Mathematical modeling of an oscillating gene circuit to unravel the circadian clock network of Arabidopsis thaliana

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The Arabidopsis thaliana circadian clock is an interconnected network highly tractable to systems approaches. Most elements in the transcriptional-translational oscillator were identified by genetic means and the expression of clock genes in various mutants led to the founding hypothesis of a positive-negative feedback loop being the core clock. The identification of additional clock genes beyond those defined in the core led to the use of systems approaches to decipher this angiosperm oscillator circuit. Kinetic modeling was first used to explain periodicity effects of various circadian mutants. This conformed in a flexible way to experimental details. Such observations allowed a recursive use of hypothesis generating from modeling, followed by experimental corroboration. More recently, the biochemical finding of new description of a DNA-binding activity for one class of clock components directed improvements in feature generation, one of which revealed that the core of the oscillator is a negative-negative feedback loop. The recursive use of modeling and experimental validation has thus revealed many essential transcriptional components that drive negative arms in the circadian oscillator. What awaits is to more fully describe the positive arms and an understanding of how additional pathways converge on the clock.

Keywords: Arabidopsis thaliana, circadian clock, mathematical modeling, light signal transduction, temperature acclimation, hormone signal integration, metabolic signal integration, stress signal integration
FIGURE 1 | Graphical outline of the mathematical Arabidopsis thaliana clock models in historical order, showing the development from a simple positive-negative feedback model (Locke et al., 2005a) toward more complicated interconnected feedback loops (Locke/Zellinger 2006 model; Locke et al., 2006; Zeilinger et al., 2006). These models took into account the period lengthening and shortening behavior of mutations in genes defined in these models, and were often capable of recapitulating the transcript misexpression levels of genes in the clock in various reciprocal mutant combinations. Both models came to similar conclusions of how the multiple interconnected feedback loops are constructed (Locke/Zellinger 2006 model; Figure 1; Locke et al., 2006; Zeilinger et al., 2006). These working hypotheses were predictive for future experiments, and subsequent molecular-genetic tests have often conformed to mathematical predictions. One key observation rhythms (Millar et al., 1995; Scarpini et al., 1999). By analyzing circadian parameters of mutants, genetic approaches have uncovered a number of genes in the clock, and these will be described below.

THE Arabidopsis thaliana CLOCK MODELS

The first mutants defective in clock function provided a platform toward understanding the components of this oscillating gene network (Millar et al., 1995). One break-through in this approach led to the hypothesis that the morning acting clock genes CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY; Schaffer et al., 1998; Wang and Tobin, 1998) repress the evening acting transcriptional regulator TIME OF CAB EXPRESSION (TOC1; Strayer et al., 2000), which was then found to activate CCA1 and LHY expression (Alabadi et al., 2001). This defined a so-called negative-positive feedback loop as the core oscillator (graphically illustrated in the Locke et al., 2005a model; Figure 1). Early genetic models of the higher plant clock realized the lack of construction features of this oscillator (McWatters et al., 2001; Staiger, 2002). Mathematical modeling of this network revealed that the clock network must be more complicated (Locke et al., 2005a). Nevertheless, this “one-loop” model provided a critical conceptual framework that guided a decade of molecular-genetics research. From this core-loop hypothesis, many other clock genes were described and placed to the clock circuitry.

Experimental data on the clock accumulated rapidly and exceeded the conceptual capacity to “understand” the network. Several landmark papers in systems biology resolved this dilemma. Firstly, it was mathematically hypothesized that the oscillator is composed of interconnected loops (Locke et al., 2005b model; Figure 1). Then work from the Doyle and Millar groups separately defined similar kinetic models that incorporated most available molecular-genetic data (Locke et al., 2006; Zeilinger et al., 2006). These models took into account the period lengthening and shortening behavior of mutations in genes defined in these models, and were often capable of recapitulating the transcript misexpression levels of genes in the clock in various reciprocal mutant combinations. Both models came to similar conclusions of how the multiple interconnected feedback loops are constructed (Locke/Zellinger 2006 model; Figure 1; Locke et al., 2006; Zeilinger et al., 2006). These working hypotheses were predictive for future experiments, and subsequent molecular-genetic tests have often conformed to mathematical predictions. One key observation...
predicted was that the cca1 lhy toc1 triple mutant, which lacks the core oscillator, would be arrhythmic. That was indeed experimentally observed (Ding et al., 2007; Ito et al., 2007b). Taken together, the seminal hypothesis of Doyle and Millar that the oscillator is a set of interconnected feedback loops defined for the first time a rational view of how plants tell daily time (Locke et al., 2006; Zeilinger et al., 2006). These explicit mathematical hypotheses have largely stood experimental tests (Shin and Davis, 2010), with some small additions and modifications described below, and one very large one (see below on TOC1 as a repressor).

The clock has been proposed as an interconnected feedback loop with morning, mid-day, evening, and night elements. The first practical models of the clock are illustrated in the Locke/Zeilinger 2006 model (Figure 1, Locke et al., 2006; Zeilinger et al., 2006). In this, the morning expressed PSEUDORESPONSE-REGULATOR 9 and 7 (PRR9, PRR7) proteins repress CCA1 and LHY expression, whose proteins in turn activate the former. This is the so-called morning arm. TOC1 protein in turn represses its activator “Y,” an element whose activity is at the end of the day, and this can be partially ascribed with the GIANTANEA protein (GI). This has been defined as the evening arm. These models served the clock community well, although some conflicts could be noted. For instance, the Locke/Zeilinger 2006 model predicted that Y/G1 transcript levels would elevate in the toc1 null, but this could not be experimentally observed (Martin-Tryon et al., 2007). One explanation for this was that decreased GI expression in toc1 loss-of-function mutants is not direct (Martin-Tryon et al., 2007), and this hypothesis still awaits testing. Finally, several groups have concluded that GI has biochemically separable roles in its ability to integrate light signals, work in the clock, and control flowering time (Misra et al., 2005; Martin-Tryon et al., 2007; Olivero et al., 2007). How GI transcriptionally fits into the clock has not been particularly well resolved, but it has been proposed to additionally work in the clock as a hub of a protein destruction complex (Kim et al., 2007).

The Locke/Zeilinger 2006 models considered multiple interconnections in the oscillating circuit. One reason for this was based on the observation that none of the founding clock components in this model were arrhythmic when mutated to loss-of-function. Genetic ablation of any one loop leads to the persistence of other loops; rhythms thus persist. In an interconnected circuit, reduction of paths reduces flux. As such, cca1 lhy, and toc1 mutants were short period because there were less paths in the circuit (Locke et al., 2005a; 2006; Zeilinger et al., 2006). This could be extended in the Pokhilko et al. (2010) model (Figure 1). These described models thus did not allow for loss of function in any one gene to lead to arrhythmicity.

The notion of a single gene in the clock leading exclusively to periodicity defects needed to be reevaluated with the finding that the early flowering 4 (ELF4) gene (Doyle et al., 2002) was core to the oscillator, and that when mutated, the oscillator stopped (Kolmos and Davis, 2007; McWatters et al., 2007). The ELF4 gene was found to be both necessary and sufficient to promote CCA1 and LHY, and repress TOC1 (McWatters et al., 2007). This led to a preliminary hypothesis that ELF4 worked directly on these genes (Kolmos and Davis, 2007). That hypothesis could quickly be refuted. If CCA1/LHY levels were low and TOC1 levels were high in elf4, then it was a simple expectation that PRR9, PRR7, and GI levels would also be low. Experimentally the reverse was found for all cases (Kolmos et al., 2009). The Locke et al., 2006 model helped to solve this contradiction. Using parameter fitting of the observed levels of PRR9, PRR7, and GI in elf4, it was found that the oscillator would stop and that CCA1 levels would collapse and that TOC1 levels would be constantly high without rhythm. This is indeed exactly what is seen in the elf4 mutant (Kolmos et al., 2009).

Partial function alleles at ELF4 conform to this finding (Kolmos et al., 2009). Thus, in vitro hypothesis testing of the Locke et al., 2006 model provided the first correct placement of an evening complex (EC) component into the oscillator (Kolmos et al., 2009 model). What this work did not address was the placement of ELF4 in mathematical terms to this oscillating circuit.

PLACING THE ELF4 AND LUX EVENING COMPONENTS INTO THE CLOCK MODEL

The multiple interconnected feedback loops were made in part to accommodate the phenotypic effects from loss-of-function data. Arrhythmic mutants could not simply be defined in the original Doyle and Millar models (Locke et al., 2006; Zeilinger et al., 2006). Arrhythmic mutants exist at three loci and these are at the EC-components ELF4, ELF3, and LUX (Hazen et al., 2005; McWatters et al., 2007; Thines and Harmon, 2010). None of these genes had been conceptualized in the core-oscillator mechanism. ELF4 association to ELF3 directs LUX action in the clock (Herrero and Davis, 2012; Herrero et al., 2012), and this complex was termed the EC (Nausinov et al., 2011).

Using LTI modeling, ELF4 and ELF3 were concluded to directly target PRR9 and PRR7 (Herrero et al., 2012 model; Figure 1). Indeed, the elf4 mutant was found to be responsive to loss-of-function as it showed increased levels of these transcripts, especially in darkness, but not to such an extent under light (Kolmos et al., 2011). The elf4 mutant had a larger effect on transcript misexpression phenotypes than elf3, and this was especially seen for the increase of PRR7 transcript levels in elf4 during the light phase, whereas in darkness, both PRR9 and PRR7 were similarly increased in elf4 (Kolmos et al., 2009). Also, epistasis experiments showed that both ELF3 and LUX act downstream of ELF4 (Herrero et al., 2012). Consistent with that, whereas the ELF4 and ELF3 proteins have both been shown capable associated to the PRR9 promoter (Dixon et al., 2011; Herrero et al., 2012), as can LUX (Helfer et al., 2011), only ELF4 has been shown to directly bind to the PRR7 promoter (Dixon et al., 2011). Notably ELF4 over-expression resulted in attenuated PRR7 accumulation to a reduced extent than that of ELF3 over-expression (Herrero et al., 2012). ELF4 thus appears to have more targets in the clock than ELF3 and LUX.

A systems analysis of the EC led to a new kinetic model that agreed with the LTI modeling of ELF4 and ELF3. Here a “repressor hypothesis” was created with sequential waves of repression first by the transcription factors CCA1 and LHY, then by the PRRs, and finally by the EC, with LUX as the as the DNA-binding component of this complex (Pokhilko et al., 2012 model; Figure 1). Notable here was the biochemical finding that all PRRs directly associate to DNA (Gendron et al., 2012) and direct repression at their targets (Gendron et al., 2012; Huang et al., 2012). It is thought...
that all three EC genes are evening expressed because of direct repression by CCA1 and LHY (Kolmos et al., 2007; Lu et al., 2012; Pokhilko et al., 2012). The EC in turn is known to repress PRR9 directly (Dixon et al., 2011; Helfer et al., 2011; Herrero et al., 2012), and genetically, can repress PRR7 and perhaps GI (Kolmos et al., 2009, 2011; Herrero et al., 2012). It is currently unclear if the EC can directly repress GI, as mathematically predicted in one study (Kolmos et al., 2009), or if this is indirect through effects at PRR9 and PRR7, as mathematically predicted in another (Herrero et al., 2012). In the one test of this latter mathematical hypothesis, the perrperr7 mutant was not found to have altered mean transcript levels of GI (Salome et al., 2010). Therefore, how GI fits in the clock is not particularly well understood within a transcriptional context.

CONCEPTUAL USES OF MODELS: WEATHER PATTERNS AS AN EXAMPLE

Having a firm and experimentally validated model at hand allows for future optimization to test the robustness of the complex circadian clock network hypotheses. Here, the Millar group asked which environmental cues and following downstream regulations demanded such a highly complex clock network and followed an in silico approach to test whether the proposed oscillator is plausible in contexts of environmental variation seen in nature. For instance, they selected for networks that correctly predicted particular phases of the day under a light/dark cycle (Trioin et al., 2009). The general conclusion was that changes in environmental cues demand for a high complexity in the clock network in order to encompass the details of environmental perturbations typical of daily weather or annual photoperiod variation. This finding provided a validation for the benefits of modeling. Tests of mathematical models often show their limitations. Community willingness to flexibly perform molecular-genetic and biochemical tests of these models has allowed for new model generation to account for such proven discrepancies.

While the existing models reveal a rational view of how an oscillating circuit can resist weather-related environmental changes, and still be sensitive to daily entrainment cues, the models have also been insightful into placing the vernalization effect on clock cellular coordination of said processes (Wenden et al., 2012). Its expression correlates with period length, as a consequence of metabolic changes (Dalchau et al., 2011), and investigating effects of feedback signals from further downstream investigations of the phase change of the clock by light pulses. This consideration of light as an input factor to the clock when testing the phase change of the clock by light pulses. This allowed for current modeling efforts to define numerous negative–negative feedbacks. In the current Pokhilko et al. (2012) kinetic model, only CCA1 and LHY are defined as positive elements. This model also predicts that the EC controls CCA1/LHY and TOC1 expression through the multi-loop of PRRs, which is consistent with the previous experimental observation of higher transcript levels of the CCA1, LHY, TOC1, and PRR9 in the elf3, elf1, and lux mutants. Consequently, the Pokhilko et al. (2012) model shows the importance of repression of TOC1 and PRR9 by the EC for robust anticipation of dawn.

The recent work of Pokhilko et al. (2012) additionally considered the role of light as an input factor to the clock when investigating the phase change of the clock by light pulses. This model predicted that the acute activation of CCA1/LHY expression by light is required for the observed phase advance or delays at a given time during the night. The ability of the model to predict such an observation emphasizes the importance of incorporating input signals to circadian modeling. From this, we need to start investigating effects of feedback signals from further downstream processes, such as hormonal signaling (Hannou et al., 2006) and as a consequence of metabolic changes (Dalchau et al., 2011), and cellular coordination of said processes (Wenden et al., 2012).

A general conclusion of recent kinetic and linear models could lead one to consider the clock network as “solved.” This is not the case. Two-component limit cycle oscillators can exist if at least one component is “autocatalytic” and there is also a negative feedback ( Tyson, 2002; Novak and Tyson, 2008). Here, “limit cycle” means that every cycle is the same, and thus, there is no dampening or noise. If the plant circadian oscillator is not built in such a way, to make this oscillator, the circuit is anticipated to have a minimum of three components and positive and negative arms must exist within it: representer networks need activators (Sprinzak and Elowitz, 2005). It is thus plausible that the current model of the plant clock lacks adequate activators to be rationally defined. How such activators fit into the clock system of course
Another important consideration is to define the details of feedback signals from metabolic rhythms (Stitt and Zeeman, 2012) and their role in the redox circadian oscillator (Edgar et al., 2012). The whole clock community awaits those integrative results (van Ossejen and Millar, 2012).

MODEL NEEDS AND PROPOSED FURTHER USES

There are several areas where modeling has yet to place the clock in a signal context of observed findings. This seems relevant as numerous transcription factors fine-tune clock parameters, implying massive signal interconnections of divergent and disparate signaling systems to and from the clock (Hanano et al., 2008). One example is that multiple phytohormones have direct effects on clock parameters (Hanano et al., 2006; Yin et al., 2007), but to date, modeling has not explained how this feedback is plausible. Here, current efforts to reciprocally link the stress hormone abscisic acid (ABA) to the clock seem particularly relevant (Legnaioli et al., 2009). This could relate physiological connections to drought and salinity on the physiology of clock performance. Additionally, as auxin signaling rises with increasing warmth (Gray et al., 1998), and as auxin application phenocopies the effect of warmth to create more stochastic noise in the oscillator (Hanano et al., 2006), this thermal-dampening mechanism could relate to an ability of increasing temperatures to increase auxin signaling flux as a modulator of circadian amplitude. Modeling this hypothesis could direct the plausibility of this. Other hormones have distinct effects on phase and period, and these could act on light signaling to the clock, but that is not yet described in mathematical terms. Modeling signaling cross-talk to and from the clock seems ripe for future investigation.

Light has two main modes to set the clock. Light intensity increases lead to periodicity decreases (Somers et al., 1998a). This speeding up of the clock by increased light perception leads to an eventual phase shift of the clock back to a correct resonance, and this is called parametric entrainment. In contrast, the discontinuous nearly immediate setting of the clock happens at dawn and needs extended light far beyond that which activates light-regulated gene expression (Millar and Kay, 1996), and this sudden clock setting is called non-parametric entrainment. In some manner, the photoreceptors and clock genes have a role in these setting mechanisms (Somers et al., 1998a; Deslín and Kay, 2000). Interestingly, a photoreceptor complex (Mas et al., 2008) is genetically interactive in clock function (Deslín and Kay, 2000). A mechanistic hypothesis for photoreceptor input to the clock has not yet been generated. Although, it has been shown biochemically that light controls degradation of PRR7, PRR9, TOC1, and GI proteins (Mas et al., 2003; David et al., 2006; Farre and Kay, 2007; Ito et al., 2007a). These photic effects then act on outputs within a diurnal context that changes in duration throughout the season (Davis, 2002; Salazar et al., 2009; Troin et al., 2009; Guerrier et al., 2012; Song et al., 2012).

Low-fluence rate UV-B light has been shown to control development, promote photomorphogenesis, and drive gene expression (Heijde and Ulm, 2012). UVR8 and COPI are crucial for physiological UV-B responses and entrainment of the clock by UV-B light (Feher et al., 2011). Although under supplemented UV-B light, COPI induces ELONGATED HYPOCOTYL 5 (HY5) and HY5 HOMOLOGY (HYH), HY5 and HYH are not required for clock entrainment by UV-B (Feher et al., 2011). With the identification of UVR8 as the UV-B receptor (Heijde and Ulm, 2012), this is another input signal to the oscillator that must also be mathematically defined as an input cue.

Interestingly, under far-red (FR) light the otherwise arrhythmic elf3 and elf4 mutants regain rhythmicity (Kolmos et al., 2011; Wenden et al., 2011). Phytochrome A (phyA) has been shown to be required for controlling clock-regulated gene expression under these conditions (Wenden et al., 2011), yet the effect of FR-light input to the clock is not well understood. As shade alters the red/FR ratio, this observation suggests a different clock entrainment under these environmental conditions. Here, genetics could profit from mathematical modeling.

Ambient temperature effects on the clock work in two discreet ways. In one sense, the oscillator resists changes in mean ambient temperature to run at about 24 h over a fairly wide range of temperatures. Modeling has been able to explain this as effects at transcript abundance of clock genes (Gould et al., 2006). In contrast, the daily oscillation of daytime warmth with evening coolness can set the oscillator (Somers et al., 1998b; Boikoglou et al., 2011). This form of entrainment is completely unresolved (McClung and Davis, 2010). Modeling efforts have not yet been conducted to predict inputs generated from temperature entrainment. Another point is that stress temperatures of cold can lead to oscillator arrest (Biemanska et al., 2008). How stress temperature stops the clock is as yet non-explored in a systems sense (McClung and Davis, 2010).

Stress and metabolic signals enter the clock. Redox effects by photosynthesis, and alterations in sucrose and starch have been connected to normal oscillator function (Dalchau et al., 2011). Relations to ABA signaling appear to intersect here (Sanchez et al., 2011). How redox and carbon as photosynthesis-related processes enter the clock are not known. Modeling is likely to add useful hypotheses to this point. Other metabolites can act on oscillator parameters, including cyclic ADP ribose (cADPR), and this has been modeled (Dodd et al., 2007). Primary metabolites could act as energy intermediates, and trehalose-6-phosphate has been hypothesized to signal in homeostasis (Schliepmann et al., 2012). In contrast, secondary metabolites, such as glucosinolates that act on clock parameters (Kerwin et al., 2011), are more difficult to rationalize as just a metabolic effect. Numerous secondary compounds are perhaps probable as direct signaling molecules in clock fine-tuning. Placing all of these metabolic effects to the clock will likely be aided by informatics and systems approaches.

Clock genes in A. thaliana display extensive sequence variation manifested in quantitative variation within a population (Swarup et al., 1999; Boikoglou et al., 2011; Undurraga et al., 2012) and this is also seen in the monocot barley (Stracke et al., 2009; Faure et al., 2012). Furthermore, ploidy changes that are prevalent in plants also act on clock behavior at a physiologic (Ni et al., 2009) and genomic scale (Lou et al., 2012). Future clock models should be able to predict how subtle allelic variants lead to expressed-trait
effects on clock parameters. As the analysis of clock-gene expression in barley did not exactly follow that of A. thaliana (Campoli et al., 2012), models need to be generated that consider the evolutionary divergence between monocots and dicots. Finally, modeling is also likely to be useful in predicting how the assembly of a larger nucleus in new polyploids, and the effect of larger gene dosage, is buffered.

Moving beyond transcription, numerous clock proteins are subjected to post-transcriptional and post-translational regulation (Staiger and Koster, 2011). Phosphorylation and regulated protein degradation can be a forcing force for the input of environmental signals to the oscillator (Herrero and Davis, 2012). Together, these dynamics at the protein level need to be considered in new modeling efforts.

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Perhaps such an approach could lead to an improved placement of GI into the clock.

**CONCLUSION**

Mathematical models of the A. thaliana circadian oscillator have motivated hypothesis-driven experimental studies that have largely resolved this system. In this way, the plant circadian network serves as an example for how other plant-signaling systems can profit from interactive modeling-experimental efforts.

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