MYB transcription factors in the Arabidopsis circadian clock

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Abstract

The LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED (CCA1) genes encode closely related MYB transcription factors, which regulate circadian rhythms in Arabidopsis. LHY and CCA1 verify some of the properties of oscillator components, since (i) expression of their transcripts and protein exhibits circadian oscillations; (ii) their constitutive expression abolishes overt rhythmicity and (iii) they function as components of a negative transcriptional feedback loop. LHY and CCA1 have been proposed to function in conjunction with the pseudo response regulator TOC1, as components of the circadian oscillator. The regulation of their respective transcripts and protein levels in response to light signals suggests that these proteins may also mediate the regulation of circadian rhythms by light. This review discusses experimental evidence for these hypotheses.

Key words: Arabidopsis, circadian clock, light, MYB transcription factors, temperature.

Introduction

The impact of circadian rhythms on physiology is better characterized in plants than in any other system (Harmer et al., 2000), but the molecular mechanism of the underlying oscillator is not yet understood. Genetic approaches in model organisms, such as Neurospora, Drosophila, mice, and cyanobacteria, have identified the molecular components of their respective circadian clocks (reviewed in Dunlap, 1999; Bell-Pedersen, 2000; Young, 1998; Scully and Kay, 2000; Whitmore et al., 2000; Reppert and Weaver, 2000; Kondo and Ishiura, 2000). Similar approaches have been undertaken more recently in Arabidopsis, and at least 20 genetic loci have been shown to regulate the function of the circadian clock (see Roden and Carré, 2001, for a review). A number of the cognate genes have been cloned (Schaffer et al., 1998; Wang and Tobin, 1998; Fowler et al., 1999; Strayer et al., 2000; Somers et al., 2000). However, so far, none of these genes encode orthologues of clock components from any other system and it is not known at present how they interact to generate circadian rhythms.

Conceptually, the circadian system can be divided into three components. Circadian time-keeping is mediated by a central oscillator, which is thought to consist of a biochemical feedback loop. To be useful as a clock, this oscillator has to be set to local time. The environmental cues that set the phase of the clock are usually light and temperature, and the signal transduction pathways that reset the clock in response to these signals are known as input pathways. Downstream of the oscillator, other signal transduction pathways, known as output pathways, mediate the control of overt rhythms. Many of the loci that regulate the function of the Arabidopsis clock appear to function in light input (Roden and Carré, 2001; Devlin, 2002), but so far only three genes have been proposed to function as components of the circadian oscillator. The TIMING OF CAB 1 (TOC1) gene has also been described as ARABIDOPSIS PSEUDO RESPONSE REGULATOR 1 (APRRI). It comprises a sequence that is related to the receiver domain of two component-signalling systems of plants and animals, and a region that is similar to the flowering time regulator CONSTANS (Strayer et al., 2000; Makino et al., 2000). The two other putative oscillator genes encode closely related transcription factors of the MYB family, LHY and CCA1. This paper describes the experimental approaches that enabled the identification of these candidate clock genes and the rationale used to probe
their functions in the *Arabidopsis* circadian clock. This review aims to be accessible, rather than exhaustive, and apologies are extended to those whose work has not been mentioned here. More detailed overviews of the plant circadian field have been published elsewhere (McClung, 2001; Roden and Carré, 2001).

**Identification of circadian clock mutants in Arabidopsis**

The isolation of mutants in any process requires a phenotype that can be scored easily, reliably and in a non-invasive manner. *Arabidopsis* mutants with deficient circadian clocks have been identified by different methods. First, transgenic plants were produced that carried a firefly luciferase reporter gene (luc) under the control of the circadian-regulated *CAB* (*CHLOROPHYLL a/b-BINDING PROTEIN*) promoter. The activity of the luciferase reporter gene was rhythmic in these plants, and could be measured *in vivo* using a photon-counting camera (Millar et al., 1992). These transgenic plants were then mutagenized using EMS and their M2 progeny was screened for abnormal rhythms of bioluminescence in constant light (LL). This screen resulted in the isolation of a number of long and short period mutants (Millar et al., 1995). The *toc1* mutant was particularly interesting as its circadian phenotypes were completely independent of illumination conditions (Somers et al., 1998). The mutation shortened the free-running rhythm of *cab:luc* expression to similar degrees under all fluences of light. When plants were grown under 24 h light/dark cycles, the peak of *CAB* gene expression was advanced in *toc1* relative to the wild type. This phase defect was argued to reflect the abnormal function of the central oscillator, rather than aberrant light input to the clock, because a similarly altered phase was observed in constant light under 24 h temperature cycles.

Another set of mutants was identified by their abnormal flowering time phenotypes. Many plants and animals enter their reproductive phase at specific times of the year, and the perception of changes in seasons relies primarily on the measurement of daylength, or photoperiod. The measurement of photoperiod is thought to be mediated by a circadian clock (Carré, 2001), therefore a subset of mutations that cause abnormal floral responses to daylength were expected to cause abnormal circadian rhythms. For example, the *early flowering 3* (*elf3*) mutant flowered much earlier than wild-type plants under short-day photoperiods (8L16D, the alternation of 8 h of light and 16 h of darkness), *elf3* was a very good candidate for a clock mutant, since it flowered at the same time and at the same developmental stage, regardless of photoperiod. The *cab:luc* reporter gene was introduced into the *elf3* mutant by genetic crossing, and its expression was monitored under different light/dark conditions. Interestingly, *cab:luc* expression was arrhythmic in *elf3* plants under constant light conditions, suggesting that the function of their circadian clock was disrupted (Hicks et al., 1996). But *elf3* mutant plants exhibited rhythmic *cab:luc* expression under diurnal light/dark cycles, and these oscillations persisted for one to two cycles following transfer to constant darkness (DD). This result indicated that *ELF3* function was not required for circadian oscillations. Since the arrhythmic phenotype of the *elf3* mutant was conditional upon constant illumination of the plants, the mutation was proposed to alter the regulation of the circadian clock by light (Hicks et al., 1996; McWatters et al., 2000).

Like *elf3*, the *late elongated hypocotyl* (*lhy*) mutant exhibited daylength-insensitive flowering, but its flowering time was intermediate between that of wild-type plants grown under short or long days (Schaffer, 1997). Circadian rhythms of leaf movements were abolished by the *lhy* mutation, and *cab:luc* expression was arrhythmic in LL and in DD. In addition, the *lhy* mutation disrupted the rhythmic expression of the *CCR2* (*COLD-CIRCADIAN RHYTHM-RNA-BINDING*) gene, which normally peaks in the evening, several h after *CAB* (Schaffer et al., 1998). Since the *lhy* mutation disrupted several overt rhythms and the oscillation of genes that peaked at different times of the day, it was unlikely to disrupt the function of a specific output pathway. This suggested that the *LHY* gene may either mediate or regulate the function of the circadian oscillator.

**LHY and the closely related gene CCA1 may encode redundant components of a negative transcriptional feedback loop**

The *lhy* mutation was caused by the insertion of a transposon (*Ds*) within the 5′ untranslated region (5′UTR) of the *LHY* gene. The *Ds* element comprised a strong promoter (35S, from the cauliflower mosaic virus) which caused the overexpression of the adjacent open reading frame. Transformation of wild-type plants with a cosmid clone comprising the *Ds* element and the adjacent open reading frame was sufficient to reproduce the effect of the *lhy* mutation in transgenic plants. These results, and the dominant nature of the *lhy* mutation suggested that it resulted from a gain of function allele, caused by the overexpression of the *LHY* gene (Schaffer et al., 1998).

The amino acid sequence of *LHY* revealed homology to the MYB family of transcription factors. However, *LHY* encoded an unusual member since its DNA-binding domain comprised a single MYB repeat instead of two or three as in most MYB proteins (Jin and Martin, 1999). It showed a high level of similarity to *CCA1* (*CIRCADIAN CLOCK-ASSOCIATED-1*), a protein that bound to a conserved light-regulated element of the *Arabidopsis* *CAB* (*Chl-b*) gene (Wang et al., 1997). *LHY* and *CCA1* have virtually identical DNA-binding domains, and show strong homology throughout their protein sequences (Schaffer et al., 1998). *CCA1* was also shown to play a role similar to that of *LHY*, in the regulation of circadian rhythms. As with *LHY*, overexpression of the *CCA1***
transcript under the control of the 35S promoter abolished the rhythmic expression of CAB, CCR2 and CATALASE 3 (CAT3) (Wang and Tobin, 1998).

The LHY and CCA1 transcripts were both expressed rhythmically, and peaked shortly after dawn. These rhythms persisted in constant light and in constant darkness, showing that both genes were under circadian control (Schaffer et al., 1998; Wang and Tobin, 1998). The rhythmic expression of the LHY and CCA1 mRNAs was abolished in lhy1 and CCA1-overexpressing (CCA1-ox) plants, respectively. Since the constitutive expression of these transcripts correlated with arrhythmic phenotypes, the rhythmic expression of LHY and CCA1 was suggested to be required for the expression of circadian rhythmicity.

Intriguingly, LHY and CCA1 regulated their own expression, as well as each other’s. Plants that carried a transgenic, overexpressed copy of either gene exhibited arrhythmic expression of the endogenous LHY and CCA1 transcripts. Furthermore, the expression of both endogenous genes was repressed to the trough level of wild-type plants. These results suggested that LHY and CCA1 may function redundantly as components of a negative feedback loop (Schaffer et al., 1998; Wang and Tobin, 1998).

**LHY and CCA1 exhibit properties of circadian oscillator components**

Oscillatory feedback loops have been found at the core of all circadian clock mechanisms examined so far (Dunlap, 1999). Negative feedback loops are known to give rise to oscillations and are thought to constitute the timing component of circadian systems. Therefore, the observation that LHY and CCA1 negatively regulated the expression of their own transcripts was provocative, since it suggested that they may both function as part of the circadian oscillator. However, plants that carried loss of function mutations in the LHY or CCA1 genes exhibited robust rhythms of gene expression and leaf movements (Green and Tobin, 1999; Mizugochi et al., 2002). The period of the rhythms was shortened by 2–3 h, but circadian rhythmicity was otherwise not impaired. Thus, neither LHY nor CCA1 were required for the function of the circadian oscillator. However, the similarities between these two proteins suggested that they might be able to substitute for each other’s function. To test this hypothesis, double mutants lacking functional copies of both genes were constructed. These lhy-11 cca1-1 plants exhibited diurnal rhythms of gene expression (lhy:luc, cca1:luc, and ccr2:luc) with an abnormal phase, but the amplitude of these rhythms was gradually reduced and no significant oscillations of transcript levels were detected on RNA blots after 3 d in constant light (Mizugochi et al., 2002). A similar loss of circadian rhythmicity was observed after transfer to constant darkness, suggesting that this phenotype did not reflect aberrant light input to the clock (Hae-Ryong Song and Isabelle Carré, unpublished results).

When grown under light/dark cycles, the lhy-11, cca1-1 and double knock-out mutants also exhibited an early phase of expression of both morning (LHY, CCA1) and evening (CCR2) genes. As previously described for toc1, a similar phase alteration was observed under temperature cycles. These results indicated that the lhy-11 and cca1-1 mutations altered the properties of the central oscillator, rather than its regulation by specific input pathways.

**Reciprocal regulation between LHY/CCA1 and TOC1**

What is the function of LHY and CCA1 within the circadian clock? When a construct comprising the coding region of LHY fused to the green fluorescent protein (GFP) was transformed into onion or Arabidopsis cells, fluorescence was localized to the nucleus (Fig. 1). The nuclear localization of LHY is consistent with its proposed function as a transcription factor. Furthermore, LHY and CCA1 were shown to bind specifically a DNA sequence that is found within the promoter of many rhythmic genes that peak near dusk (Alabadi et al., 2001; Harmer et al., 2000). Interestingly, this 'evening element' (EE) was present within the promoter of the putative oscillator component TOC1. This suggested that LHY and CCA1 might regulate TOC1 expression in a direct manner.

In wild-type plants, expression of the TOC1 transcript exhibited circadian oscillations (Strayer et al., 2000). Maximum transcription of TOC1 at dusk coincided with minimal expression of LHY and CCA1 proteins. In plants that overexpressed either LHY or CCA1, TOC1 expression was arrhythmic in constant light. Most importantly, it was repressed to a level close to the trough of the wild-type rhythm. This result suggested that LHY and CCA1 inhibit transcription from the TOC1 promoter (Alabadi et al., 2001).

The reciprocal regulation of clock-associated genes is central to the function of all circadian oscillators (Dunlap, 1999). It was therefore important to test whether TOC1 regulated transcription from the LHY and CCA1 promoters. Expression of both LHY and CCA1 transcripts was reduced in a presumed loss-of-function allele, toc1-2, suggesting that TOC1 may function as a positive effector of LHY and CCA1 expression (Alabadi et al., 2001). It is not known at this point whether this regulation is direct or not. The TOC1 protein does not exhibit any recognizable feature that would mediate its binding to DNA. It is therefore likely that its function requires one or more protein partners.

**A working model for the Arabidopsis circadian clock**

The results discussed above suggested that the MYB transcription factors LHY and CCA1 might function as the negative elements of a transcriptional feedback loop, a role similar to those of PERIOD (PER) and TIMELESS (TIM) in Drosophila, or FREQUENCY (FRQ) in Neurospora. TOC1 might function as a positive element, a role similar
to those of CLOCK (CLK) and CYCLE (CYC) in Drosophila, or WHITE COLLAR 1 and 2 (WC1 and WC2) in Neurospora. Alabadi and colleagues (2001) proposed that the reciprocal regulation between TOC1 and LHY/CCA1 might define the basic framework for the clock mechanism in Arabidopsis (Fig. 2). Expression of the negative elements (LHY and CCA1) in the morning would repress expression of the positive element (TOC1), leading to a decay in LHY and CCA1 transcript levels in the course of the day. Decreasing levels of CCA1 and LHY protein would mediate the derepression of the TOC1 gene and allow its transcription to resume in the early evening. The subsequent accumulation of TOC1 protein at night would result in increased transcription from the LHY and CCA1 promoters, through an unknown mechanism.

**Entrainment to light/dark cycles**

In natural conditions the phase of circadian oscillations is locked to that of environmental cycles, a phenomenon known as ‘entrainment’. The entrainment of circadian clocks to light/dark cycles is generally mediated by light-induced changes in the level of a component of the oscillatory feedback loop. Thus, in Neurospora, light induced increases in the level of FRQ transcript. By contrast, the effects of light on the Drosophila clock were mediated by light-induced decreases in the level of TIM protein. Thus, if LHY and CCA1 function as elements of the circadian oscillator, light-induced increases in their expression levels would be expected to reset the phase of the clock.

CCA1 was originally identified as a protein that mediated the regulation of CAB transcription by light (Wang et al., 1997). In etiolated seedlings, expression of the CCA1 transcript was induced by red light, and transgenic plants that carried an antisense CCA1 construct exhibited reduced induction of their endogenous CAB gene. A similar pattern of light induction in etiolated plants was reported for LHY (Martinez-Garcia et al., 2000). However, in light-grown plants, the regulation of LHY expression by light was mainly post-translational (Jae-Yean Kim, Hae-Ryong Song and Isabelle Carré, unpublished results). In plants that carried a constitutively transcribed copy of the gene, the LHY protein accumulated in response to light. This accumulation was caused by the increased rate of accumulation of the protein, rather than by its accelerated turnover. The translational induction of LHY by light was only observed at specific phases of the circadian cycles, however, as it was limited to times when the transcript was already present (around dawn). It is not known yet whether the effects of light on expression of the CCA1 protein are gated in the same manner.
Further levels of complexity

If LHY plays a role in the resetting of the clock in response to light signals, maximum phase-shifting responses should coincide with times when LHY expression is most inducible. Light pulses perceived early in the night caused phase advances, and similar signals given late in the night induced phase delays (Covington et al., 2001; Jae-Yean Kim, Hae-Ryong Song and Isabelle Carré, unpublished results). The greatest responses were observed in the middle of the night (CT18), a phase clearly distinct from that when LHY expression was responsive to light (ZT1). These results suggest that the induction of LHY by night does not mediate entrainment of the clock to light/dark cycles. Light-induced increases of LHY levels at dawn may serve to maintain high amplitude oscillations of the central pacemaker. Alternatively they may serve to regulate the amplitude of oscillation of output genes such as CAB, that exhibits dual regulation by light as well as by the clock.

The model proposed in Fig. 2 for the mechanism of the Arabidopsis clock is satisfying at first sight, because it fits well within the framework established for circadian clock mechanisms of other organisms. But less than a year after its publication by Alabadi and colleagues, new data indicate that it is insufficient to explain mutant phenotypes and to describe the regulatory interactions between known clock components. For example, the constitutive over-expression of TOC1 would be expected to cause an arrhythmic phenotype, as previously shown for LHY and CCA1. Yet, overexpression of TOC1 in transgenic plants did not abolish circadian oscillations, although the amplitude of gene expression rhythms was reduced (Makino et al., 2002). The rhythmic phenotype of TOC1-overexpressing plants might be explained, if TOC1 function requires association with a protein partner that is also expressed rhythmically. However, such a partner remains to be identified. What is more confusing is that expression of LHY and CCA1 transcripts in these plants was repressed to the trough of the wild-type rhythm, instead of increased as predicted by the model. On the other hand, plants that lacked LHY or CCA1 function or both, displayed reduced (rather than increased) levels of expression of lhy:luc and cca1:luc reporter fusions (Bethan Taylor, Hae-Ryong Song and Isabelle Carré, unpublished...
results). These results do not necessarily rule out the hypothesis that the LHY1, CCA1 and TOC1 exhibit the cross-regulation described in Fig. 2, but they suggest that the core mechanism of the plant circadian oscillator comprises additional levels of regulation.

The circadian oscillator of Arabidopsis might comprise multiple loops, mediating positive as well as negative feedback. Such interlocked feedback loops have been described within the circadian clocks of fungi, flies and mammals (Lee et al., 2000; Shearman et al., 2000; Glossop et al., 1999). Thus, the damped circadian oscillations in lhy1-1 cca1-1 double mutants may represent the function of a feedback loop that would normally interact with the LHY/CCA1/TOC1 feedback loop, but that is capable of oscillatory behaviour in its absence. Faced with such complexity, it may be time to throw away all of the preconceptions of how clock genes should behave, and focus on analysing the network of regulatory interactions between clock-associated genes. The recent identification of several of these genes provided the first tools to approach these questions at the molecular level, and it is now possible to begin assembling the pieces of the puzzle.

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References


