Published in final edited form as:

Curr Opin Plant Biol. 2010 February ; 13(1): 83. doi:10.1016/j.pbi.2009.09.007.

***Arabidopsis* circadian clock and photoperiodism: time to think about location**

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Summary of recent advances

Plants possess a circadian clock that enables them to coordinate internal biological events with external daily changes. Recent studies in *Arabidopsis* revealed that tissue specific clock components exist and that the clock network architecture also varies within different organs. These findings indicate that the makeup of circadian clock(s) within a plant is quite variable. Plants utilize the circadian clock to measure day-length changes for regulating seasonal responses, such as flowering. To ensure that flowering occurs under optimum conditions, the clock regulates diurnal *CONSTANS* (*CO*) expression. Subsequently, *CO* protein induces *FLOWERING LOCUS T* (*FT*) expression which leads to flowering. It is emerging that both *CO* and *FT* expression are intricately controlled by groups of transcription factors with overlapping functions.

Introduction

In order to adapt to daily fluctuations in the surrounding environment, most organisms have acquired a circadian clock, a vital mechanism that regulates time-dependent biological events. Therefore, plants that are unable to synchronize internal events with external daily changes suffer a severe loss of environmental fitness [1]. The earth's tilted rotational axis causes day length to vary throughout the year. These differences in length are especially dramatic at higher latitudes. Organisms have evolved ways of optimizing their behaviors and development to align with these changes. To anticipate seasonal variation, organisms measure alterations in day length using their circadian clocks and by sensing light and temperature conditions [2]. In many plants, changes in day length regulate the most important developmental transition, from a vegetative growth phase to a reproductive phase [2]. Our understanding of the molecular mechanisms of circadian clocks and day-length-dependent (photoperiodic) flowering time regulation has advanced very rapidly in recent years. Here I introduce recent findings regarding the circadian clock and day length measurement mechanisms in *Arabidopsis thaliana*.

Molecular circuit of the circadian clock

Computational analyses predict that the *Arabidopsis* clock is composed of at least three interlocking loops [3,4] (Figure 1). In this model, morning-expressed CIRCADIANT CLOCK-ASSOCIATED 1 (*CCA1*)/LATE ELONGATED HYPOCOTYL (*LHY*) and PSEUDO RESPONSE REGULATOR 7 (*PRR7*)/*PRR9* comprise one loop, while evening-peaked

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TIMING OF CAB EXPRESSION 1 (TOC1) and hypothetical clock component Y form another loop. The CCA1/LHY-PRR7/9 loop and the TOC1-Y loop are connected by the originally proposed negative feedback loop, which is formed by CCA1/LHY and TOC1 [5]. Most TOC1 proteins are degraded by the photoreceptor F-box protein ZEITLUPE (ZTL) before dawn [6, 7]. Because TOC1 protein likely induces *CCA1* and *LHY* expression indirectly in the morning, TOC1 regulates *CCA1/LHY* expression via hypothetical component X [8]. Although this model does not contain all known clock components, it provides a testable framework of the plant circadian clock architecture.

It was proposed that a candidate for Y could be GIGANTEA (GI) protein [3,8]. However, GI by itself does not fulfill the entire role of Y, mainly due to the lack of a clear genetic connection in the transcriptional regulation between *GI* and *TOC1* [9,10] (Figure 1). On the other hand, GI protein increases the amplitude of the daily TOC1 protein oscillation by stabilizing ZTL protein [7]. Since GI enhances the peak expression of TOC1 protein, GI could still contribute a part of Y activity. Thus it is implied that multiple factors constitute Y activity, including GI.

Closing the CCA1-TOC1 loop

Because of the unknown molecular nature of TOC1 function, the mechanism by which TOC1 induces *CCA1* and *LHY* expression remained elusive until recently. CCA1 HIKING EXPEDITION (CHE), which is a TCP (TEOSINTE BRANCHED1, CYCLOIDEA, and PCF)-class transcription factor, functions as a TOC1-associated regulator of *CCA1* [11••]. TOC1 physically interacts with CHE, which acts as a transcriptional repressor of *CCA1*, and both proteins associate with a similar *CCA1* promoter region. Reciprocally, CCA1 binds to the evening elements in the *CHE* promoter and inhibits its expression. In addition, *CHE* overexpression weakens the long period phenotype caused by elevated levels of *TOC1* expression. Although the molecular relationship between CHE and TOC1 in *CCA1* expression regulation is not completely understood, these results indicate that TOC1 regulates *CCA1* expression in part by forming a complex with CHE, and that CHE and CCA1 form an additional feedback loop (Figure 1). Since CHE does not regulate *LHY* expression, and because *CCA1* is still expressed in *che* mutants, it is likely that other factors participate in the regulation of *CCA1* and *LHY* induction. As the identification of CHE partially closes the CCA1/LHY-TOC1-X loop, X activity (like Y activity) seems to comprise multiple proteins.

In addition to CHE, several new components that function within or adjacent to the circadian oscillator(s) have been identified within the last two years. These include FIONA1 (FIO1) [12], XAP5 CIRCADIAN TIMEKEEPER (XCT) [13], LIGHT-REGULATED WD1 (LWD1) and LWD2 [14]. Although the mechanisms by which these proteins affect circadian rhythms remain unknown, plants harboring mutations in these genes have altered period length and different clock-associated physiological responses, such as flowering time regulation and photomorphogenesis. These results further confirm the importance of proper daily time-keeping mechanisms in the regulation of physiological responses. The next challenges regarding these newly identified clock factors are to understand their molecular roles within the currently proposed circadian clock circuits and to elucidate the molecular connection between the circadian oscillator and the physiological processes it controls.

Structural plasticity of the clock circuit

A previous report has suggested that plants may have different circadian clocks in their aerial parts than in their roots [15]. A recent report provided molecular evidence that in certain tissues not all clock components participate in forming a functional clock, even though they are expressed in the same cells. In mature roots grown in light-tight boxes, genes in the CCA1/LHY-PRR7/PRR9 loop oscillate diurnally. However, evening-expressed clock genes such as *TOC1*, which have the evening elements (=CCA1/LHY binding sites) in the promoters, are

expressed at similar levels throughout the day in the roots when the whole plants were incubated under constant light conditions [16••]. Under these conditions, the timing of the peak expression of *CCA1*, *LHY*, *PRR7* and *PRR9* is different between the aerial parts and roots. In addition, the *toc1* mutation does not affect period length in the roots, but causes short-period phenotype in the shoots, indicating that TOC1 does not participate in period-length regulation in the mature roots [16••]. Since the *toc1* mutant roots from young seedlings show the short-period phenotype [15], the degree of TOC1 involvement in the root clock could possibly be age-dependent. Interestingly, in the *prp9 prr7* mutant roots, *TOC1* shows circadian oscillation [16••]. These results suggest that the uncoupled evening-phased loop can be connected to the morning-phased loop under certain circumstances. Two different clock-regulated genes, both expressed in the same tissues but with one having a morning peak and the other an afternoon peak, responded to temperature shifts differently [17]. Based on current observations, the two clock outputs might be regulated by different transcriptional loops (Figure 1), which under some conditions are uncoupled from each other. Moreover, a photosynthesis-related signal, possibly sucrose, may entrain the root clock [16••]. As photosynthesis activity is regulated by the circadian clock [1], this result indicates the presence of a mechanism that couples circadian clocks between different organs.

Tissue-specific circadian clock

Many circadian clock output genes that control various physiological events are expressed in specific tissues or cells. For instance, the expression of clock-regulated genes that are involved in photoperiod measurement is often restricted in vasculature tissues (Figure 1), while the expression of the well-known clock output gene, *CHLOROPHYLL A/B-BINDING PROTEIN 2* (*CAB2*, also known as *LHCB1*1*), largely occurs in leaf mesophyll cells. However, it was uncertain whether plants possessed tissue-specific clock(s) to regulate tissue-specific signaling pathways. Recent findings suggest that the expression of some clock components is indeed tissue specific. The evening-expressed clock gene *PRR3* is mainly expressed in aerial vascular tissues [18••]. Both *PRR3* and *TOC1* proteins are highly phosphorylated at night and the phosphorylation enhances the direct interaction between *PRR3* and *TOC1* [19]. This interaction, which may primarily occur in vascular tissues, protects *TOC1* protein from ZTL-dependent degradation [18••]. This explains the stronger circadian clock phenotype in the vascular tissues compared to the mesophyll cells of *prp3* mutants [18••]. Spatial expression pattern analysis revealed that *PRR9* might also be strongly expressed in the vascular tissues [18••], implying that the *prp9* mutant would also show a stronger phenotype if circadian rhythms in vascular tissues were analyzed. These studies suggest that the circadian clocks in vascular cells and cells in the rest of the leaf may consist of different components. Notably, the core day length-sensing mechanism regulating flowering time resides in leaf phloem [20]. Also, phloem and xylem are essential for daily transport of sugar, water and minerals. Thus, plants may need specific sets of clock proteins in the vasculature to precisely coordinate the daily traffic of solute transport as well as seasonal measurement.

Photoperiodic flowering regulation

Plants defective in the day-length sensing mechanism can not properly time flowering in the natural environment [21•]. Circadian clock regulation of *CONSTANS* (*CO*) gene expression and light-dependent regulation of CO protein stability are important components in the monitoring of changes in day length [20]. This biphasic regulation of CO ensures that its target, *FLOWERING LOCUS T* (*FT*), is induced specifically in long days. FT protein is synthesized in the leaf vasculature and then translocated to the shoot apical meristem, where it interacts with the bZip transcription factor FD to initiate the transcriptional cascade of floral transition [22,23]. Recent evidence indicates that FT protein is a major part of the long-sought floral inducer ‘florigen’ [24]. There are several recent reviews that focus on the florigen molecule

[25–27]. Here I summarize new insights into the molecular mechanisms by which plants measure day length changes in leaves (Figure 1).

Circadian clock-dependent *CO* regulation

The circadian clock regulates *CO* transcription partly by degrading *CO* repressors at a specific time of day in long days (Figure 2). CYCLING DOF FACTOR1 (*CDF1*) is one of these *CO* repressors [28]. Multiple morning-phased Dof transcription factors, which are homologous to *CDF1*, are also involved in the repression of *CO* [29••]. Overexpression of *CDF2*, *CDF3*, and *CDF5* in phloem causes late flowering in long days. In the *cdf1 cdf2 cdf3 cdf5* quadruple mutant, daytime *CO* expression level is elevated throughout the day and the mutant no longer responds to changes in day length. This indicates that *CDF1*, *CDF2*, *CDF3*, and *CDF5* redundantly repress *CO* expression during the day.

In the afternoons of long days, *CDF1* expression is strongly repressed by *PRR9*, *PRR7* and *PRR5* [30]. At the same time, *CDF1* and *CDF2* proteins are degraded by the GI and FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (*FKF1*) complex [29••,31]. *FKF1*, a ZTL homolog, is a photoreceptor that possesses ubiquitin ligase activity [28,32]. The interaction between *FKF1* and GI is induced by blue light, which is perceived by *FKF1* [31] (Figure 2).

At night, GI is degraded via the E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENESIS 1 (*COP1*) [33•]. The *COP1*-GI interaction requires EARLY FLOWERING 3 (*ELF3*), a circadian gating factor [34], which functions as an adaptor protein in-between [33•] (Figure 2). *FIO1*, *LWD1* and *LWD2* seem to be involved in the suppression of *CO* in the dark, although it is not certain whether these proteins by themselves directly influence *CO* transcription [12,14]. These results clearly demonstrate that plants utilize multiple circadian-regulated factors to precisely control the diurnal *CO* expression pattern.

FT transcriptional regulation

We are obtaining more evidence that various clock-regulated components act coordinately to regulate *FT* expression throughout the day and also throughout plant development (Figure 1). Once *CO* transcript is expressed in the afternoon in long days, *CO* protein is stabilized by phytochrome (*phy*) and cryptochrome (*cry*) photoreceptor signals to ensure induction of *FT* [35]. In the dark, *CO* protein is degraded by the SUPPRESSOR OF *PHYA*-105 1 (*SPA1*)-*COP1* complex [36–38]. Cryptochrome regulates flowering time via other mechanisms than control of *CO* stability as well. *Cry2* binds to the CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX 1 (*CIB1*) transcription factor under blue light. *CIB1* binds to the *FT* promoter and *CIB1* overexpression elevates the *FT* expression level without affecting the *CO* expression pattern [39•]. Thus, *cry2* regulates *FT* expression through both *CIB1* and *CO*. *CO* may induce *FT* by forming a DNA binding complex with HEME ACTIVATOR PROTEIN (*HAP*) proteins [40,41]. The *CO* homolog CONSTANS-LIKE 5 (*COL5*) may also participate in *FT* induction. *COL5* overexpression increases *FT* expression without changing *CO* expression and induces early flowering in short days [42].

Many transcription factors, such as TEMPRANILLO 1 (*TEM1*), *TEM2*, TARGET OF *EAT1* (*TOE1*), *TOE2*, SCHLAFMÜTZE (*SMZ*), SCHNARCHZAPFEN (*SNZ*), SHORT VEGETATIVE PHASE (*SVP*) and FLOWERING LOCUS C (*FLC*), all seem to function as *FT* [43–47]. *TEM1* and *TEM2*, both of which belong to the RAV (Related to *ABI3*/VP1) transcription factors, bind to the *FT* promoter [43]. During seedling growth, as *TEM1* expression levels decreases, *FT* expression levels increase. The spatial expression patterns of *TEM1* change throughout leaf development. *TEM1* is expressed evenly in young leaves. As leaves grow, expression gradually decreases from the proximal part of the leaves toward the periphery [43]. *APETALA2* (*AP2*), and the *AP2*-related genes *TOE1*, *TOE2*, *TOE3*, *SMZ*, and

SNZ transcripts are all targets of microRNA172 (miR172); TOE1, TOE2, SMZ, and SNZ redundantly repress *FT* [45,48,49]. Among these, at least SMZ directly associates with *FT* chromatin [45]. In addition, GI regulates miR172 expression, which increases in long days [44]. FLC and SVP MADS-box transcription factors form a heterodimer that directly represses *FT* expression in leaves [46,47]. CCA1 and LHY decrease SVP protein stability, possibly through protein-protein interaction via ELF3 under continuous light conditions [50,51].

In addition to these transcription factors, chromatin remodeling factors are also involved in *FT* repression. The polycomb repressive complex 2 (PRC2) components mediate histone H3 lysine 27 trimethylation on the *FT* chromatin region to maintain repression of *FT* expression and prevent precocious flowering [52]. In sum, we have now identified many regulators that affect *FT* expression. It will be fascinating to decipher how exactly these various transcription factors developmentally orchestrate *FT* expression in time and space.

Circadian clock regulation of diurnal growth and metabolism

The circadian clock controls not only seasonal development but also daily growth and metabolism. When seedlings are grown under daily light-dark cycles, hypocotyl growth mainly occurs around dawn [53•]. This diurnal growth pattern is regulated by the interaction between light signaling and the circadian clock [53•]. Diurnal growth is in part attributed to the clock regulation of *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) and *PIF5* transcription and their protein stability [53•,54]. A study using natural phenotypic variation also led to the identification of the TANDEM ZINC KNUCKLE/PLU3 (*TZP*) protein that affects diurnal growth [55]. *TZP* expression oscillates with a morning peak and *TZP* regulates the expression of auxin-related genes, which possibly contribute to morning-specific growth. In addition, the results of genome-scale gene expression analyses imply that the timing of diurnal stem growth likely correlates with the increase in transcription of various hormonal synthesis and signal transduction pathways around dawn [56,57]. Recently, a direct molecular connection linking the light and gibberellin (GA) pathways to regulation of stem growth was elucidated. DELLA proteins, repressors of the GA pathway, repress the transcriptional activity of *PIF3* and *PIF4*. This repression is released by GA-mediated DELLA protein degradation [58,59]. Therefore, the time-dependent degradation of DELLA proteins may participate in dawn-specific growth mechanisms.

More broadly, metabolomic analysis reveals that the PRRs may represent the link between the circadian clock and coordinated daily changes in metabolism. In the *prp5 prp7prp9* triple mutant, the amounts of tricarboxylic acid (TCA) cycle intermediates and abscisic acid (ABA) are elevated, suggesting that these PRRs have output function in mitochondrial and chloroplastic metabolism [60•]. PRR5, PRR7 and PRR9 proteins also mediate metabolic changes accompanied with cold-, salt- and drought-stress responses [61].

Conclusions and perspective

The molecular clock architecture in plants is more diverse and flexible than previously thought. The presence of multiple circadian clocks had been predicted in several reports, and it is emerging that the molecular makeup of plant circadian clocks varies in specific tissues. In addition, as we saw in the case of the root specific clock, the network architecture of the circadian clock seems to be rather plastic. Thus, in the future, an area of great interest will be the composition of the clocks in specific organs, tissues, and even cells. This will enable us to more accurately understand the molecular connections that allow the circadian clock to regulate many physiological responses.

In the regulatory pathway of photoperiodic flowering, there is already strong evidence for multiple layers of functionally redundant factors in the regulation of *CO* and *FT* transcription.

One of our challenges in the near future will be to understand the complex mechanisms by which these factors coordinate to regulate seasonal flowering response. Also, it would be intriguing to note how much similarity there is in the molecular levels of the clock and photoperiodic pathways between various plant species.

Acknowledgments

I thank Drs Stacey Harmer, Eva Farré, and Marcelo Yanovsky for critical reading of the manuscript. I apologize to colleagues whose work was not cited due to space constraints. Preparation of this review was supported by the National Institute of Health (R01GM079712).

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Figure 1.

A model for the circadian clock and the photoperiodic flowering pathway in *Arabidopsis*. The most recently proposed clock architecture comprises three feedback loops (depicted in three different colors within the pale green circle that indicates the circadian clock). In the computational model CCA1 and LHY were treated as one functional unit, rather than two different proteins. Recent studies show that CCA1 and LHY form a functional unit by dimerization *in vivo* [62,63]. The CCA1/LHY-PRR7/9 loop is the only one that oscillates in mature roots under constant light condition [16••]. Several known clock components are not in the diagram due to their undetermined positions in the circuit. The diagram that is outside the pale green circle area denotes the clock-regulated photoperiodic pathway. Oval symbols depict proteins and overlapping ovals indicate protein complexes. Thick solid lines denote direct physical interactions, while thin solid lines denote genetic interactions. The dotted lines depict transcription and translation. The leaf cartoon indicates gene products that may be expressed mainly in leaf vascular tissues, based on studies of promoter-driven β -glucuronidase (GUS) reporter lines. The proteins that are directly involved in *CO* and *FT* gene expression and have gene and/or protein expression that is regulated by the circadian clock are shown with the clock cartoon symbol. The expression of genes which lack published gene or protein expression data under constant light conditions was analyzed using the publicly available

microarray data at the DIURNAL project website [64]. Note that only a subset of the *HAP* genes shows daily oscillation in expression. All components in this diagram are likely expressed in the vasculature; therefore, this diagram may depict the connections between the circadian clock and photoperiodic flowering pathways in vascular tissues.

Figure 2.

Schematic representation of the diurnal regulation of *CO* expression. In the morning, the expression levels of *CDF1*, *CDF2*, *CDF3*, and *CDF5* genes are high. These CDF proteins likely bind to the *CO* promoter and are involved in suppression of *CO* transcription. The transcription of afternoon-phased *GI* and *FKF1* is thought to be directly repressed by *CCA1* and *LHY*. In the afternoon, the expression of these four *CDF* genes declines, and at least *PRR5*, *PRR7* and *PRR9* participate in the down-regulation of *CDF1* expression. Decreasing levels of *CCA1* and *LHY* release the repression *FKF1* and *GI* and allow them to be expressed at high levels. Once *FKF1* observes blue light, *FKF1* forms a complex with *GI*. This complex is involved in the degradation of *CDF1* and *CDF2*, and possibly other CDFs. Removal of *CO*

repressors facilitates the expression of *CO*. At night, GI is degraded by a COP1 complex that contains ELF3. COP1 directly interacts with ELF3 to control ELF3 protein turnover [33•]. COP1 forms a complex with GI via ELF3 and also regulates GI stability. GI is required for the stabilization of FKF1 protein [29••], although it is not certain whether FKF1 is also degraded by COP1. Symbols have the same meaning as in Figure 1. Ub stands for ubiquitin.