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The Intracellular Dynamics of Circadian Clocks Reach for the Light of Ecology and Evolution

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Abstract

A major challenge for biology is to extend our understanding of molecular regulation from the simplified conditions of the laboratory to ecologically relevant environments. Tractable examples are essential to make these connections for complex, pleiotropic regulators and, to go further, to link relevant genome sequences to field traits. Here, I review the case for the biological clock in higher plants. The gene network of the circadian clock drives pervasive, 24-hour rhythms in metabolism, behavior, and physiology across the eukaryotes and in some prokaryotes. In plants, the scope of chronobiology is now extending from the most tractable, intracellular readouts to the clock's many effects at the whole-organism level and across the life cycle, including biomass and flowering. I discuss five research areas where recent progress might be integrated in the future, to understand not only circadian functions in natural conditions but also the evolution of the clock's molecular mechanisms.

Contents

Behavior (in an organism): the genome's way of coping with the environment; it centers on movement in animals but on biochemistry and development in plants

Behavior (in a dynamical system): the form of a system's change over time, such as oscillation

Circadian clock: in a narrow sense, a small or minimal set of components that generate circadian rhythms; the term can also overlap with circadian system

Photoperiod: the duration of light within the day-night cycle

1. INTRODUCTION

Understanding how a particular molecular regulation contributes to the physiology, behavior, and selective advantage of a complex organism in a relevant environment is extremely difficult. Convincingly linking molecular regulation from the genotype to a whole-organism phenotype is hard enough. The progress in five areas of research suggests that, in the case of the plant circadian clock, we can extend our understanding of molecular regulation from the simplified conditions of the laboratory to environments that are ecologically relevant, and from the most tractable intracellular readouts to the clock's many effects across the plant life cycle. We might then also understand how the environment controls the plant clock on a much longer timescale, through the evolution of the clock's molecular regulation, and consequently how rational manipulation might fine-tune biological timing in future.

Biological clocks are thought to have evolved in response to the Earth's 24-hour rotation, which drives the predictable day-night cycle of light and temperature in the environments of almost all organisms. Photosynthetic organisms face a particular challenge because sunlight—the energy supply for their metabolic systems—is predictably unavailable for half of that cycle. Adding complication, the duration of daylight within the cycle can vary widely across the seasons. The seasonal variation in photoperiod (day length) depends on the organism's latitude, changing by only minutes at the equator but from constant light to constant darkness at the poles.

This circuit of understanding, from genotype to phenotype and back, crosses all the life science disciplines, from molecular and cell biology to physiology, ecology, evolution, and population genetics. What makes chronobiology, the study of biological timing, a useful thread to connect these disparate fields? First, the clock is a pervasive regulator. It drives so many 24-hour rhythms

that it affects many aspects of plant physiology, including important crop traits (Section 2). Second, clock function depends intimately on environmental signals, which ensure that the endogenous biological rhythms are set appropriately to local time. The circuit of gene regulation that creates the circadian clock (Section 3) is therefore likely to be affected by present and future climate change, which will in turn affect all the downstream clock-regulated processes. Third, the plant clock system has recently been tested for natural genetic variation (Section 4). Clock gene variants have also been selected by plant breeders in the past to expand the geographical range for particular crops, allowing the plants to thrive in new environments. Fourth, the circadian clock has evolved to address biological challenges that are ubiquitous, affecting most organisms in most environments (Section 5). Circadian biologists therefore routinely compare results among species and expect that the concepts learned in one species will apply broadly to many others. The comparisons are made easier because our focus is on a single dimension (time) with an obvious and universal coordinate system (the day-night cycle). This broad view is one of the major attractions of chronobiology, justifying the inclusion here of examples from other taxa, especially where we lack comparable results from plants. Finally, chronobiology has routinely included formal mathematical models, giving this area a head start on the multiscale models that are now required to link intracellular dynamics to the whole-organism scale and beyond (Section 6).

I refer below to a few of the recent reviews on each specific area. Broader treatments are included in a retrospective from Colin Pittendrigh, a British American founder of the field (113), and in a comprehensive textbook (40). An overview of the historical consensus on transcriptional clock mechanisms in laboratory model species (155) also presents a comparison across all taxonomic groups, which is now rare.

Clock-regulated processes: conceptually, pure outputs of the clock that do not feed back to alter circadian timing; in practice, clock outputs might feed back indirectly

Mathematical model: the description of a biological system or process formalized in a mathematical representation, such as ordinary differential equations

Laboratory model species: a species for which experimental protocols, tools, and resources have been developed to facilitate laboratory studies

Circadian rhythm: a self-sustaining biological rhythm with a temperature-compensated period close to 24 hours, which is normally entrained to the day-night cycle

2. PERVASIVE DAILY RHYTHMS

Early observations on plants first identified endogenous circadian rhythms (24). Research on many plant species has contributed to the field and continues to do so (88). The most noticeable rhythms in plants included rhythmic movements of leaves and petals (38), rhythmic emission of floral fragrance (48), and rhythmicity in photosynthesis (34) and in the responses to day length (133). A shared vocabulary describes the properties of circadian rhythms in all organisms (**Figure 1**; see also sidebar Rhythmic Properties).

2.1. Clock-Controlled Gene Expression

The diversity of plant rhythms suggests that the clock mechanism must control many different biochemical or molecular processes. The potential breadth of rhythmicity was most dramatically revealed by the first large-scale study of the rhythmic transcriptome in any eukaryote (60), which is also among several advances from research on the laboratory model species *Arabidopsis thaliana* (mouse ear cress). The rhythmic genes functioned in a huge variety of metabolic, developmental, and stress response pathways, which were apparently identified by reading several hundred papers, because the *Arabidopsis* genome sequence, published in the same year, had only sparse annotation. Genes that functioned together were often expressed at the same phase, suggesting many more biological processes that might be rhythmically controlled. What proportion (and which) of these RNA rhythms are physiologically relevant remains largely an open question.

More recent analyses have confirmed this temporal clustering, with at least 30% of transcripts showing circadian clock control in *Arabidopsis* under constant conditions and up to 90% of transcripts scored as rhythmic under diel light and/or temperature conditions (23, 89). Similar results are now available from ten plant species and two green algae (reviewed in 54, 109) (see sidebar

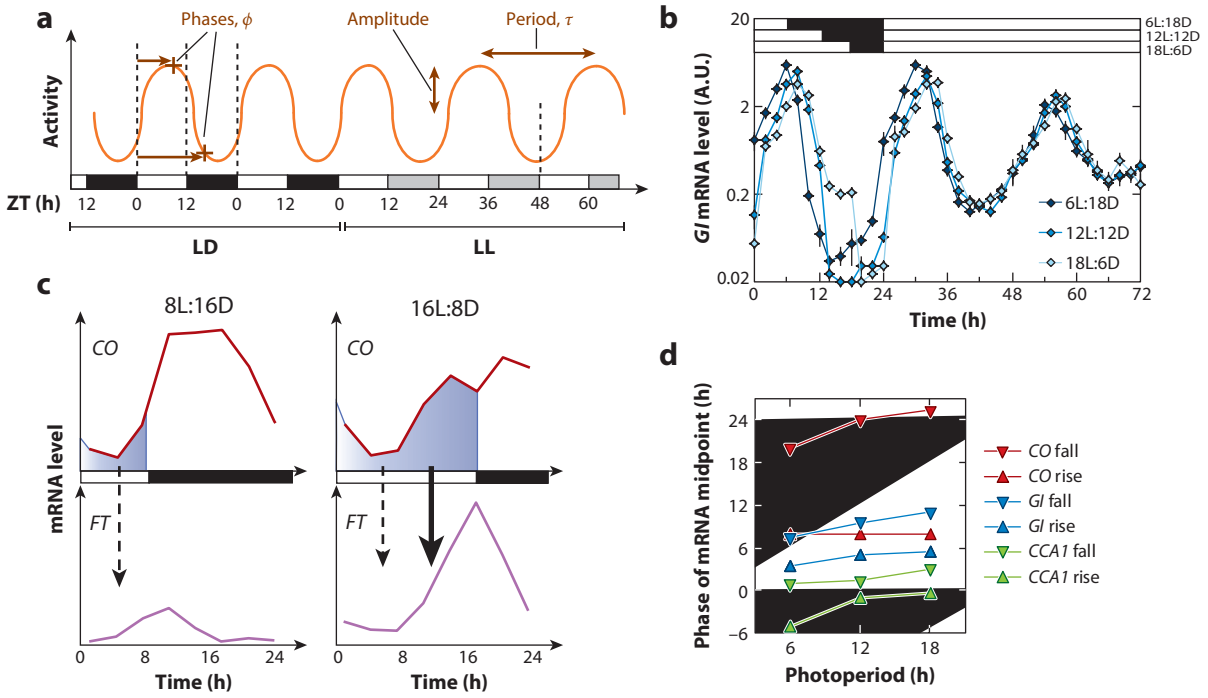

Figure 1

Circadian rhythms in *Arabidopsis*. For additional discussion of these concepts, see the Rhythmic Properties sidebar. (*a*) Basic properties of circadian rhythms under light:dark cycles (LD) and constant light (LL). The activity of a rhythmic, biological process is plotted on the *y* axis. The Zeitgeber time (ZT) on the *x* axis is measured in hours from the last onset of light. The white bars represent light, the black bars represent dark, and the gray bars represent predicted darkness during LL. (*b*) Rhythmic *GI* mRNA [in arbitrary units (A.U.)] from quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays of seedlings under 6L:18D, 12L:12D, and 18L:6D followed by LL. The error bars show the ranges of biological duplicates. Data replotted from Reference 41 on a logarithmic scale, to show the high amplitude of rhythmic regulation in LD and the lower amplitude in LL. (*c*) Graphs showing that an external coincidence between light and rhythmic *CO* mRNA (*shaded area*) induces *FT* expression and flowering under long photoperiods. The darker shading and thick arrow denote greater induction in the late day, when FKF1 (not shown) is expressed. (*d*) Graph showing that the midrise and midfall phases of mRNA waveforms under LD cycles change with photoperiod. The white and black areas show the light and dark intervals, respectively. A dawn-tracking rhythm would follow the horizontal line of dawn (dusk sensitivity = 0); a dusk-tracking rhythm would follow the diagonal of dusk (dusk sensitivity = 1). The various dusk sensitivities of these RNA markers form part of the *Arabidopsis* clock's dynatype (26). Data replotted from Reference 41; the phases for *GI* were taken from plots of the data in panel *b*, on a linear scale.

Online Resources for Plant Chronobiology). Promoter sequences that are enriched among genes expressed at the same phase include the Evening Element, which is bound by the clock proteins LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) (see Section 3). The rhythmic transcriptional regulators of the clock mechanism provide one mechanism for pervasive rhythmic control, as chromatin immunoprecipitation studies show that these proteins individually interact with hundreds to thousands of downstream genes (69). We still need to understand how these rhythmic regulators interact on their target promoter sequences in order to explain the earlier observations that several regions of each promoter can support rhythmic expression (1, 60). Designing synthetic promoters that target expression to any desired phase will then validate that understanding.

RHYTHMIC PROPERTIES

Our vocabulary for biological rhythms supplements the mathematical description of oscillations with properties that are shared by the circadian rhythms of all organisms. **Figure 1*a*** illustrates the basic properties. Time is usually measured from the last onset of light, defined as Zeitgeber time 0 (ZT0). A Zeitgeber ("time-giver") is an entraining signal. The absolute amplitude is measured as peak level minus midpoint, as shown in the figure, but for assays that return arbitrary units, relative amplitude (peak divided by trough) is more often cited. Phase refers to a point on the waveform and is measured from a reference point, usually ZT0 under a light:dark cycle (LD, a diel cycle). The 12-hour photoperiod illustrated in **Figure 1*a*** is denoted 12L:12D. Period refers to the time between successive occurrences of the same phase. For a stably entrained clock, as in LD, the clock's period is by definition the same as T, the period of the entraining diel cycle. The endogenous period τ is measured under constant conditions, such as constant light (LL). This period is rarely exactly 24 hours, so the phase progressively changes with respect to the predicted times of dawn and dusk (compare with the dashed lines at ZT0 and ZT48 in **Figure 1*a***).

The data in **Figure 1*b*** illustrate how light directly affects clock components to entrain the clock, in this case increasing mRNA expression of *GI* in *Arabidopsis*. The logarithmic scale shows the >100-fold relative amplitude of regulation in LD, which progressively decreases in LL. The fall in relative amplitude is referred to as damping.

Figure 1*c* illustrates the concept of external coincidence in the photoperiod response pathway (reviewed in 133). Under short days of 8L:16D, little *CO* mRNA is present during the light interval. The shading indicates that the CO protein is also unstable in the early day, so little expression of CO's target, the flowering gene *FT*, is activated. Under long days of 16L:8D, *CO* mRNA is abundant in the extended day and the CO protein is also stabilized, inducing high *FT* levels (and hence rapid flowering).

Figure 1*b* and **Figure 1*d*** show that the waveform of the clock components also reflects particular entraining signals, in this case 6L:18D, 12L:12D, or 18L:6D. The effect on the clock's phase is detectable in subsequent LL, for example, in the *GI* peak at approximately 54 hours in **Figure 1*b***. The change in phase under different photoperiods is termed dusk sensitivity (41). The observed change in phase under LL is small compared with the change in photoperiod, so the *Arabidopsis* clock is termed dawn-dominant; a larger phase change was observed in constant darkness (DD) (41). The waveforms under LD combine direct (acute) environmental responses and feedback from other clock components (see **Figure 2**, below), such that the midrise and midfall phases are delayed by several hours under long photoperiods (**Figure 1*d***). These dynamic behaviors of the clock system can be referred to collectively as the system's dynatype, by analogy with the phenotype (26).

New approaches to studying the global transcriptional network are now contributing to our understanding of the output network downstream of the clock in *Arabidopsis*. First, systematic analyses of DNA binding site preferences have gone beyond focused studies on specific clock proteins (55, 63) or promoter sequences (120). Clock-relevant proteins have been analyzed in broad-scale studies using protein-binding microarrays, including for CCA1 and REVEILLE 1 (RVE1) among over 30 *Arabidopsis* proteins (51) and for *Arabidopsis* LUX ARHYTHMO [LUX, also known as PHYTOCLOCK 1 (PCL1)] and *Ostreococcus tauri* transcription factors among more than 1,000 other eukaryotic transcription factors (147). Second, nuclease-sensitivity assays coupled with high-throughput sequencing (134) are now extending, genome-wide, the footprinting methods first used to identify protein binding on the early examples of clock-regulated promoters (1). Importantly, neither of these approaches requires specific antibodies against the clock proteins or epitope-tagged proteins in transgenic plants, which are time-consuming to produce. Instead, rhythmically changing nuclease sensitivity at a particular promoter sequence may implicate a specific set of potential DNA-binding factors, each with an associated probability. The mutant lines available in *Arabidopsis* for these binding factors, or inducible misexpression constructs, can then

ONLINE RESOURCES FOR PLANT CHRONOBIOLOGY

- Reference 49 describes publicly accessible time-series data for clock gene RNAs in multiple conditions and genotypes.
- Diurnal (**http://diurnal.mocklerlab.org**) is a database of transcriptome time-series data for several plant species, scored for RNA expression phase and waveform (94).
- Plant Systems Biology Modelling (PlaSMo; **http://www.plasmo.ed.ac.uk**) is a repository of models for plant growth and systems biology, including circadian clock models (28).
- The Biological Data Repository (BioDare; **http://www.biodare.ed.ac.uk**) is a portal for time-series data other than transcriptomes, with tools to document, analyze, and visualize rhythmic data (49, 95).
- The Arabidopsis Information Portal (AraPort; **http://www.araport.org**) and The Arabidopsis Information Resource (TAIR; **http://www.arabidopsis.org**) are databases for the model organism *Arabidopsis thaliana* that also link to many related resources.
- The Nottingham Arabidopsis Stock Centre (NASC; **http://www.arabidopsis.info**) is the European *Arabidopsis* seed and DNA stock center.

more easily be tested for misexpression of the target genes in order to validate the prediction. Hit-and-run regulators that only transiently bind to a promoter should still be identified by this approach (110).

Two challenges will remain. First, the levels of the relevant transcription factors are as important as their relative binding affinities, but the absolute protein abundance (protein copy number per cell) is unknown for these proteins, with only indirect estimates in the alga *O. tauri* (141). Second, resolving the contributions of multiple, overlapping, or redundant DNA-binding proteins—for example, by using multiply-mutant plants (67), multifunctional artificial microRNAs (125), or model species with smaller genomes (22)—remains laborious. Nonetheless, there is now a realistic prospect of connecting clock-controlled genes to a genome-wide network for higher plant transcription. The gene network could then be linked down to mechanistic clock models that explain rhythmic dynamics (see Section 3), while coregulated genes in putative functional pathways are linked up to physiological processes in the whole plant (see Section 5).

2.2. Canonical Outputs from the Plant Clock

The first clock-controlled genes studied in plants were highly expressed, encoded abundant proteins, and were initially identified for their strong regulation, for example, by light [*LIGHT-HARVESTING COMPLEX* (*LHC*) and *RUBISCO ACTIVASE* (*RCA*)] or cold [*COLD AND CIRCADIAN-REGULATED* (*CCR*), also known as *GLYCINE-RICH PROTEIN* (*AtGRP*)]. Most interest now focuses on the genes that regulate key aspects of clock-controlled physiology, where the canonical examples include cold signaling (54), the daily control of hypocotyl growth and light-regulated gene expression (136), and the regulation of flowering by day length (photoperiod) (133). In each case, the clock controls the expression of intermediate transcription factors that integrate abiotic and biotic environmental signals with temporal information from the clock (**Figure 1*c***). In the flowering pathway, intermediate transcription factors of the B-box and DNA-binding with one finger (DOF) families, notably *CONSTANS* (*CO*) and the *CYCLING DOF FACTOR* (*CDF*) genes, converge to regulate *FLOWERING LOCUS T* (*FT*) (**Figure 1*c***). In hypocotyl elongation, basic helix-loop-helix factors of the *PHYTOCHROME INTERACTING FACTOR* (*PIF*) family are most strongly implicated, along with basic leucine zipper proteins such as LONG HYPOCOTYL

5 (HY5) (136). In both the output pathways and the clock mechanism (see Section 3), light input is mediated by transcriptional regulation and by posttranscriptional mechanisms, notably protein degradation via the CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) ubiquitination complex (119). These mechanisms, together with the interaction of the PIF and CO/CDF pathways downstream of the clock, were recently represented in an integrated mathematical model that included both canonical and genome-wide clock targets (127). Environmental regulation of RNA processing is one of the modifying mechanisms that should be integrated in the future (103, 130).

2.3. A Broader Set of Clock Targets

Circadian control of plant metabolism has long been documented but is now known to extend well beyond photosynthesis (34), including secondary metabolites such as floral fragrance and defense compounds. In several cases, this regulation is likely mediated by rhythmic transcription of the relevant enzymes (48, 56, 77). A fascinating example is the clock-mediated prediction of the time of dawn, which sets the degradation rate of transient starch in the chloroplast at dusk (58). The mechanism of starch regulation in the chloroplast might be posttranslational, although this does not preclude transcriptional clock regulation of the posttranslational modifier (reviewed in 126). Intriguingly, circadian regulation has been discovered in fundamental cellular systems such as ribosome biogenesis and translation (76), in addition to the cell cycle (see Section 3). The clock's relationship with central and ancient aspects of cellular function and possible nontranscriptional clock outputs are both gaining new relevance as our view of the circadian clockwork itself expands to consider potentially ancient nontranscriptional mechanisms.

3. THE CLOCK'S TIMING MECHANISMS

The circadian system of each organism includes a phylum-specific gene regulatory network that is required for most rhythmicity and is intimately connected to the environmental day-night cycle (155), as well as one or more nontranscriptional oscillators that are less well characterized in eukaryotes.

3.1. The Transcriptional-Translational Feedback Loops

In plants, the clock gene network has been best studied in *Arabidopsis thaliana*, revealing a complicated circuit of highly connected negative regulators (**Figure 2**) as well as positive regulators that mediate light input to the clock and, more recently, rhythmic activators (reviewed in 68). Expression of the MYB-related transcription factors LHY, CCA1, and RVE8 begins rising at midnight and peaks at dawn [Zeitgeber time 0 (ZT0); see sidebar Rhythmic Properties], when LHY and CCA1 inhibit the expression of later-expressed genes, including *PSEUDO-RESPONSE REGULATOR 5* (*PRR5*) and *TIMING OF CAB EXPRESSION 1* (*TOC1*, also called *PRR1*). The biochemical mechanism of inhibition includes interaction with general TOPLESS repressor proteins (145), DE-ETIOLATED 1 (DET1) (82), and chromatin modification at the target promoters (reviewed in 4). The DNA-binding pseudo-response regulator genes *PRR9* and *PRR7* are expressed after dawn (ZT2–6). LHY and CCA1 bind to the promoters of these two genes, but the genetic evidence that this results in activation of *PRR9* and *PRR7* (46, 123) has not yet been reconciled with the fact that LHY and CCA1 are repressors for other well-studied targets. Expression of PRR9 and PRR7 and then of PRR5 and TOC1 ends the morning phase. The PRR proteins bind to and inhibit *LHY* and *CCA1* expression, as demonstrated by experiments (55, 69, 101) and predicted by modeling (115, 116). Moreover, the data suggest that later-expressed PRR

Circadian system: the biological components that mediate circadian rhythms; it is conceptually divided into input, oscillator, and output components, although in practice a component may contribute to multiple functions

Oscillator: the biological components that mediate self-sustaining rhythms, entrained by the input components and regulating the output components of the circadian system

Pseudo-response regulator: a protein with homology to the receiver domain of bacterial histidine-aspartate phosphorelay proteins but lacking the phospho-accepting aspartate

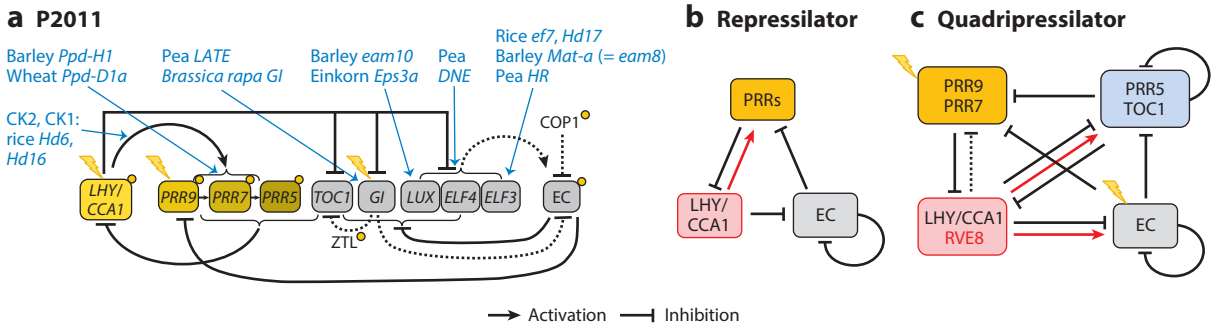

Figure 2

Simplified schemas of the *Arabidopsis* clock gene circuit. (*a*) The clock genes (*rectangles*) as they were connected in the P2011 model (115). LHY and CCA1 are combined into one component; yellow flashes and dots denote transcriptional and posttranscriptional light inputs, respectively; and dashed connections denote posttranslational regulation. Such circuits and their associated biochemical parameter values have been termed the clock's chemotype (26). Note that inter-regulation of the PRRs was modeled as a forward, activation cascade, for want of experimental data. Current results support a reverse, repression cascade, shown in simplified form in panel *c*. Homologous genes that control crop flowering are shown above the schema. Einkorn refers to *Triticum monococcum*. (*b*) The clock circuit of the P2011 model, oversimplified as a repressilator (115). The red arrow indicates the proposed activation of early *PRR* genes by LHY and CCA1, previously termed the morning loop; negative autoregulation of the Evening Complex (EC) forms an evening loop independent of *LHY* and *CCA1*. (*c*) A four-component quadripressilator circuit, abstracted from current understanding (see Sections 3.1 and 3.2). This circuit retains the ring of repressors, with RVE8 as an activator (*red*); the inhibition of *PRR9* and *PRR7* by LHY and CCA1 remains to be demonstrated (*dashed line*) (50). In panels *b* and *c*, the few activating links are highlighted in red.

proteins in general inhibit the earlier-expressed *PRR* genes (reviewed in 15, 50, 115), providing a mechanism to explain the distinct timing of each *PRR* gene expression and hence to explain the temporal wave of *PRR* expression within the day.

Falling LHY and CCA1 levels derepress expression of *EARLY FLOWERING 3* (*ELF3*), *ELF4*, and *LUX*, allowing these transcripts to peak at approximately ZT10. RVE8 [also called LHY/CCA1-LIKE 5 (LCL5)] protein also accumulates (several hours after its peak RNA abundance, owing to an enigmatic delay) and activates evening-expressed genes (67), interacting with NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED (LNK) proteins (112). GIGANTEA (GI), a large plant-specific protein, is rhythmically expressed under LHY/CCA1 control but functions at a posttranslational level through, for example, stabilization of the TOC1-degradation factor ZEITLUPE (ZTL) (79). The protein products of *ELF3*, *ELF4*, and *LUX* or its homolog *BROTHER OF LUX ARHYTHMO* (*BOA*, also called *NOX*) interact to form another repressor, the Evening Complex, at ZT12–14 (32, 63, 64). The Evening Complex ends the day phase, as it binds to and inhibits expression of *PRR9* and *PRR7*. *ELF4* and *LUX* are also targets of the Evening Complex, forming an autoregulatory feedback loop that ends *ELF4* and *LUX* expression several hours before the rise of their other major repressors, LHY and CCA1. The resulting rhythmic waveforms of the clock genes are robust across plant age, growth conditions, and multiple laboratories (49). A range of mathematical models have helped elucidate how the clock's dynamic behavior, or dynatype, arises from this complicated circuit (reviewed in 11, 50) and from its parameter values (see below), or chemotype (26).

Waveform: the temporal profile of a biological rhythm over one cycle; it includes phase, period, and amplitude information and the more complicated properties of wave shape

Parameter: a usually constant value in a mathematical model that affects the dynamics of the model components, such as the maximum rates and binding affinities in biochemical reactions

3.2. Abstracting to Understand

The known clock circuit does not obviously divide into modules (50, 67). However, simplification provides an intellectual framework to organize detailed results, and in the future should allow

the abstraction of simpler mathematical models from our current, fine-grained versions. Starting from the observation that later-expressed components often inhibit earlier-expressed components, **Figure 2** shows two simpler networks. A three-loop structure was previously described (84) in which a morning loop and an evening loop were coupled around a repressilator (100, 115) (**Figure 2*b***). The repressilator is a ring of three inhibitors in which the expression of each gene inhibits the earlier-expressed gene, as noted above. The circuit was implemented in *Escherichia coli* in a canonical example of synthetic biology (reviewed in 118). However, James Locke and David Rand (personal communication) have pointed out that, in contrast to the repressilator, the plant clock has sufficient components to separate the control of the rising and falling phases of each gene expression peak.

Slightly extending the scheme suggests a unidirectional ring of four inhibitors surrounding two diagonal interactions (**Figure 2*c***). Each group of genes is regulated by two or three inhibitory interactions, allowing separate control of rising and falling phases. *LHY/CCA1* and the late *PRR* genes (*PRR5* and *TOC1*) are mutual inhibitors across one diagonal, and the Evening Complex inhibits the early *PRR* genes (*PRR9* and *PRR7*) across the other diagonal. The four-inhibitor ring or the two-member mutual inhibition motif would naturally have toggle-switch equilibria rather than oscillations (140), suggesting that the combination of both, along with the asymmetric elements, may be important for oscillation. It will be fascinating to discover how these dynamics are altered in larger genomes, for example, in crop plants (see Section 4). In the minimal genome of *O. tauri*, where homologs of LHY, TOC1, and a LIGHT, OXYGEN, AND VOLTAGE (LOV) domain–histidine kinase are the proposed clock components, either a simple negative feedback model (96, 137) or a repressilator (105) has been proposed to account for the data. The closely related chlorophyte *Chlamydomonas reinhardtii* includes clock genes that differ from the plant sequences (104). The growing resources in further algal and diatom species will likely reveal much more diversity among clocks in the green lineage (reviewed in 104). In addition, several processes and components identified in *Arabidopsis* remain to be fully integrated into our view of the circuit.

3.3. Environmental Inputs and Modifying Kinetics

Posttranscriptional, translational, and posttranslational regulation, often modified by protein interactions, modulate the clock's dynamics by changing the rate or strength of the connections in the transcriptional-translational feedback loop (reviewed in 80, 112, 128). These quantitative dynamics are critical in a timing system. Except in the Kai oscillator of cyanobacteria (see below), it is unclear what biochemistry allows the slow, 24-hour circadian timescale to emerge from the normally faster dynamics of the molecular processes involved. Measuring the absolute values of the relevant biochemical parameters for the plant clock (as in 108) has taken more time than in animal systems, where a larger field and a context of pharmaceutical interest maintains a greater focus on biochemistry.

Environmental control of these parameter values allows both entrainment under diel cycles (**Figure 1**) and the buffering of circadian timing over a range of constant temperatures (temperature compensation). Multiple effects of light and temperature have now been documented (reviewed in 68). Mathematical modeling is essential to integrate their joint effects and to understand how they allow particular behaviors, such as dusk sensitivity (**Figure 1**) and temperature compensation (57). Further work remains to test the models' proposals. For example, the most recent detailed clock models (50, 115) include light-dependent degradation of the Evening Complex based on a model of hypothetical COP1-containing protein complexes (117), which was also adopted by leading researchers of COP1 function (129). Further aspects of that model might now be validated as dynamic regulation of the Evening Complex components is identified (144)

and linked to the relevant phytochrome, cryptochrome, and LOV-domain photoreceptors (135). These environmental responses of the clock genes and proteins link the intracellular regulation of all the clock-regulated processes to the anticipated changes in the global environment. The challenge now is to measure their quantitative contributions to plant performance (see Section 6).

3.4. The Ecology of Oscillators

In addition to nontranscriptional modulators of the canonical clock gene circuit, there is steadily accumulating evidence for 24-hour rhythms that are independent of rhythmic gene expression (121). The cyanobacterial KaiABC oscillator is the canonical example of such timing (reviewed in 73), although convincing reports came decades earlier from measurements of rhythmic photosynthesis in giant algal cells from which the nucleus had been removed (for references, see 150). Recent results point to rhythms involving calcium levels in several organisms, the calcium-release factor cyclic ADP-ribose in plants, and cyclic AMP in animal cells (reviewed in 142). The most direct evidence for the nontranscriptional oscillator in eukaryotes, including *Arabidopsis*, now comes from rhythms in the oxidation of peroxiredoxin, a highly conserved, redox-sensitive enzyme that detoxifies hydrogen peroxide. This posttranslational modification was shown to be rhythmic in nontranscribing cells, using human erythrocytes and dark-adapted *O. tauri* (106, 107, 141). The key result has now been replicated independently in each system (10, 20). Redox regulation has also gained further prominence, for example, interacting with the clock gene network during salicylic acid–mediated pathogen defense (158).

There has consequently been renewed interest in the multiple links between circadian rhythms, metabolism more generally (reviewed in 62, 66), and other rhythmic processes. The best-established link between the clock and another oscillator is with the cell cycle in eukaryotes and cyanobacteria, based on the observation that many types of cells divide at a characteristic circadian phase. This regulation was often described as the clock creating a permissive phase for cell division, or gating the cell cycle, for example, in the green algae *C. reinhardtii* (102) and *O. tauri* (97). Recent mathematical analysis based on imaging single cells, by contrast, has shown that the mammalian circadian clock and the cell cycle are phase locked (47). Neither oscillator waits for, or gates, the other; rather, they progress together as a coupled system. These observations support earlier results showing that canonical cell-cycle genes such as that encoding the protein kinase Wee1 are not only clock regulated but also affect the pace of the clock as much as the canonical clock genes (157). Such clock coupling to the cell cycle has not been so clearly documented in higher plants and seems to be quite different in root primordia. The root apical meristem maintains a dawn phase in constant darkness, leaving its progeny cells to progress later in phase (52). As multiple cell divisions form a new meristem in lateral roots, the clock is dramatically reset (143).

The evolutionary breadth of the discussion is slowly extending to species that lack obvious circadian rhythms, such as the yeast *Saccharomyces cerevisiae*. Yeast chemostat cultures show that a well-documented metabolic cycle, which separates oxidative from reductive metabolism, controls cell division and the expression of all yeast genes. Manipulation of the chemostat conditions suggests that its period might extend from less than 6 hours (in the initial reports) to as long as 24 hours (44). A recent study showed that the peroxiredoxin oxidation state changes with the yeast metabolic cycle and, intriguingly, that inhibition or deletion of the homologs of the mammalian clock-related protein kinases Casein Kinase 1 (CK1) and Glycogen Synthase Kinase 3 (GSK3) alter the yeast cycle's period (16). Although the effects could result from conserved metabolic regulation, they might also reflect an ancestral timing mechanism that linked these cellular systems, which are now most often studied separately.

4. VARIANTS IN CLOCK GENES

Notwithstanding tantalizing speculation on the roles of nontranscriptional oscillators, the demonstrated physiological roles of the clock so far relate to its gene circuits. Clock mutants in *Arabidopsis* have been important in identifying many plant clock genes (see Section 3) and now have further importance in understanding the genetic variation in natural and breeding populations.

4.1. Crop Varieties and Breeding Lines

Bünning's pioneering work of the 1930s in *Phaseolus multiflorus* was perhaps only one generation away from identifying the first genetic variant in a clock-affecting plant gene (data reanalyzed in 91). The genetic variation in the breeding germplasm of many crop species also includes many clock-affecting loci, as **Figure 3*a*** illustrates for vegetable brassicas (122). A subset of flowering-time variation in crops is due to allelic variation in clock gene homologs—for example, in pea (148), sorghum (98), rice (72), and wheat (53, 92)—although many other crop flowering traits are clearly due to alleles of downstream, clock-regulated genes, such as the *B-BOX 19* (*BBX19*) homolog that controls *FT* floral regulators in beet (25). The northward progress of barley cultivation over several millennia, from the Fertile Crescent to Scotland and Scandinavia, was accompanied by selection for mutants in the homologs of *PRR7*, *ELF3*, and *LUX* (7, 156), which are also known as single-gene mutations that alter flowering time (13). The *PRR7* homolog *Photoperiod H1* (*Ppd-H1*) (12), however, did not affect clock gene expression rhythms in barley leaf RNA samples, although it did affect expression of *CO* and *FT* homologs (14). *Ppd-H1* is therefore specialized either for a nonclock flowering function (139) or at most for the clock of a minor cell type. The vascular phloem companion cells provide the critical timing signal for photoperiodic flowering in *Arabidopsis* (21, 45), so a specialized clock in these cells remains a possibility, as proposed in *Ipomoea* (61).

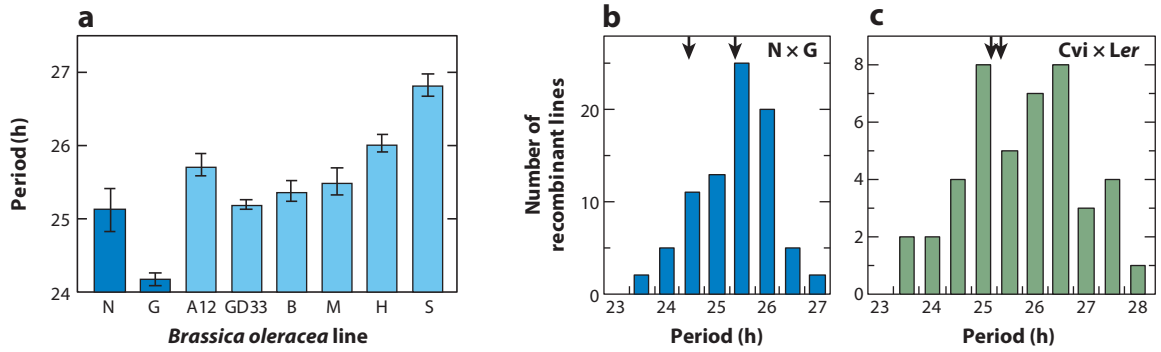

Figure 3

Genetic variation in the plant clock. (*a*) Circadian period of leaf movement under constant light in *Brassica oleracea* lines (*blue*) (122). Genetic variation among the lines alters the circadian period by almost 3 hours, as much as the strong, single-gene clock mutants in *Arabidopsis*. The parental lines N (var. *botrytis*, cauliflower) and G (var. *gemmifera*, Brussels sprout) are shown in dark blue. (*b*,*c*) Graphs showing the distribution of circadian period among recombinant progeny lines, compared with the periods of their parental varieties (*arrows*). The wider, transgressive variation in the progeny compared with the parents indicates that the parents carried allelic variants at multiple clock-affecting loci. Segregation of these alleles leads to longer and shorter periods among the progeny lines. Transgressive variation is modest in the N × G population of *B. oleracea* (122) (panel *b*; *blue*) but large in the Cape Verde Islands (Cvi) × Landsberg *erecta* (L*er*) *Arabidopsis* population tested at 12°C (43) (panel *c*; *green*). Although the parent *Arabidopsis* lines had nearly identical periods, the progeny segregated for a major clock-affecting quantitative trait locus; the underlying gene or genes remain to be identified.

4.2. Natural Genetic Variation

The many accessions of *Arabidopsis thaliana* distributed from community seed stock centers (see sidebar Online Resources for Plant Chronobiology) with known variation in flowering time presented a potential gold mine of genetic variation to identify new clock genes (reviewed in 3). Indeed, crossing different accessions resulted in progeny lines with very wide variation in circadian properties, indicating that the rhythms in each parent line were due to a combination of clock-lengthening and clock-shortening alleles. The first circadian quantitative trait loci (QTLs) were located within five years after the first induced clock mutants were created. However, the difficulty of identifying the causal gene for any subtle, quantitative trait was compounded by the relatively laborious assays for circadian rhythms [by using leaf movement (43, 122) or whole-plant movement (151) or by transforming many lines with firefly luciferase (*LUC*) reporters (9, 27, 81)]. Only two such genes were identified in the following 15 years [*ELF3* (2) and *FLOWERING LOCUS C* (*FLC*)], along with a suggested metabolic input (77) (see below). Natural variation at *FLC* is well studied (83); its effects on the clock circuit include slightly altered *LUX* expression (42) and genetic interaction with *LHY* and *CCA1* (154). Very recently, natural variation in *Brassica rapa* was mapped to an allele of *GI* (151). A study of *Arabidopsis* transformed 77 accessions with a *GI:LUC* reporter and performed QTL analysis in a Liepowiez 0 (Lip-0) × Columbia (Col) recombinant breeding population. The photoreceptor *PHYTOCHROME B* (*PHYB*) was identified as the causative gene for an altered expression phase under long photoperiods (30), similar to the result of an earlier mutant screen (124). The QTL did not affect circadian period in constant conditions but did alter light induction of *GI* transcription, emphasizing the clock genes' function as integrators of environmental signals.

Thus, the three most prominent nuggets so far from the gold mine turned out to be known regulators. Each study has also revealed several other QTLs that remain to be identified (**Figure 3*c***) and might indeed be additional clock components. Streamlined phenotypic assays will be important to tap this genetic resource, perhaps including the single-time-point transcriptome approach to estimate phase, which suggested that the glucosinolate biosynthetic pathway could alter the clock (77). The second hope in the studies of natural variation was that the accessions' habitat of origin would yield new insight into the ecological opportunities and constraints for circadian timing. Understanding the role of circadian timing in nature, however, depends on background knowledge of circadian physiology and of ecological contexts.

5. PHYSIOLOGICAL IMPORTANCE AND ENVIRONMENTAL CONDITIONS

5.1. Advantages of Circadian Timing

The physiological benefit of circadian timing initially seems to be a mystery. Plants are necessarily exposed to light:dark cycles (dormant seeds, tubers, and polar residents excepted) and have multiple photoreceptors. One might ask, if it were advantageous to carry out some biological process in the daytime rather than at night, couldn't the photoreceptors activate that process directly? If a delayed rather than an immediate response were required, couldn't that be achieved with simpler biochemistry than a full-blown oscillator that is self-sustaining even in constant light? Timing a process to the daylight hours is an example of external coordination, one of the two conceptual benefits that have long been proposed for circadian timing, which is discussed in more detail below. Internal coordination, by contrast, is simply providing a temporal sequence to control internal events, but direct evidence to validate this benefit is limited. The sequence

could avoid conflicts, such as separating oxygen-sensitive nitrogen fixation in the subjective night from oxygenic photosynthesis in a unicellular cyanobacterium (75), and gain synergies, such as the coregulation of multiple steps in a biosynthetic pathway (see Section 2). Internal coordination by the circadian clock is therefore an alternative to the direct regulation of one biological process by another, to avoid (or promote) co-occurrence. The yeast metabolic cycle (see Section 3.2) is presumably an example of pervasive internal coordination. The cycle seems to occur in individual, unsynchronized yeast cells as well as in chemostat cultures (131), so this type of regulation may be more general than was first thought. However, an experimental test for internal coordination by the circadian clock in the cyanobacterium *Synechococcus elongatus* found little benefit, as an arrhythmic *kaiC* mutant suffered no competitive disadvantage under constant light (149). Most plant biologists therefore focus on the clock's advantages for external coordination.

Simulation: in this context, a mathematical representation of a biological process as a result of numerically solving a mathematical model of that process in a given set of conditions

The clock offers three benefits compared with a simple, direct environmental response, namely multiple phases, the integration of multiple signals, and, crucially, history. First, clock components are expressed throughout the day and night (see Section 3.1) and can directly regulate output processes at many different phases, not only following the light:dark transitions. The output processes can, of course, be diverse, including the rhythmic gating of other signaling pathways at particular phases. Second, the clock is sensitive to changing conditions (e.g., temperature and metabolism) even though circadian timing is maintained with little alteration in a range of constant conditions (reviewed in 74). Most important, circadian rhythms continue to predict the time of day even if such environmental inputs are altered, because the clock is self-sustaining. That prediction is modulated by past environmental inputs, such as photoperiod, and updated by present inputs, through the inputs' entraining effects on the clock components (**Figure 1*b*,*d***). The clock's history dependence and network structure can reduce the effects of environmental variability, such as variation in the daily light intensity (36). Temperature history also affects circadian rhythms in *Arabidopsis* (9).

The extent of the history effect varies among clock systems and was recently tested using *LUC* reporter genes in the transition from the short-day to the long-day state. The expression profiles of an indicator clock component readjusted over three transient cycles in the complicated clock of *Arabidopsis* (31, 33), whereas the markers available for *O. tauri* adjusted on the second cycle (31). These results agreed with theoretical expectations, which show at the extreme that the simplest timers have no history. The level of sand in a running hourglass determines when the timer will run out, for example, irrespective of the events that led to that sand level. By combining history and multiple phase outputs, clocks can time events in sophisticated ways, for example, by controlling phase with intermediate dusk sensitivity rather than dawn or dusk tracking (**Figure 1*d***). The result is that clocks offer robust but evolvable timing across many conditions and seasons (114).

5.2. Clocking the Environment

These conceptual advantages of circadian timing lead to an evolutionary perspective, because they predict how the clock mechanism will eventually evolve to reflect the particular timing challenges (and opportunities) of the organism in its ecological context. For example, the slow re-entrainment of an intricate clock gene circuit might reflect a selective advantage that was conferred by a prolonged effect of the clock's history. Simulations can readily show these effects. Model gene circuits that were evolved in silico to anticipate the time of dusk in simple light:dark conditions had very simple circuits that functioned as sand timers: No clock was required. Self-sustaining clocks with complicated feedback circuits evolved only when the time of dusk varied unpredictably and the photoperiod also changed, simulating weather and seasons, or when they were evolved

to match a year of measured natural weather and seasons (138). Extreme circadian evolution has led to loss of rhythmicity in some animals that temporarily (reindeer in the Arctic summer) or permanently (Mexican cave fish) lose daily environmental Zeitgeber signals (5, 85), although not in others (75). In plants, loss of rhythmicity has been documented in Norway spruce (59) and in chestnut and *Arabidopsis* at low temperature (6). Most evidence for the physiological importance of plant rhythms, however, has been derived during ongoing environmental cycles.

Experimental settings can simulate extreme conditions, such as life on a different planet with a non-24-hour rotation, known as a T-cycle experiment. Classic studies of particularly sensitive species, such as tomato, showed many-fold more growth in 24-hour cycles compared with 12- or 48-hour cycles (65). In *Arabidopsis*, comparisons of 20-, 24-, and 28-hour cycles showed that wild-type plants favored 24-hour cycles, whereas *toc1* and *ztl* mutants with 20- and 28-hour periods grew better in their resonant T-cycle than in the opposite T-cycle (35); a similar study detected an altered allele ratio in a subsequent generation, indicative of a selective advantage (152). A straightforward mechanism was suggested because a resonant T-cycle restores normal phase relationships, relative to the environmental cycle, in such period mutants. While replicating the earlier results, subsequent work also showed that both 20- and 28-hour mutants grew better in 24-hour days than in either altered T-cycle (58), indicating that another mechanism in addition to circadian phase was involved (see Section 6).

The most persuasive data from altered natural environments involve latitudinal and altitudinal clines, where the input signals change progressively. The signatures of evolution can then be detected in common-garden studies in a single condition. Early collections of *Arabidopsis* were likely too disparate in origin to reveal such changes in circadian properties. Even an extensive study of 150 accessions revealed only a correlation of circadian period with midsummer day length (90), a nonlinear transformation of the latitude of origin that has since been mis-cited as a latitudinal cline. A study of 27 accessions across three temperatures found no obvious relationship with latitude (43). Now that many more *Arabidopsis* accessions have been collected with more detailed habitat information, a second limitation has come into play, namely the laborious assays required to test circadian phenotypes. The phase of *CO* expression in European aspen (*Populus tremula*) trees was shown strikingly to delay with higher latitudes of origin in the handful of varieties tested (8), where the photoperiodic induction of cold-hardy buds is a crucial overwintering adaptation. Several studies have implicated *PHYB* and the clock genes *LHY1* and *LHY2* in photoperiodic bud set in *Populus*, with some evidence for clinal variation (70, 86). Bud set in Norway spruce and Siberian spruce has been linked to clinal variation in clock gene homologs such as *PRR3* as well as in *FT*-related genes (17, 18). A key challenge now is to understand the dynamics of the clock network of any plant species in ecologically relevant conditions, preferably in species where we also find tractable genetic variation and ecological understanding.

6. UNDERSTANDING THE RHYTHMIC PHENOTYPE

6.1. Plant Rhythms in the Field: Which Rhythms Are Selected?

The data that directly measure rhythms in field conditions are limited but now growing. Ecologically relevant species have been tested; for example, gas exchange rhythms in tropical tree species have been evaluated under constant conditions (39). Opening of stomatal pores in anticipation of dawn can be clock regulated (e.g., 35). The resulting nighttime transpiration in field conditions in cotton and bean, tested using large transpiration balances, is claimed to be of similar magnitude to the expected change in transpiration due to climate change (29). Linking these responses with

clock gene circuit dynamics, however, will depend on transcriptional profiling. Following early transcriptomes that mapped the seasonal responses of free-living poplar (132), a landmark study has used hundreds of RNA microarrays to test the rice transcriptome over two growing seasons, with variable time resolution, in a field site close to a national meteorological station (99). The many rhythmic gene profiles were then tested for correlation to weather data, first for the best single correlating factor and then for multiple meteorological factors (87). Rhythmically modulated responses to temperature were a widespread explanatory factor, although circadian clock genes such as *OsLHY* were strikingly temperature insensitive. We likely have much still to learn from these data sets. *Nicotiana attenuata*, by contrast, offers exceptional ecological background information on plant-insect interactions in field research sites and is now being developed as a circadian system (78, 153). Circadian regulation of plant defense can clearly alter insect herbivory (56). It is possible that the clock evolves to optimize such defenses and, if so, that adaptations of circadian timing will be identifiable in *N. attenuata*. As in the trees and crop species, the circadian regulation of photoperiodic flowering time and possibly of seed germination (111) also very likely mediate part of the clock's contribution to fitness in natural settings.

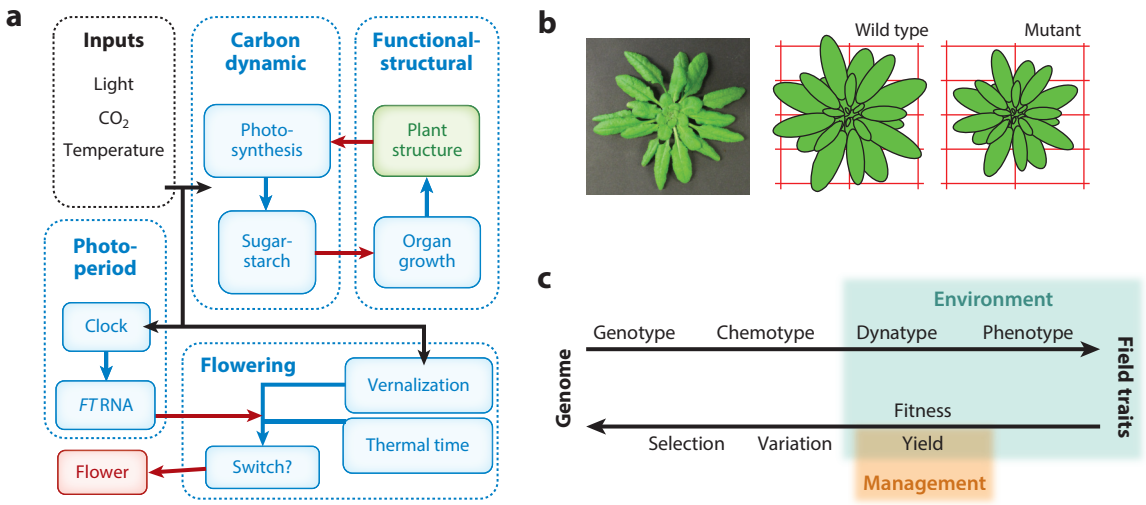

Figure 4

A community model to understand plant circadian systems. Panel *a* shows a highly simplified schema of the first *Arabidopsis* Framework Model, which predicted the biomass and area of each leaf (panel *b*) in a mature wild-type rosette under standard conditions, to within experimental error (19). The model combined four earlier models of various types (*blue dashed outlines*), representing the processes depicted in shaded rectangles, with links among models (*red arrows*) and from environmental inputs (*black arrows*). One model component (in *green*) represented the rosette and root structure. The clock model only regulated photoperiod-responsive flowering, although the clock controls many processes in the plant. The present challenge is to understand (explain and predict) complex, whole-plant phenotypes such as the pleiotropic effects of circadian clock mutants, quantitatively and based on the underlying mechanisms. Such understanding would link the plant's genotype, via chemotype and dynatype, to its phenotype in a given environment (panel *c*). The multiscale Framework Model promises to make this link. For example, the right-hand plant model in panel *b* shows the predicted growth defect caused by a simulated mild defect in starch mobilization, compared with the wild-type plant model in the center (Y.H. Chew, A. Zardilis & A.J. Millar, unpublished results). If the clock model also controlled starch degradation (see Section 2.3), then the model might predict the growth defect of a simulated clock mutant. Our ambition is to go further, starting from the genome sequence and extending to relevant field traits (panel *c*). It should then be possible to link the field traits to yield, for a given approach to agricultural management, or to fitness in natural conditions. Combined with an understanding of (natural or breeding) genetic variation, we may then hope to understand the selection that shaped the sequences and interconnections of the plant clock genes (**Figure 2**).

6.2. A Community Model to Study Complex Traits

The flowering and bud set traits discussed above are among the physiological processes that contribute to phenology, the temporal sequence of plant development, and thus to the plant's life history. Phenological traits are significant at a global scale because they affect how the vegetation component of the biosphere contributes to the Earth system. Indeed, Earth system models include simple phenology models for plants in various functional groups. Will it be possible to build on our molecular understanding of the circadian system to understand, for example, how the clocks of particular deciduous trees will alter the timing of bud set under future climates? Mathematical models will be crucial to cross the necessary scales of biological organization from the intracellular to the ecological (**Figure 4**). One recent example is the *Arabidopsis* Framework Model (**Figure 4*a***), which combined simple models of carbon metabolism, plant structure, flowering time, and a clock-driven photoperiod sensor (19). This model predicted whole-plant and leaf-level biomass and area based on existing models with minimal reparameterization, suggesting that mechanistic models of additional processes could be incorporated in a community approach, to which multiple groups can contribute. Similar optimism is shared among marine biologists (93) and in the fields of ecology and evolution, such that one might hope to understand the evolutionary feedback from the macroscopic, whole-organism scale of the phenotype back to the molecular scale of the genome (**Figure 4*c***). A recent review noted that "no. . .model has incorporated all of the components discussed [in the review], but such integration is possible and would be a powerful approach for studying ecological and evolutionary outcomes" (37, p. 75). It seems entirely possible that we will develop noninvasive, time-resolved assays to understand clock effects in the field, together with the cognate models, in order to link our molecular understanding of biological rhythms up to larger scales in the coming decade.

SUMMARY POINTS

1. The biological clock in higher plants is a tractable example of a complex, pleiotropic regulator that affects many processes across the plant life cycle.
2. Clock-regulated transcription has been studied in many individual plant genes; in *Arabidopsis*, rhythmic regulation might next be integrated into a genome-wide transcriptional regulatory network.
3. The mechanism of the clock gene circuit in *Arabidopsis* comprises a set of interlocked feedback loops; I outline a simplified schema named the quadripressilator.
4. Nontranscriptional, 24-hour rhythms have been demonstrated in eukaryotes, as well as in cyanobacteria, as part of the cell's ecology of oscillators; the mechanisms and contribution of nontranscriptional oscillators to daily timing in eukaryotes await further study.
5. Homologs of the *Arabidopsis* clock genes control photoperiodic, seasonal rhythms in crops and trees. Genetic variation in these genes can contribute to crop domestication, and latitudinal variation has been described in some species.
6. Circadian rhythms are sensitive to the environment, and plant rhythms are now being measured in detail under natural conditions. A current challenge is to understand the link from circadian timing to physiological traits in the field.

7. Mathematical models are essential to define and test the quantitative, mechanistic links from molecular regulation to field traits. The *Arabidopsis* Framework Model suggests a modular, community-based approach to their construction.

FUTURE ISSUES

1. If synthetic promoters can direct plant gene expression to predictable phases—or, better, to predictable waveforms in diel cycles—then can synthetic genomics reengineer the temporal expression of all genes for a metabolic or signaling pathway in order to test the contribution of their rhythmic control to plant growth?
2. Why are the known gene circuits of plant clocks more complicated than the clocks of animals with comparable genome sizes (fungal and cyanobacterial clocks might be genome limited)? Does this result from an evolutionary requirement, an experimental artifact (confounding multiple tissue-specific clocks, for example), or a preference of the scientists involved? Our genetic screens aspired to identify all discoverable *Arabidopsis* clock genes, as in cyanobacteria. By contrast, Konopka's rule, based on his discovery of the fly *period* mutants in an earlier era, advises, "If you don't find it in the first two hundred, then quit" (146).
3. How and when does a cell's "ecology of oscillators," including the nontranscriptional oscillator that controls peroxiredoxin, support or supplant rhythmic regulation by the canonical clock gene circuit?
4. What new mathematical methods will allow us to analyze and understand the dynamic coupling of multiple oscillators to biological processes, without abstracting so much that we lose contact with measurable biochemical entities?

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