrong links in previous models. For example, previous models claimed that TOC1 was an activator of CCA1/LHY and now it is proposed that it is a repressor. This is valuable information, but the question is: if previous models got it wrong, what guarantees do we have that this model is correct? How do we know that the next version will not change everything again?

Our work is based on very recent experimental results, which necessarily drive the field to revise its understanding. In the case of TOC1, we first noticed that our earlier model disagreed with recent data on the *ztl* mutant (Baudry et al, 2010). The next logical step was that, in terms of protein structure, TOC1 (PRR1) is one of the PRR gene family, which were known to function as inhibitors of *LHY* and *CCA1*. So we changed the sign of TOC1 action in the model and were surprised to find that this improved not only description of the *ztl* mutant, but also of the *prr9 prr7* double mutant, which we were struggling to describe in the previous model. Very recently, we have preliminary indication of TOC1's inhibitory function from more direct experiments: the details of this inhibition are a subject of our future studies. Thus our model reflects rapid progress in experimental studies of the plant clock. Importantly, integrating the results in the form of the model provides experimentalists with a tool to design future investigations (our previous models were likewise proven to be useful in stimulating new experiments).

There are several problems with finding detailed models and drawing conclusions from them.

1) Lack of knowledge of hidden variables. At any given time, only part of a network components are known and/or measured. Models are typically build with those variables and perhaps a few more to better explain the data. When new components are discovered they are added to the model, which means that existing parameters may change substantially. Conclusions drawn from properties of current models (such as repression, activation, network topology, etc) may be incorrect.

We agree that hidden variables affect the structure. However, this work substantially decreased the number of hidden variables and increased the model's robustness to parameter variation. We added related a comment at the end of p.13 (end of the first paragraph):

*"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."*

We agree that there are always uncertainties, which come from the experimental field, but modelling helps to address them. It is difficult to predict the behaviour of a system with multiple feedback loops without having at least hypothetical structure: this uncertainty can lead to unnecessary experiments. We are encouraged that our current model, although unpublished, is already in use by some of our collaborators. This is dynamic process: models help to plan experiments and new data change the models. The proposed structure of the clock is a more realistic basis for this research than the previous models, as explained in the text.

2) Large number of parameters. In most cases, data are not rich enough to correctly identify parameters for large dimensional systems, such as the one in the paper. What this means is that there are probably several parameter sets that fit the data. If they are enough far apart, then different parameters may lead to different models. Even finding suitable parameters is hard, since it typically involves solving a nonlinear optimization problem. Global optima parameters are almost impossible to find as the optimization tends to get stuck in local minimums. The set of parameters is very large and as the models get more complex the search for "good" parameters gets harder and harder. In this paper, several parameters are fixed from previous models. This facilitates finding other parameters. However, there is no guarantee that the fixed parameters are accurate. They were a good fit for a previous model, which does not mean it is a good fit for this model.

We agree that uncertainties in parameter values of big models can be an issue, and we address this in response to Editor's comment 1 (please see above). However, there are different kinds of big models. In some cases the biochemical pathways and the structure of the system are more or less well known, so only the regulatory steps and parameter values are in question. But in many cases like ours, the structure of the system is unknown. This presents a higher level of complexity. For such systems the absolute parameter values may bring little information about the behaviour of the whole system. This was discussed, for example, in Gutenkunst et al., PLoS Comp. Biol. 2007, p.1871, where the authors suggested based on analysis of many different

published models that collective fitting of model behaviours to multiple timeseries datasets is more informative at this stage than the precise values of parameters.

We used some parameter values from the previous model because they provided a match for the data in the current model also, but they were not fixed during the fitting. As outlined in response to Editor's comment 1, we described the parameter values in more detail and also corrected Table 1 by giving specific references to the relevant data that were used to constrain these parameters. In addition, a few parameters were directly measured or estimated in our previous paper from timeseries data.

Also, regarding the mutant data, unless they correspond to gene knockouts, it is not clear at what level parameters should be changed. Perhaps the corresponding parameters should also be variables. For *cop1*, *n5* was reduced? Why by 10%?

Only one non-null mutant was used, *cop1-4*, because null *cop1* mutant is lethal. Yes, we varied *n5*. We described this in more detail on p. 8 of the Supplementary Information:

*"Mutations of the clock genes were simulated by decreasing the rate of the genes transcription to zero for all the null mutants used in our study. The only non-null mutant we used was cop1, which was simulated by reducing the COP1 transcription rate n5. Values of n5 in the range of 1-30% of its WT value gave very similar simulated circadian periods in constant light conditions, between 22.4 and 22.8 h. We chose to use 10% of the WT value of n5, which matches the level of COP1 protein in the viable cop1-4 mutant allele (McNellis et al, 1994)".*

3) Data comes mostly from oscillations. When modeling oscillatory systems, measured signals tend to be oscillations. The problem is that, in the frequency domain, such signals have most of their energy centered at around  $1/T$ ,  $T=24h$ . Thus, there is little information at high and especially at low frequencies. Thus, models can only explain this particular range of frequencies and not the whole spectrum.

We agree with this comment. However the  $1/24h$  frequency has crucial evolutionary significance for the clock. Mutations that perturb the clock cause only slight deviations from the 24h period (up to 6 hours), indicating the stability of the system's frequency.

Along these lines, it may be true that TOC1 is a repressor of CCA1/LHY. However, just because the existing model seems to favor this particular property, it is also possible that, by including other still unknown species, the model may change to be an activator. Or, it is also possible that there are other sets of parameters with a good fit to the data where we have an activator. I find it very hard to believe one can decide activation versus repression solely based on oscillating data. To be sure, ideally we would need data where feedback loops were disrupted to remove oscillations in TOC1 and CCA1 and then TOC1 expression would either be increased or decreased. The effect in CCA1 would then be clear.

We agree that arrhythmic lines are useful but they are rare. We have previously expended much effort in screening for such mutants. The multiple-loop structure of

the clock explains this, so that only simultaneous disruption of several parts of the system results in arrhythmicity. At the moment we continue to cross the arrhythmic mutants with other clock mutants to investigate detail mechanisms of the new connections in the clock (as we did in Dixon et. al. 2011, for the *lhy/cca1/elf3* triple mutant).

Finally, I think the word repressilator in the title is misleading. Repressilators are much simpler circuits which offer very little robustness. As stated in the main text, the network here is much more complex with additional feedback loops. Probably other networks that do not resemble the repressilator could be constructed by picking other components of the system. I suggest that the authors remove this analogy from the paper.

The Editor's comment 2 also stressed this point, which we addressed as described above.

2nd Editorial Decision

09 November 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your revised manuscript. As you will see from the referees' reports, the third reviewer has important remaining concerns with your work that we feel are substantial enough to preclude publication of this manuscript.

The key remaining issue is the conclusiveness of the demonstration that TOC1 acts to repress, rather than activate, morning genes. Reviewer #3 argues that it is impossible to determine whether TOC1 acts as a repressor or activator from oscillatory data (this reviewer's report is attached as a pdf file). S/he presents a simple example of the difficulty in assessing activation or repression from two oscillatory patterns, showing that altering assumptions about the delay in TOC1 action can flip the apparent sign of TOC1's activity. The model fitting performed in your work is clearly much more complex than this simple example: you show that changing TOC1's sign improves the ability of the model to fit a variety of previous experimental results and allows you to remove the hypothetical component X. Nonetheless, reviewer #3 is not convinced that these results provide convincing evidence regarding the actual biological activity of TOC1, and his/her comments suggest that this improvement in model performance could result from a variety of potentially arbitrary conditions in the complex nonlinear optimization process. Given that the proposed change in TOC1 activity represents a key point of novelty in this work, and is the main evidence supporting the ability of this model to generate new mechanistic insights into the Arabidopsis circadian clock system, we feel that this issue is of fundamental importance, and is sufficient to prevent publication of this work at Molecular Systems Biology.

In general, journal policy only allows a single round of major revision; however, given the positive assessments by the other two reviewers, and our hope that you may be able to address this issue, we would like to offer you an additional chance to revise this work. Any revised work would need to provide further, convincing support for the biological relevance of the proposed TOC1 repression of morning gene expression. Naturally, the most direct way to address this issue would be with new experimental evidence demonstrating TOC1 repressive activity. We note that you have written that you have obtained preliminary results in this direction. Full elucidation of the molecular mechanism underlying the repressive activity would certainly lie outside the scope of this work, but any supportive experimental evidence would certainly be very helpful in addressing this important issue.

The third reviewer is also still concerned about the repressilator concept, but in this regard we do not feel that additional revision of the manuscript is needed. We note that the other two reviewers clearly found the repressilator structure embedded in the Arabidopsis clock to be very interesting. Given the recent observations of a repressilator structure in the mammalian clock, we agree that it is appropriate to highlight this motif.

If you feel you can satisfactorily deal with this issue you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have altered the manuscript in response to the reviewers' concerns. We reserve the right to send any revised work back to some or all of the reviewers, but we will make every effort to expedite the process as much as possible. As you probably understand, we can give you no guarantee at this stage that the eventual outcome will be favorable.

Sincerely,

Editor - Molecular Systems Biology  
msb@embo.org



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Referee reports

## Reviewer #1 (Remarks to the Author):

My major concerns with the manuscript have been addressed well. The new version of the paper is well-structured, and I think the authors were wise to re-focus the paper around outstanding biological questions and emphasize experimental data throughout. The addition of information about model limitations and parameter sensitivity to the supplementary information add much-needed context to the claims being presented. Emphasizing the repressilator as a clock design motif (as in Figure 7D) sets up important points of contact with other biological clock systems, and I highly recommend the publication of this manuscript in *Molecular Systems Biology*.

I only have an even minor concern (a little bit editorial one). The manuscript in the current form may be acceptable, but it will be improved if the following point is addressed.

## Minor Minor Comment:

The only remaining suggestion is with the discussion of the PRC calculations in section 4. I had to read this section over a few times before I really felt like I understood it; a large part of the reason is that "acute light response" seemed never to be defined in the main text. The explanation in the supplementary information (light-induced transcriptional activation by an as-yet-unidentified dark-accumulating activator) made this much clearer; defining "acute light response" somewhere near the beginning of section 4 would help dramatically. This is somewhat different from the ways in which the transcriptional effects of light have been modeled in mammals, for example. It might also help to emphasize that, although light acts on the system at numerous sites (as illustrated in Figure 1), the dominant contribution to the PRC (though not necessarily to light response generally, as is clear from Fig. 6D) comes from the light-induced activation of LHY/CCA1.

## Reviewer #2 (Remarks to the Author):

The paper has been revised appropriately.

## Reviewer #3 (Remarks to the Author):

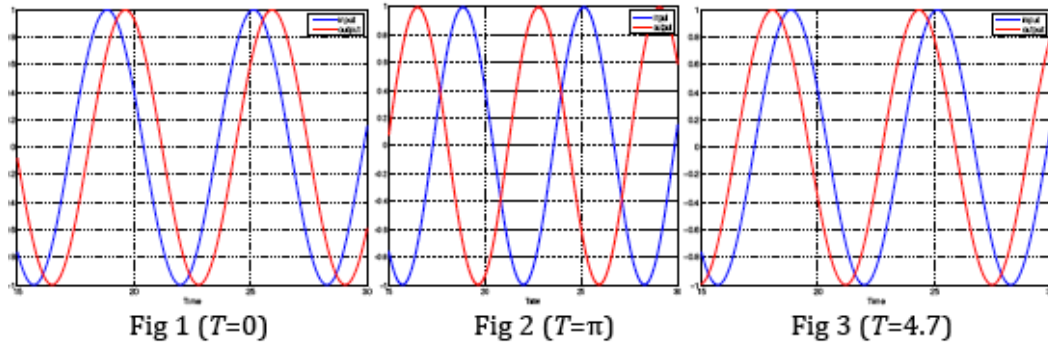
Please see pdf file sent by email

The authors have made some changes to the paper but most of my comments have not been addressed. Thus, I cannot recommend that the paper be accepted. The authors emphasize that the EC genes were not present in previous models and that TOC1 is a repressor. Furthermore, they claim, “Our combination of computational and experimental approaches to integrate these recent advances brings *significant* new understanding”. While there is an improvement of the mathematical model by including the EC genes, it is nevertheless just an updated version of previous models (and surely not the last). This is an increment of past work, which I do not see as a significant step in understanding. As for the result that TOC1 is a repressor, I am convinced that there is not enough information in this data to make such a conclusion. Finally, I still do not agree with the analogy to a repressilator.

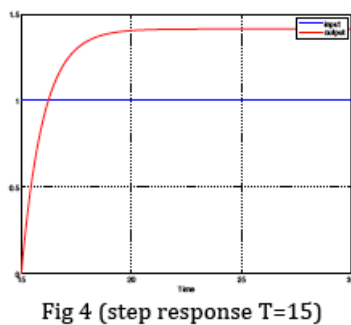
*“So we changed the sign of TOC1 action in the model and were surprised to find that this improved not only description of the ztl mutant, but also of the prr9 prr7 double mutant, which we were struggling to describe in the previous model.”*

As I explained before, there are many reasons why such a change in sign would improve some modeling aspects. First there is the fact this is a nonlinear optimization problem. Second there are hidden states and so parameters adjust to fit the data as best as possible to counteract the missing states, possibly providing very different values and leading to wrong conclusions. Third, and possibly more important, from oscillation data is not possible to conclude about activation or repression. I will illustrate that with an example.

Take a very simple linear system (one can think of a linear system as a linearization of a nonlinear system) give by its transfer function  $1/(s+1)$ . In this case, the “1” in the numerator is positive so it refers to an activation. Note that the Hill coefficient  $h$  in the nonlinear system would appear in the numerator of the linear system transfer function (just take the derivative at an equilibrium point). This linear system does not obviously represent the oscillator (as this needs to be nonlinear) but it can represent the map from some specie  $u$  to another specie  $y$  in the oscillator. Assume also that the input  $u$  is delayed but some time  $T$ . The delay can be a pure delay or be a simplified representation of a complex (hidden) system. If desired, one could approximate the delay with an arbitrary dimensional linear system. We also scale the system by a constant  $\sqrt{2}$  to make it easier to observe the responses. So, the overall system is  $\sqrt{2} \cdot \exp(-sT)/(s+1)$ . Now, by varying  $T$ , one can be led to make different conclusions about the activation/repression nature of the system. Lets simulate the system and observe it after all transients have disappeared for  $T=0, \pi, 4.7$ . When  $T=0$ , it seems to suggest an activation (Fig 1). In fact, the output follows the input with a small lag. However, for  $T=\pi$ , the data seems now to suggest exactly the opposite. It feels like it is a repression, which is wrong. Moreover, the simulation in Fig 2 matches exactly that of the system  $-\sqrt{2}/(s+1)$ , which is a repressor. Hence, there is no way to know for sure which one is it in the presence of delays or hidden species. Finally, Fig 3 is for  $T=4.7$  which now seems to suggest that the output is activating the input.



In fact, by varying  $T$  I could have placed the output anywhere with respect to the input. Technically, this is explained by the fact that the delay adds phase to the Bode plot. Now, if I could excite the d.c. frequency with for example a step I would have known immediately whether I had an activation or repression, independently of  $T$  (see Fig 4). If the output is positive in steady state then it is activation (as in Fig 4) and if the output is negative in steady state then it is a repressor.



The problem is that the data are oscillatory and contains very little information at zero frequency. Ways to get around that include disrupting feedback loops or consider cases where TOC1 goes arrhythmic and then somehow change the concentration of TOC1.

*Very recently, we have preliminary indication of TOC1's inhibitory function from more direct experiments: the details of this inhibition are a subject of our future studies.*

I then suggest then that a complete study is conducted that perhaps includes some of the suggestions above before publishing these preliminary results.

*We agree with this comment. However the 1/24h frequency has crucial evolutionary significance for the clock. Mutations that perturb the clock cause only slight deviations from the 24h period (up to 6 hours), indicating the stability of the system's frequency.*

I think my point here was not made clear. I was refereeing to modeling and not to robustness of the oscillations. As the above example shows, it is very hard to fit some system parameters when data has most of their energy around the 1/24h frequency. Finally, the title still includes the word "repressilator" and this

is still one of the major aspects of the paper. As I explained in the first review, I disagree with such a strong analogy. Perhaps at the most one small paragraph at the end of the paper could mention that at some level the system could be represented as a repressilator.

## Reply to reviewer comments

We are very grateful for the Editor's and reviewers' further comments, which we address below, quoting both from the comments and *from the revised paper*.

## Editor and Reviewer #3's Major Comments

1. *Given that the proposed change in TOC1 activity represents a key point of novelty in this work, and is the main evidence supporting the ability of this model to generate new mechanistic insights into the Arabidopsis circadian clock system, we feel that this issue is of fundamental importance ....*

The change in the sign of TOC1 function is indeed a novel aspect of the model that we do not wish to diminish. It will attract attention due to its revision of previous understanding in the field. However, we stress that the major novelty and source of new mechanistic insight come from our inclusion of three additional clock genes (ELF3, ELF4 and LUX), for the first time. It is the formation of a new repressor by their products (the Evening Complex, EC) and the known light regulation of the ELF3 protein that allow us to revise the model's circuit. Including the EC is unquestionably novel. The experiments that identified the EC were published only a month before our submission, whereas TOC1's original function was proposed a full decade earlier.

Including the EC gives both mechanistic and conceptual advances. To see the EC's importance, consider that our previous model (Pokhilko et al., MSB 2010) included three PRR proteins that inhibited *LHY/CCA1* transcription (PRR9, 7 and the NI). Their homologue TOC1 now extends this function that was already present. In contrast, the EC introduces entirely new connections among the clock components and to light inputs. These increase the new model's repertoire of dynamic behaviour, matching further the experimental data and leading to our major mechanistic insights (Figures 2, 3, 6 and Supplementary Figures 5, 7, 11), including the first accurate explanation for the observed phase response curve (Figure 7). Feedback of the EC to inhibit expression of the EC genes provides a mechanistic basis for the evening loop. The known light regulation of ELF3 protein allows us to revise the light inputs to the evening loop, and to introduce for the first time the mechanism of light regulation by the COP1 protein degradation system. Together, these allow us to remove *Y* from the model. *Y* was a cornerstone of previous models, with four regulatory connections including a strong light input. Removing *Y* is both a mechanistic and a conceptual advance. Likewise including the EC's known regulation of *PRR9*, which revealed the repressilator motif.

The change of TOC1 sign allows us to remove *X* from the model. This has obvious heuristic benefits from simplifying both the model and also has practical effects as experiments to find *X* are no longer required. However, the only function of *X* in the earlier models was to provide a delay before TOC1 activated *LHY/CCA1*. *X* had no light input and no other regulatory connections, so it was less important than *Y*.

The change of TOC1 sign also simplifies the interpretation of PRR protein function: all are now repressors, rather than TOC1 being the unique activator. The model with the altered sign is simpler, matches more experimental data than the earlier ones, and resolves apparently paradoxical results (such as the lower *LHY/CCA1* expression under LL in both the *toc1* mutant and the TOC1 overexpressor). Thus the change in the sign of TOC1 can be justified by Occam's razor alone, shifting the 'burden of proof' to hypotheses that would keep TOC1 as an activator. Nevertheless, the revised manuscript includes new experimental data that support the change in sign.

2. *Any revised work would need to provide further, convincing support for the biological relevance of the proposed TOC1 repression of morning gene expression.*

To address this, we performed additional experiments and simulations, shown in new Figure 5. Such experiments face the major difficulty that TOC1 is only one of at least four PRR's that inhibit *LHY*

and *CCA1* expression (the fifth, PRR3, may be cell-type-specific). This leads to the prediction that the effect of TOC1 on the level of *LHY* and *CCA1* expression will be most easily detectable over a limited time interval at the end of the night. The comparison of *LHY* and *CCA1* expression levels between the *TOC1* overexpressing line and *toc1* mutants over the relevant time interval clearly demonstrated the negative effect of TOC1 on *LHY* and *CCA1* expression, as explained in the revised text at the end of p.11:

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. Figures 5A,B show that *LHY* and *CCA1* mRNA levels rise more slowly in the TOC1-ox plants compared to WT, whereas the rise of *CCA1* is accelerated in the *toc1* mutant. The model simulations of TOC1-ox and *toc1* matched our experimental observations (Figure 5C), supporting the proposed repressive function of TOC1 towards *LHY* and *CCA1* expression.

Thus multiple lines of evidence including our new data support the negative sign of TOC1 in the clock. However, the exact mechanisms of the regulation of *LHY* and *CCA1* by TOC1 remain a subject for further studies, as we noted in the text at the end of p. 11:

*This change compared to 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.*

Reviewer #1 Minor Comment:

*The only remaining suggestion is with the discussion of the PRC calculations in section 4. I had to read this section over a few times before I really felt like I understood it; a large part of the reason is that "acute light response" seemed never to be defined in the main text. The explanation in the supplementary information (light-induced transcriptional activation by an as-yet-unidentified dark-accumulating activator) made this much clearer; defining "acute light response" somewhere near the beginning of section 4 would help dramatically. This is somewhat different from the ways in which the transcriptional effects of light have been modeled in mammals, for example. It might also help to emphasize that, although light acts on the system at numerous sites (as illustrated in Figure 1), the dominant contribution to the PRC (though not necessarily to light response generally, as is clear from Fig. 6D) comes from the light-induced activation of *LHY/CCA1*.*

To address this point, we amended the text as suggested on p. 14:

“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 (Locke 05, Kim 03).”

Acceptance letter

13 February 2012

Thank you again for sending us your revised manuscript. We are now satisfied with the modifications made and I am pleased to inform you that your paper has been accepted for publication.

Before we can send this work to production we request that you provide a new Supplementary Information pdf file with some minor changes:

- In the Table of Contents at the beginning of this document, please also list the SBML model file and Supp. Table 1, listing them as "separate files".

- Please incorporate the *Toc1*-ox description you previously provided into this document.

We did note the recently published work by Gendron et al, and, naturally, we will make every effort to see that your work is published in an expedited fashion. If you would like to add a reference to the work by Gendron et al, you may provide an updated main document now -- this could save time over making changes during the proofing stage

Thank you very much for submitting your work to Molecular Systems Biology.

Sincerely,

Editor - Molecular Systems Biology  
msb@embo.org

Reviewer #5 (Remarks to the Author):

The authors present a new model of the Arabidopsis clock. This model represents a considerable advance on previous versions as it incorporates three new components, ELF3, ELF4 and LUX, which together form the Evening Complex. This new model also addresses one of the glaring inconsistencies in our earlier conceptions of the Arabidopsis clock. Genetic studies had suggested TOC1 as an activator of LHY/CCA1. However, more recent work from Baudry et al (2010) showed that TOC1 expression increases in *ztl* mutants, which have a long period phenotype. At the same time, LHY and CCA1 expression in *ztl* plants shows reduced amplitude. This 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.

In addition, emerging biochemical characterization of PRR5, PRR7, and PRR9 has established them as DNA binding proteins that repress TOC1 transcription. It seemed contradictory to suggest TOC1 as an activator when its close homologs were repressors. Recasting TOC1 as a repressor reconciled these contradictions.

However this recasting of TOC1 required experimental support. Accordingly, the authors measured CCA1 and LHY mRNA accumulation in *toc1* mutant and TOC1-ox plants. In each case, overexpression of TOC1 reduced the accumulation of CCA1 and LHY mRNA in the pre-dawn hours (Figure 5). Loss of *toc1* function resulted in more rapid and greater accumulation of CCA1 (although not of LHY) mRNA (Figure 5). Thus, both loss and gain of TOC1 function affected CCA1 and LHY mRNA accumulation in a manner consistent with TOC1 serving as a repressor of CCA1 and LHY expression. This provides satisfying experimental validation of the revised role of TOC1 in their model.