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The short-period mutant, *toc1-1*, alters circadian clock regulation of multiple outputs throughout development in *Arabidopsis thaliana*

David E. Somers[1], Alex A. R. Webb[2], Mark Pearson[2,*] and Steve A. Kay[1,†]

[1]NSF Center for Biological Timing, Department of Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
[2]Department of Biological Sciences, Institute of Environmental and Natural Sciences, Lancaster University, Bailrigg, Lancaster, LA1 4YQ, UK
*Present address: 1 Scarborough Rd., Wickersley, S. Yorks, S66 2H6, UK
†Author for correspondence (e-mail: sak5y@virginia.edu)

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SUMMARY

The coordination of developmental and physiological events with environmental signals is facilitated by the action of the circadian clock. Here we report a new set of circadian clock-controlled phenotypes for *Arabidopsis thaliana*. We use these markers together with the short-period mutant, *toc1-1*, and the clock-controlled *cab2::luciferase* reporter gene to assess the nature of the circadian clock throughout development and to suggest the position of *TOC1* within the circadian clock system. In dark-grown seedlings, the *toc1-1* lesion conferred a short period to the cycling of *cab2::luciferase* luminescence, as previously found in light-grown plants, indicating that the circadian clocks in these two divergent developmental states share at least one component. Stomatal conductance rhythms were similarly ~3 hours shorter than wild type in *toc1-1*, suggesting that a cell-autonomous clockwork may be active in guard cells in 5- to 6-week-old leaves. The effect of daylength on flowering time in the C24 ecotype was diminished by *toc1-1*, and was nearly eliminated in the Landsberg *erecta* background where the plants flowered equally early in both short and long days. Throughout a 500-fold range of red light intensities, both the wild type and the mutant showed an inverse log-linear relationship of fluence rate to period, with a 2-3 hour shorter period for the mutant at all intensities. These results indicate that TOC1 acts on or within the clock independently of light input. Temperature entrainment appears normal in *toc1-1*, and the period-shortening effects of the mutant remain unchanged over a 20°C temperature range. Taken together our results are consistent with the likelihood that *TOC1* codes for an oscillator component rather than for an element of an input signaling pathway. In addition, the pervasive effect of *toc1-1* on a variety of clock-controlled processes throughout development suggests that a single circadian system is primarily responsible for controlling most, if not all, circadian rhythms in the plant.

Key words: Circadian clock, *toc1*, *Arabidopsis*, Flowering time, Entrainment

INTRODUCTION

Close coordination of developmental and physiological processes with the environment is essential to successful plant growth. Although many of the key events in plant development depends on receiving the appropriate environmental signals at the right time, plants also possess internal timekeeping mechanisms that allow them to keep pace with and anticipate cyclic events in their environment. By alternating between restrictive and permissive phases, such an internal timekeeper can act to both gate the effect of an environmental stimulus, and to modulate the extent to which physiological processes occur. The best understood of these endogenous clocks is the one based on a 24 hour (circadian) pacemaker.

From sleep/wake cycles in mammals (Moore, 1997) to the transcriptional control of gene expression in cyanobacteria (Golden et al., 1997), a circadian clock facilitates the coordination of behavioral, developmental, and physiological events with the daily cycles of light and dark. A genetic approach towards understanding the clock was initiated more than 25 years ago in *Drosophila*, through a screen for circadian clock mutants (Konopka and Benzer, 1971), and has since been extended to algae (Bruce, 1972), fungi (Dunlap, 1996), mouse (King et al., 1997), cyanobacteria (Golden et al., 1997) and higher plants (Millar et al., 1995a). All have been successful in recovering single gene mutations that disrupt the function of the circadian system (Dunlap, 1996).

The goal of most studies has been to identify the primary components of the central oscillator of the clockwork. The

conceptual framework guiding this pursuit has simplified the system into three domains (Block et al., 1993). The input, or entrainment pathway, is defined by the series of processes by which environmental signals are transduced to synchronize the clock with the outside world. These signals terminate at the central oscillator, which consists of a series of processes that generate an oscillatory feedback loop that sets the period length and phase of the overt rhythms. Finally, the output pathway constitutes a second signaling cascade that couples the activity of the central oscillator to the observable rhythms (i.e. the 'hands of the clock').

To qualify as a bona fide component (state variable) of the central oscillator certain criteria must be met that will distinguish an oscillator element from input or output components. First, the activity or amount of the component should itself oscillate. Second, constitutive expression (or complete loss) of the component should stop all oscillations (arrythmicity). Third, a transient change in the level of the component should phase shift the clock. Rigorous application of these criteria requires close control of expression level, most easily obtained by placing a cloned gene under the control of an inducible promoter. The product of the *frequency* locus in *Neurospora* meets these criteria (Aronson et al., 1994), and the *Drosophila period* protein has been tested similarly (Roshbash et al., 1996; Sehgal et al., 1996). However, no single criterion alone is sufficient to identify a pacemaker element; outputs cycle by definition and disruption or constitutive expression of an output element may mask the activity of the oscillator, giving the impression of arrythmicity. Similarly, changes in the level or pulse applications of an input element, such as light, can affect period length and phase of the clock.

The semi-dominant *toc1-1* mutant was recovered in a screen for period length mutants in a line of *Arabidopsis* expressing a *cab2*::luciferase (*cab2::luc*) reporter gene (Millar et al., 1995a). Initial characterization of *toc1-1* showed that the period length in two clock-controlled outputs, cycling of *cab2*:: *luciferase* (*cab2::luc*) activity and the rhythmic movements of expanding cotyledons and leaves, is 2-3 hours shorter than wild-type (WT) (Millar et al., 1995a). The mRNA abundance of two closely related RNA-binding proteins has also been shown to cycle with a shorter period in *toc1-1* (Kreps and Simon, 1997). Both reports were limited to describing the effects of *toc1-1* in young, light grown seedlings. Demonstration of aberrant clock function throughout development in a *toc1-1* background would have two important implications. First, it would support the notion of a single, central clock mechanism operating throughout the life of the plant in a diversity of tissues. Second, it would demonstrate the importance of *TOC1* in controlling the activity of that clock.

We describe here a detailed characterization of both the general aspects of the circadian clock in *Arabidopsis* and the position of *TOC1* within that system. By examining the effects of *toc1-1* on a variety of clock-controlled processes throughout development, we suggest that a single system is primarily responsible for controlling most, if not all, circadian phenotypes in the plant. In addition, we present evidence that *TOC1* is a principle component of the central oscillator in *Arabidopsis*.

MATERIALS AND METHODS

Plant material and growth conditions

toc1-1 was originally isolated in the C24 ecotype as described in Millar et al. (1995a). F_3 populations backcrossed twice (*toc1-1* F3-25, F3-59, F3-79) or 3 times (*toc1-1* F3-73, F3-74) were selected for shortened period length using an in vivo luminescence assay (Millar et al., 1992). Lines introgressed twice (*toc1-1*/Laer F3-64, F3-6, F3-11) or 3 times (*toc1-1*/Laer F3-32, F3-13, F3-8, F3-10) into Landsberg *erecta* (Laer) were similarly selected for use in flowering time experiments. *cab2::luciferase* luminescence assays confirmed that *toc1-1* acts semi-dominantly in the Laer ecotype (data not shown). Unless otherwise noted, seeds were surface sterilized and sown onto MS (Sigma) agar plates + 3% sucrose as previously described (Anderson et al., 1997).

Light grown seedlings used in luminescence assays were entrained under 50-60 μmol m^{-2} $second^{-1}$ white fluorescent light (WL) in 12 hour light /12 hour dark (L/D) cycles at 22°C, or under 12 hour 24°C/ 12 hour 20°C temperature cycles, constant white fluorescent light (50-60 μmol m^{-2} $second^{-1}$), for 5 days prior to commencement of imaging. For fluence rate response tests, plants were placed below the appropriate layers of neutral density filter (Roscolux #3402 or #3404) or wire screen prior to transfer to constant red light (RL) to obtain the desired fluence rate. Luminescence assays commenced with the beginning of continuous RL and plants were imaged for 25 minutes every 2 hours as previously described (Anderson et al., 1997). In temperature entrainment experiments, plants were either continued under temperature cycles throughout data collection (driven experiments) or held at 24°C (free-run experiments). Sampling time and frequency was as in fluence rate response tests. For temperature compensation tests, the plants were entrained as above and transferred to the appropriate constant temperature prior to the final dark period. Period estimates were obtained according to Plautz et al. (1997) and Millar et al. (1995b).

Dark-grown seedlings were imaged in 25-35 seedling clusters as previously described (Anderson et al., 1997) and the time between the 2^{nd} and 3^{rd} peaks after the RL pulse was determined manually.

Hypocotyl length

After stratification of seeds at 4°C for 6 days, the plates were transferred into the appropriate continuous white (50-60 μmol m^{-2} $second^{-1}$), red (20-25 μmol m^{-2} $second^{-1}$) or blue (40 μmol m^{-2} $second^{-1}$) light treatment for 8 days, when hypocotyl measurements were taken with a ruler. Plants grown under saturating far-red light (FRL) were dark germinated for 24 hours before being placed under continuous illumination.

Flowering time

Seed were stratified on agar plates and maintained in fluorescent light under long days (LD) (16 hours light :8 hours dark) (90 μmol m^{-2} $second^{-1}$) or short days (SD) (8 hours light:16 hours dark) (180 μmol m^{-2} $second^{-1}$) for 7-10 days after which they were transplanted to soil. Time to flowering was taken as the number of rosette leaves at time of 1 cm high flower bolt.

Viscous flow porometry

Surface sterilized seeds of C24 wild-type and *toc1-1* F3-59 were sown onto 0.6% agar (0.5 MS, 1% sucrose, 50 μg ml^{-1} kanamycin), stratified at 4°C for 4 days and maintained at 20±1°C; 150 μmol m^{-2} $second^{-1}$ PAR (10 hour daylength) for 1 week. Seedlings were transferred to sterile 3:1 Levington M3 Compost (Levington Horticulture, Ipswich, UK):sand for 1.5-2 weeks, repotted into 5 cm diameter pots and transferred to a controlled environment chamber. Plants were maintained on a 12-hour photoperiod (112 μmol m^{-2} $second^{-1}$) under constant temperature (20±0.1°C) and relative humidity (70±3%). Chamber CO_2 concentration was monitored using

an infra-red gas analyser (WMA-2, PP Systems, Hitchin, UK) and maintained at 355-360 μl l^{-1} by controlled injection of either pure CO_2 or CO_2-free air.

Viscous flow porometer cups similar to those described by Allaway and Mansfield (1969) and Weyers and Meidner (1990) remained attached to the leaves of 5- to 6-week-old vegetative plants (one cup per leaf per plant) throughout the experiment. Viscous conductance of each leaf was recorded for 2.5 minutes every 30 minutes for 2 day/night cycles and during continuous light.

RESULTS

TOC1 lies outside the light-signaling pathway controlling hypocotyl elongation

The long hypocotyl *hy1* mutation severely reduces the amount of spectrally active phytochrome and also increases the period length of the circadian clock in RL, relative to the wild type (Millar et al., 1995b). To determine whether *TOC1* lies within the same phototransduction pathway as that which controls hypocotyl elongation, the hypocotyl length of *toc1-1* was compared to wild type grown under different light qualities.

Fig. 1 shows the hypocotyl lengths of 3 independently isolated backcrossed lines of *toc1-1* maintained under continuous white, red, far-red and blue light. As previously reported (Young et al., 1992), different light qualities vary in their relative effectiveness at inhibiting hypocotyl expansion in WT *Arabidopsis* seedlings. At the wavelengths tested here, this spectral-dependence relationship is unchanged by the *toc1-1* mutation. Although there are statistically significant differences in hypocotyl length between wild type and some *toc1-1* lines under the different light treatments, overall there is no consistently longer or shorter hypocotyl length among the 3 *toc1-1* populations, relative to WT. The slight differences observed are presumably due to the presence of other mutations segregating in the background which have a minor effect on growth rate under some light conditions. Therefore, if *toc1-1* defines a lesion in the light signaling pathway to the clock it must be independent or in a branch downstream of components involved in the light-mediated control of hypocotyl length.

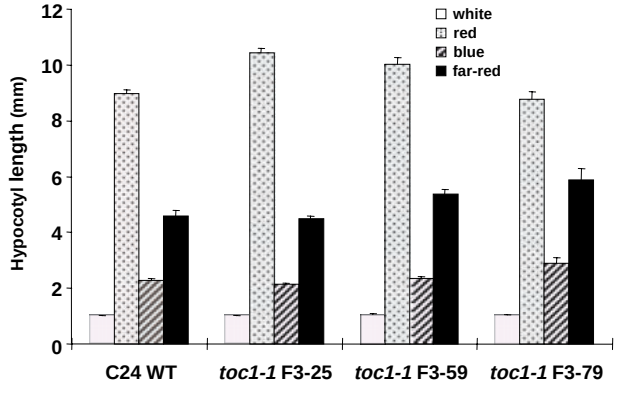

Fig. 1. Effects of light quality on the hypocotyl length of *toc1-1* and WT seedlings. Plants were maintained for 8 days under continuous illumination in the spectral range indicated. Values are a mean of 35-55 seedlings ± s.e.m.

toc1-1 alters clock activity in dark-grown plants

To test whether the influence of *toc1-1* extends beyond its effect on the clock in light-grown seedlings, we examined the effects of the mutation on the rhythm of *cab2::luc* expression in dark-grown, etiolated seedlings following a RL flash.

The kinetics and magnitude of the *cab2::luc* expression pattern during the first 8 hours after the RL flash are identical in the WT and three backcrossed lines of *toc1-1* (Fig. 2). Luminescence levels are 3- to 4-fold above background within 2 hours after the RL pulse, and return to pre-flash levels 5-6 hours later. Our earlier work demonstrated that light input through the phytochrome A and phytochrome B photoreceptors accounts for the entire extent of the rapid RL flash response (Anderson et al., 1997). These results demonstrate that the *toc1-1* mutation lies in a pathway independent of the one leading from the RL activation of phytochrome to the rapid induction of *CAB2* expression.

The timing and magnitude of the second peak (18-21 hours post flash), marking the beginning of the clock-controlled portion of the waveform (Anderson et al., 1997), are very similar in the two backgrounds (Fig. 2). The dip in the second peak in the WT was not consistently observed and conversely it was seen occasionally in all the *toc1-1* lines tested (data not shown). However, the position of the third peak (40-49 hours post flash) relative to the second peak consistently occurs approx. 3-4 hours earlier in the *toc1-1* lines (23.0, 24.5, 22.5 hour after each respective second peak) than in the WT (27.0 hours after the second peak) (Fig. 2). This effect is consistent with the shorter period length of *cab2::luc* expression seen in light-grown seedlings of *toc1-1* (Millar et al., 1995a). *cab2::luc* expression at later time points is too low to assess any differences among the lines reliably. Hence, *TOC1* is effective in controlling period length in both achlorophyllous etiolated cotyledons and in the green, differentiated mesophyll of expanded cotyledons and leaves.

Stomatal conductance rhythms are shortened by *toc1-1*

Rhythmic variation in the size of the stomatal aperture is a well-established example of how the circadian clock can control the physiology of discretely localized, highly differentiated cell types. For the first time in *Arabidopsis*, we have applied viscous flow porometry to monitor cyclic changes in stomatal aperture at very high resolution (Allaway and Mansfield, 1969). By using 5- to 6-week-old rosette plants we were also able to assay the role of *TOC1* at a stage in development much later than previously reported.

Under L/D cycles, stomatal conductance is approx. 3-fold higher in the light than in the dark, in both the mutant and WT, and the maxima and minima of conductance are coincident in both backgrounds (Fig. 3). In contrast, under continuous light the two traces become nearly 12 hours out of phase by the end of the 6-day sampling period due to the shorter free-running period of *toc1-1*. Table 1 summarizes the period estimates obtained from repeated, independent measurements of stomatal conductance on individual leaves from *toc1-1* and WT rosette plants. When the values obtained from the curve-fitting program are weighted in accordance with the confidence of the period estimate, the stomatal conductance rhythm in *toc1-1* averages 3 hours shorter than the wild type.

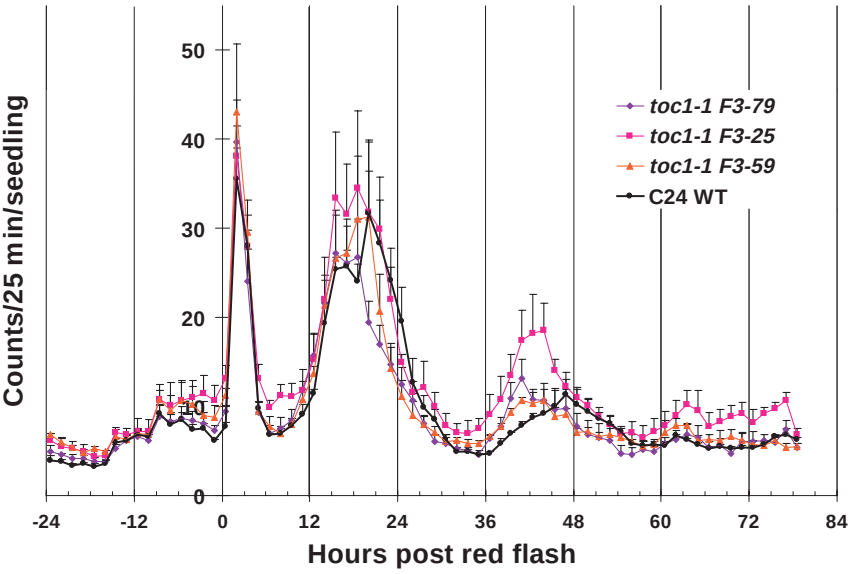

Fig. 2. Effects of a RL pulse on *cab2::luc* expression in dark-grown *toc1-1* and WT seedlings. Plants were grown 6 days in complete darkness prior to a 2 minute RL pulse at T=0. Luminescence was recorded for 25 minutes every 1.5 hours. Each line is a mean (± s.e.m.) of 3 clusters of approx. 30 seedlings.

These results are again consistent with the original report of a 3-3.5 hours shorter period in *cab2::luc* cycling in *toc1-1*. Since the size of guard cells is facilitated by the flux of ions across the plasma membrane and tonoplast, this assay shows that *toc1-1* alters the rhythm of a physiological process very different from the transcriptional control of *CAB2* expression in seedlings, and at a more advanced stage in plant development.

Alteration of photoperiodic timing in *toc1-1* is ecotype dependent

The involvement of the circadian clock in the determination of flowering time has been demonstrated in a wide variety of species (Vince-Prue, 1983). In *Arabidopsis*, a facultative long day plant, there is a 24-hour rhythm in the effectiveness that FRL pulses given over a continuous background of white light have in accelerating flowering (Deitzer, 1984). These results, together with the recent report of a circadian clock defect in the early flowering mutant *elf3* (Hicks et al., 1996), provide the only evidence associating the circadian clock with the control of flowering time in *Arabidopsis*. It was clearly of interest then, to examine the effects of the *toc1-1* mutation on the timing of the transition from vegetative to floral development.

Table 2 presents a summary of five independent experiments testing the responsiveness of *toc1-1* lines to two different photoperiods. Five randomly selected *toc1-1* lines (C24 ecotype) were grown under short day (8 hours light: 16 hours dark; SD) or long day (16 hours light: 8 hours dark; LD) regimes and rosette leaf number at flowering was recorded. The most consistent effect of the *toc1-1* mutation in this genetic background is a diminished effect of daylength differences on flowering time, relative to WT. Within each trial the *toc1-1* lines show a significantly smaller difference between the number of leaves produced in short days compared to the number produced in long days (SD-LD). This occurs through a slightly earlier flowering in SD paired with a slightly later flowering in LD (trial 1), or largely through later flowering in LD alone (trial 2).

When introgressed into the Laer background, the *toc1-1* mutation has a more dramatic effect on flowering time (Table 2). In all cases, flowering in SD is significantly earlier than in the WT, with little effect on flowering time under LD. This results in a near day-neutral plant in which the SD-LD difference is reduced to 0-3 leaves, in contrast to the 5-14 leaf difference in WT (Table 2). These results are the first to suggest that a quantitative change in the pace of the clock can affect the correct processing of daylength information and result in a change in flowering time. Further experiments are in progress to conclusively eliminate the possibility that

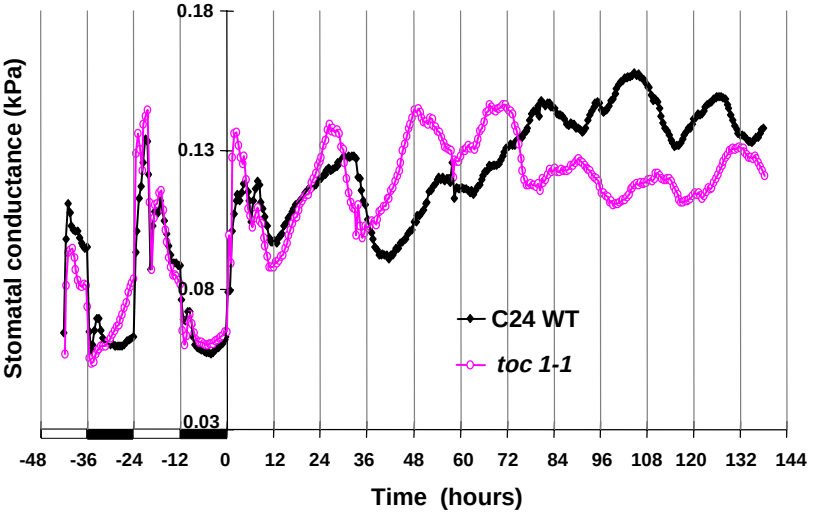

Fig. 3. Effects of *toc1-1* on stomatal conductance rhythms. Plants were grown 5-6 weeks in L/D cycles as described in Materials and Methods until T=0 after which they were held in continuous white light. A viscous flow porometer recorded conductance every 30 minutes for 2.5 minutes. Traces show the most robust examples from Table 1 (no. 12 wild type; no. 13 *toc1-1*). Black bars indicate time in darkness.

Table 1. Period estimates of stomatal conductance rhythms in rosette leaves of wild-type and *toc1-1* plants

WT (plant no.)	period (hours)	0.5 CI*	*toc1-1* (plant no.)	period (hours)	0.5 CI*
1	22.6	0.68	1	20.6	0.25
2	24.4	0.71	2	19.9	0.25
3	23.6	0.25	3	21.9	0.51
4	23.0	0.43	4	22.3	0.59
5	25.4	0.40	5	21.0	0.27
6	22.8	0.32	6	19.0	0.33
7	23.9	0.60	7	19.4	0.39
8	23.0	0.35	8	23.3	0.66
9	22.9	0.48	9	23.2	0.88
10	23.2	0.29	10	22.0	0.33
11	25.2	0.46	11	23.8	0.84
12	24.7	0.41	12	23.7	0.60
13	24.4	0.20	13	21.1	0.25
14	25.2	0.39			
var. wt. mean†	23.9			20.8	
var.wt s.d.†	0.9			1.2	
s.e.m.†	0.2			0.3	

*Approximate nonlinear asymmetric joint confidence limits (CI) were determined at the .95 level according to Plautz et al. 1997.
†Variance weighted mean and variance weighted standard deviation were determined according to Millar et al. 1995b.

these results arise from pleiotropic effects of *toc1-1* on development.

toc1-1 acts constitutively over a wide range of red light fluences

Since alterations in both the fluence rate and quality of the light input can affect the free-running period of the circadian clock (Aschoff, 1979), it is possible that *toc1-1* disrupts a component of the light input pathway. This could result in an increased sensitivity to light, causing the oscillator to run faster than wild type at some or all fluence rates. We tested the effect of light intensity on the activity of the clock by assaying the period of *cab2::luc* cycling under different RL fluence rates after entrainment in L/D cycles. We chose RL because phytochrome has been implicated in mediating the control of period length by light (Millar et al., 1995b).

In both WT and *toc1-1* backgrounds period length is inversely proportional to the logarithm of light intensity over a 500-fold range of RL fluence rates (Fig. 4). This is consistent with reports from a wide range of organisms that demonstrate a log-linear relationship between fluence rate and period length of the clock (Aschoff, 1960). At intensities below approx. 10 μmol m^{-2} s^{-1} light is clearly the limiting factor that determines period length. Higher fluence rates (10-200 μmol m^{-2} s^{-1}) do

Table 2. Effect of *toc1-1* on time to flowering under short and long day photoperiods

		Short days (8:16)		Long days (16:8)		
	Line‡	n	Leaf number†	n	Leaf number†	SD-LD
Trial 1	C24 WT	15	35.0±2.3	14	12.5±1.7	22.5
	toc1-1 F3-59	12	26.9±2.4*	12	18.0±1.2***	8.9***
	toc1-1 F3-79	13	25.3±2.0**	11	15.0±1.2	10.3**
	toc1-1 F3-25	14	25.5±1.6**	14	15.6±1.2*	9.9***
Trial 2	C24 WT	25	26.5±0.5	20	11.1±0.4	15.4
	toc1-1 F3-59	16	22.6±1.1**	15	13.5±1.1	9.1***
	toc1-1 F3-73	17	26.8±0.9	16	15.7±0.6***	11.7**
	toc1-1 F3-74	17	28.9±0.8*	17	17.2±0.8***	11.1**
Trial 1	Laer WT	13	17.9±0.8	16	8.5±0.3	9.4
	toc1-1/Laer F3-64	12	13.2±0.4***	15	10.3±0.2***	2.9***
	toc1-1/Laer F3-6	15	9.9±0.5***	13	9.1±0.3	0.8***
	toc1-1/Laer F3-11	22	10.3±0.5***	21	8.6±0.2	1.7***
Trial 2	Laer WT	19	13.2±0.5	19	8.3±0.3	4.9
	toc1-1/Laer F3-32	17	7.5±0.2***	17	7.2±0.3*	0.3***
	toc1-1/Laer F3-13	17	7.3±0.2***	17	7.3±0.2*	0.0***
	toc1-1/Laer F3-8	16	8.2±0.2***	17	7.6±0.3	0.6***
Trial 3	Laer WT	13	20.2±1.4	16	6.5±0.3	13.7
	toc1-1/Laer F3-32	16	7.6±0.3***	15	6.3±0.3	1.3***
	toc1-1/Laer F3-13	15	7.7±0.2***	15	5.9±0.2	1.8***
	toc1-1/Laer F3-10	15	9.6±0.8***	15	6.6±0.3	3.0***

‡*toc1-1* was backcrossed (C24) or introgressed (Laer) 2 or 3 times into the appropriate background and an F3 population from 5-7 independent *toc1-1* homozygous segregants were chosen randomly for subsequent trials.
†Mean rosette leaf number at 1 cm bolt height±s.e.m. Two-tailed *t* tests were performed to compare each toc1 line with the appropriate WT control. *P<.05; **P<.01; ***P<.001.

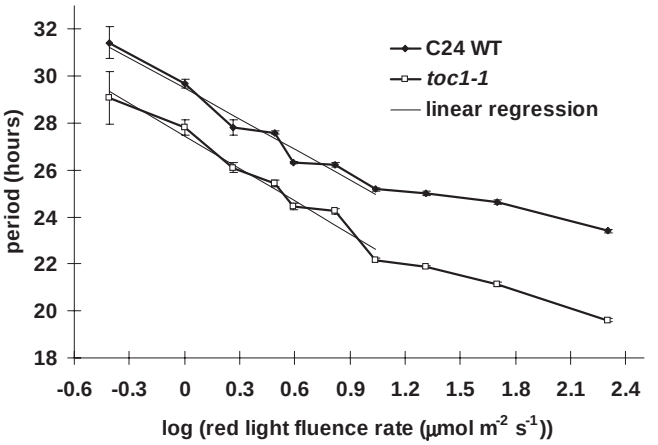

Fig. 4. Effects of red light fluence rate on free-running period length of *cab2*::*luc* luminescence in *toc1-1* and WT seedlings. Plants were entrained for 5 days in L/D cycles, then transferred to continuous RL of the appropriate fluence rate (μmol m^{-2} $second^{-1}$) for 114 hours. Mean period estimates (± s.e.m.) are from 11-20 seedlings after >100 hours in free-run. A best-fit line (linear regression) is shown for the low fluence portion (11-0.4 μmol m^{-2} $second^{-1}$) of each data set.

not saturate the response, but the relative effectiveness of RL in shortening period is much less than at lower intensities. This change in slope suggests that with increasing fluence rates there is a shift to a different RL signaling system or that some components of the low-fluence transduction pathway have changed in abundance or activity.

Consistent with the original tests in WL (Millar et al., 1995a), at high RL fluence rates the free-running period in *toc1-1* is 3-4 hours shorter than the WT (Fig. 4). The same constitutive shortening of period length (approx. 2 hours) is maintained down to the lowest fluence rates tested, as evidenced by the parallel slopes of the two linear regressions. In addition, the transition point from near saturating to limiting light (approx. 10 μmol m^{-2} s^{-1}) is identical for both the WT and mutant (Fig. 4). Together these results show that the mutation has not increased the sensitivity of the input pathway to RL, but instead suggest that *toc1-1* causes the central oscillator to run at a constitutively faster pace.

toc1-1 acts constitutively over a wide range of temperatures

One hallmark of the circadian clock is the relatively slight effect temperature has on the period length of the oscillation. Whereas a 10°C increase in temperature (Q_{10}) will typically increase a biochemical reaction 2- to 3-fold (Q_{10}=2-3), the period of the clock is compensated over a wide range of temperatures and Q_{10}s are usually between 1.0-1.1 (Lakin-Thomas et al., 1990). Some long period alleles of *period* and *frequency* disrupt normal temperature compensation, and period lengths become strongly dependent on the ambient temperature (Hall, 1997). Thus, it was of interest to test the effects of *toc1-1* on this aspect of clock function.

When grown at temperatures from between 12°C and 32°C, the free-running period of the WT varies no more than 2.5 hours (Q_{10}=1.0-1.1), demonstrating that temperature compensation is a property of the *Arabidopsis* circadian clock (Fig. 5). Throughout this range the near parallel slopes of the two best-fit lines shows that period length in *toc1-1* is consistently shorter than WT by 2-2.5 hours at all temperatures tested (Fig. 5). These results demonstrate that *toc1-1* causes no disruption of the normal temperature compensating properties of the clock, and conversely, growing temperature has no effect on the period-shortening effects of the mutation.

cab2::luc rhythms are shortened in temperature-entrained *toc1-1* plants

Although light cycles are the best characterized of the signaling inputs known to entrain the circadian clock, temperature cycles are equally effective in synchronizing the central oscillator with the external environment (Bünning, 1973). In the experiments described above, repeated L/D cycles or brief light pulses were the sole entraining stimuli presented to the plant. To further assess the position of *TOC1* within the circadian system we tested whether the *toc1-1* lesion affects the processes of temperature entrainment.

As the small s.e.m. indicates, all WT individuals (*n*=35) entrain to a 12 hour/12 hour 24°C/20°C thermoperiod (Fig. 6A). Peak and trough expression corresponds to the middle of the high (24°C) and low (20°C) temperature periods, indicating that the rise and fall of *cab2*::*luc* expression is not driven by the temperature changes, but controlled by a circadian clock entrained to the 24-hour thermoperiod. *toc1-1* plants (*n*=34) also entrain, but the sawtooth waveform and the shift in peak and trough positions to near the temperature transition points clearly indicate that the mutant processes the entraining signal conveyed by these temperature cycles differently from WT plants. When both lines are subsequently maintained in constant temperature (Fig. 6B), however, the waveform of both lines are sinusoidal with *toc1-1* plants running at a period approx. 2.6 hours shorter than the WT.

Further experiments suggested that the unusual waveform in *toc1-1* results from entrainment to a cycle with a period (T) far removed from that of its endogenous oscillator. When both the WT and *toc1-1* were entrained to a 20 hour thermoperiod (10

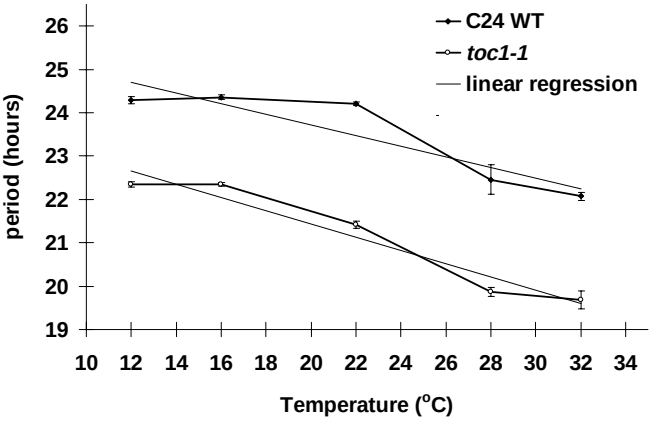

Fig. 5. Effects of ambient temperature on the free-running period length of *cab2*::*luc* luminescence in *toc1-1* and WT seedlings. Plants were entrained 5 days under 12 hour photoperiods (22°C) then shifted to the appropriate experimental temperature the day before sampling commenced. Mean period estimates (± s.e.m.) are from 18 seedlings after >100 hours in free-run. The best-fit line (linear regression) is shown for each data set.

hour/10 hour; 24°C/20°C), only the *toc1-1* lines display a sinusoidal waveform, with peaks and troughs occurring independently of the temperature transition points (Fig. 6C). In contrast, by the end of the trial, expression maxima and minima in the WT coincide closely with the temperature transitions, similar to that seen in *toc1-1* under a 24-hour thermoperiod (Fig. 6A). This complementary set of experiments shows that entrainment to a cycle that differs greatly from that of the period of the endogenous clock can result in a distortion in the normal rhythmic pattern of gene expression. This also implies that in *toc1-1* the clock runs with a short period under a 24-hour temperature cycle, and does not simply revert to a shorter period during free-run under constant conditions.

These data, together with the results from Fig. 5, strongly suggest that the *toc1-1* mutation causes no change in the temperature entrainment pathway nor in the temperature compensation properties of the clock itself. Hence, it is unlikely that the short period phenotype of *toc1-1* is due to altered temperature relations either between the environment and the clock (the input pathway) or in the interactions between components within the clock.

DISCUSSION

Circadian rhythms have been studied in a wide array of plant species, ranging from algae to higher vascular plants. The type of processes subject to clock control vary as widely as the species, including endogenous bioluminescence and phototaxis in some algae, and CO_2 assimilation, gene transcription, leaf movement and flowering in angiosperms (Sweeney, 1987). Through the use of an in vivo bioluminescence assay (Millar et al., 1992), a novel circadian phenotype was created which facilitated the isolation of circadian rhythm mutants in *Arabidopsis*. Characterization of the short period mutant, *toc1-1* (Millar et al., 1995a), now allows both a molecular genetic and comparative approach to the analysis of the plant circadian system throughout development.

The circadian clock in dark-grown *Arabidopsis*

In etiolated tissue, the relative paucity of morphological and developmental events that are clearly clock-controlled and simple to assay has meant that until recently it has been difficult to determine whether a clock is running in dark-grown plants. However, frequent monitoring of the luciferase activity of a *cab2::luc* reporter in a high luminescence mutant background (*supernova*) has shown that synchronous, rhythmic expression of promoter activity occurs with a near 24 hour period in 5-day-old etiolated *Arabidopsis* seedlings (Hicks et al., 1996). Still unaddressed has been the question of whether the same clock is operating in dark-grown and light-grown tissues. Very early in tobacco seed germination, two overlapping and out-of-phase rhythms in *CAB* mRNA levels can be induced, one set by sowing time and phytochrome-independent, and the other set by a 3-hour RL treatment. After 60 hours, only one, RL-responsive oscillation is seen, suggesting that at least one oscillator is common to both phases of development (Kolar et al., 1995).

Analogously, in *Arabidopsis*, the results of the red pulse experiments with *toc1-1* and WT (Fig. 2) now show that at least one component, *TOC1*, is common between the oscillator that regulates *cab2::luc* activity in etiolated cotyledons, and that which controls the same transgene in chlorophyllous mesophyll cells. Therefore, light-induced morphogenesis does not result in a switch to an entirely different timing system from that used in dark-grown seedlings. Although we suspect that the same clock is operating in light- and dark-grown plants, it is possible that only a subset of components are shared between two similar but different circadian clock systems, and that *TOC1* is one of those elements.

Our results also show that fully developed chloroplasts are not required for the activity of the clock that controls *CAB2* transcription. This eliminates the possibility that components of the photosynthetic apparatus or products of photosynthesis are directly required for the operation of a functional clock. However, a recent report demonstrating a circadian oscillation in chloroplastic free calcium suggests the possibility of a chloroplast-based clock that is separate from an oscillator in the cytoplasm (Johnson et al., 1995). The autonomy of this rhythm from the influence of the cytoplasm can now be determined in the *toc1-1* background.

The circadian clock in light-grown *Arabidopsis*

Stomatal conductance rhythms

A diverse range of environmental and physiological factors controls stomatal aperture size (Assman, 1993; Webb and Hetherington, 1997), and the role of the circadian clock in timing the change in stomatal opening has been demonstrated in a variety of species (Gorton, 1990; Gorton et al., 1993; Hennessey et al., 1993; Webb, 1998). Since guard cells respond to a wide range of cues, cycles in [CO_2] or photosynthesis generated from within the leaf could secondarily drive a rhythm in stomatal conductance. In the few reports comparing conductance and photosynthesis rhythms in the same plant (*Phaseolus vulgaris*), both processes exhibited similar period lengths (Fredeen et al., 1991), even when internal [CO_2] was held constant (Hennessey et al., 1993). In *Arabidopsis*, we found a similar relationship between *CAB2* transcription and stomatal conductance. In the wild type, the period lengths of the *cab2::luc* rhythm (24.7 hours; Millar et al., 1995a) and stomatal conductance rhythm (23.9 hours; Table 1, this study) were very close, and *toc1-1* also exhibited rhythms in both processes that are proportionately shorter and essentially identical (20.7 hours; Millar et al., 1995a, and 20.8 hours; this study, respectively). These results are consistent with either mesophyll-to-guard cell signaling or with separate oscillators autonomously acting in each cell. However, the persistence of a circadian rhythm in guard cell aperture in *Vicia* epidermal peels (Gorton et al., 1989) demonstrates that autonomy from the rest of the blade is possible. Furthermore, in *Phaseolus*, temperature cycles entrain rhythms in stomatal conductance but not in photosynthesis, demonstrating that these processes can occur independently of each other (Fredeen et al., 1991). Although similar studies have yet to be performed in *Arabidopsis*, our results are consistent with the hypothesis that *TOC1* acts cell autonomously in guard cells.

Photoperiodic timing

For both long day (LDP) and short day plants (SDP), there is evidence linking the circadian clock to the photoperiodic control of flowering time (Vince-Prue, 1994). In LDP like *Arabidopsis*, extension of daylength rather than interruption of the night is often the most effective way to induce flowering (Vince-Prue, 1994). In *Lolium* and *Hordeum*, however,

flowering time is accelerated by far-red light (FRL) supplements added to a background of RL and the effectiveness of this treatment is under circadian clock control (Deitzer et al., 1979, 1982). Similarly, in *Arabidopsis*, there is a 24-hour rhythm in the acceleration of flowering by 6-hour FRL additions to a background of continuous WL (Deitzer, 1984). This finding, coupled to our results showing that *toc1-1* affects a range of clock-controlled processes, clearly suggests that the dramatic acceleration of flowering time under SD in the Laer ecotype is the result of altered processing of daylength information by *toc1-1*, through its effect on the circadian clock. This also implies that the same oscillator mechanism controlling photoperiodism also controls rhythms in *CAB2* transcription, leaf movement and stomatal conductance.

The difference in the effect of *toc1-1* on flowering time in the C24 and Laer backgrounds points to significant differences in alleles at other loci in the two ecotypes that act to influence flowering through genetic interaction with *TOC1*. *FRIGIDA* (*FRI*) and *FLC* are two loci that have been shown to interact in an allele-specific way to strongly influence flowering time in *Arabidopsis* (Koornneef et al., 1994; Lee et al., 1994; Sanda et al., 1997). To test for potential interactions between these loci and *TOC1*, introgression of different *FLC* and *FRI* alleles into the *toc1-1*/Laer background is underway.

TOC1: input or oscillator component?

Because altering the level or activity of elements of the output pathway can change only the amplitude of a simple output, *TOC1* must lie within either the input pathway or the central oscillator. Hence, our effort has been to try to distinguish a component of the input signaling pathway from a bona fide pacemaker element.

Light is a universally active stimulus in setting both the phase and the free-running period of the clock. Period length is inversely proportional to the log of the fluence rate (intensity), though the slope of this relationship depends on both the organism and the light quality. In many nocturnal species (e.g. mouse, hamster) period shortens with

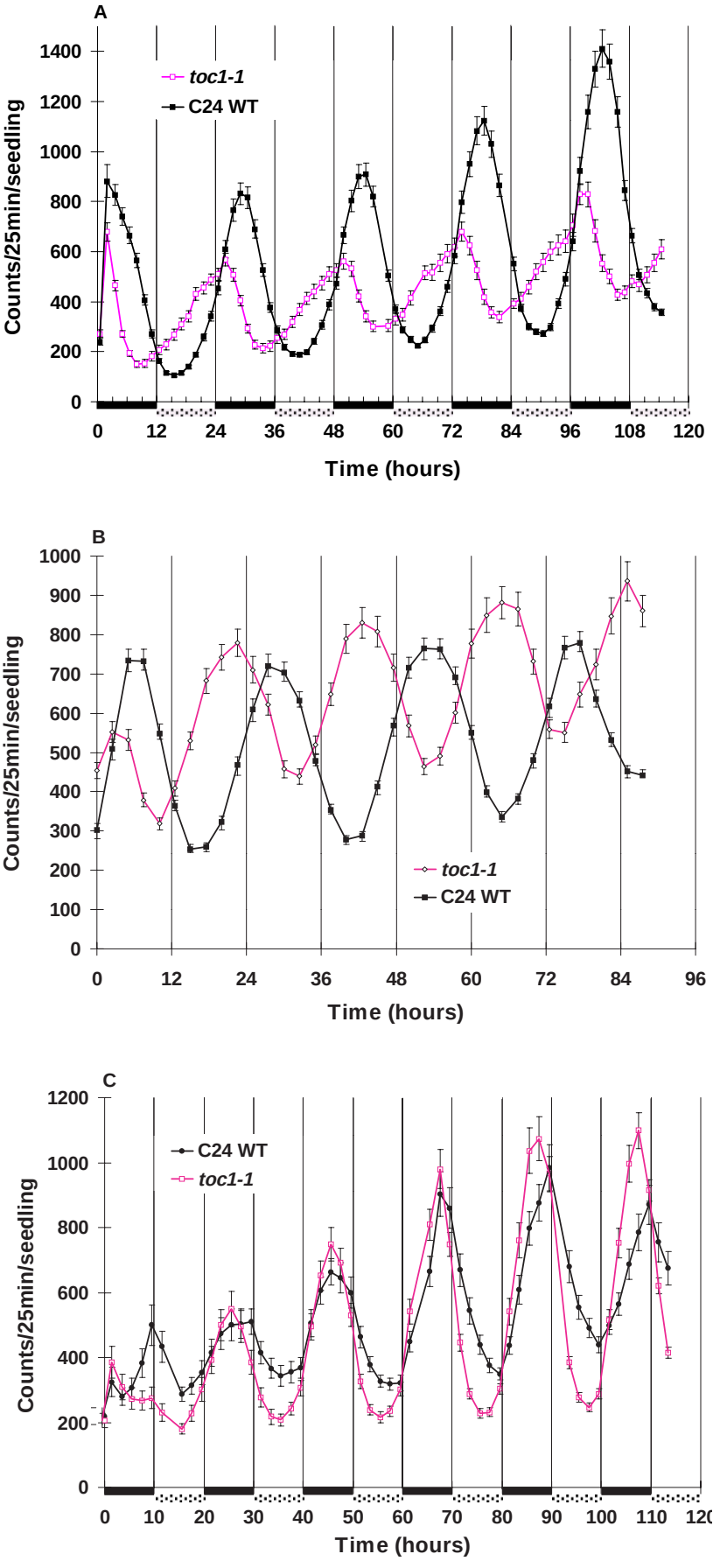

Fig. 6. Temperature entrainment of *toc1-1* and WT seedlings. (A). Luminescence from *cab2::luc* seedlings during entrainment to a 12 hour/12 hour 24°C/20°C temperature cycle. (B). Luminescence from *cab2::luc* seedlings after release from 24°C/20°C temperature cycles (T=24) into continuous 24°C. (C) Luminescence from *cab2::luc* seedlings during entrainment to a 10 hour/10 hour 24°C/20°C temperature cycle. Seedlings were maintained under continuous WL from germination. Vertical bars show ± s.e.m. luminescence values (n=18-35). The temperature regime is indicated on the x-axis in A and C: dark bars, 24°C; dotted bars, 20°C.

decreasing intensity, while period length in diurnal species (including many plants) tends to lengthen as light levels drop (Aschoff, 1960; Harris and Wilkins, 1976).

Within a species, the fluence rate response curve (FRC) can help elucidate some aspects of the light input pathway. In the bioluminescent dinoflagellate *Gonyalaux polyedra*, the slopes of the FRCs for blue and red light are of opposite sign, indicating that at least two separate photoreceptor systems are involved in setting period length (Roenneberg, 1996). In comparing the effect of different RL fluence rates on the period of *toc1-1* and WT, the parallel slopes of the two traces clearly indicate that *toc1-1* is constitutively acting to shorten period length over a wide fluence range (Fig. 4). Since signaling pathways involve amplification of the primary stimulus, a qualitative change in the activity of a component within the cascade will likely affect the final response by a multiplicative (or divisive) factor, rather than as a simple arithmetic addition or subtraction. Therefore, if *TOC1* were a component of the light input pathway we would expect a change in the slope of the red light FRC, relative to wild type (Fig. 4). That this was not observed over a 500-fold range of light intensity is consistent with our contention that *toc1-1* is a mutated clock component causing the central oscillator to run at a faster pace, independent of light input. However, it is still possible that *TOC1* is a constant factor acting on the clock in parallel to the light input pathway, or that it acts as a constitutive, fluence-independent addition to light input just prior to conjunction with the oscillator itself.

Two poorly understood but characteristic features of circadian clocks are temperature compensation and temperature entrainment. The former, demonstrated here for the first time in *Arabidopsis* (Fig. 5), is due in part to the nature of molecular mechanisms within the central oscillator. This is clear from the study of mutants of the *Drosophila period* and *Neurospora frequency* genes, both oscillator components, in which certain alleles (per^L; frq^9, respectively), have lost temperature compensation (Hall, 1997; Rutila et al., 1996). In contrast, a defect in fatty acid synthesis, the *cel* mutation, also disrupts temperature compensation in *Neurospora* (Lakin-Thomas et al., 1990). Such similar effects from such different mutations indicate that we cannot position *TOC1* within the circadian system based solely on the absence of an effect of *toc1-1* on this basic clock property.

Temperature cycles can act (like light) as an entraining stimulus to establish a stable phase relationship between peak output activity and the temperature transitions. A phase shift occurs at least once each cycle and the extent of the shift required for entrainment depends on how close the period (T) of the entraining cycle is to the endogenous period of the oscillator. The 'sawtooth' waveform displayed by the short-period (21 hour) *toc1-1* mutant under a 24 hour T cycle demonstrates the presence of an oscillator that is being rephased each cycle differently from the wild-type (Fig. 6A). The return to a 'wild-type' (sinusoidal) waveform when *toc1-1* is entrained to a 20 hour T cycle confirms this view (Fig. 6C). These results strongly suggest that *toc1-1* does not directly affect the process of temperature entrainment, but rather the oscillator mechanism with which the entraining signal interacts.

The evidence presented here suggests that *TOC1* is neither part of the light input nor the temperature input pathways. Although this leaves the central oscillator as the most likely site of TOC1 activity, there are alternative possibilities for how this oscillator fits within the entire clockwork of the circadian system. Certain aspects of phase resetting in *Drosophila* suggest a scheme in which a master oscillator drives a subordinate 'slave' oscillator (Pittendrigh and Bruce, 1959). Recent experiments with the clock-controlled gene, *AtGRP7(=CCR2)*, raise this possibility in *Arabidopsis.* Out-of-phase cycling of *AtGRP7* mRNA and protein abundance suggests that the protein may be involved in the regulation of its own message abundance. This notion is strengthened by the fact that normal cycling of *AtGRP7* mRNA abundance is eliminated when the polypeptide, an RNA-binding protein, is overexpressed in transgenic plants (Heintzen et al., 1997). However, since the cycling of other transcripts are unaffected in this background and *AtGRP7* cycles with a shorter period in *toc1-1* (Kreps and Simon, 1997), these results are consistent with a model in which *TOC1* is part of a master oscillator controlling one or more subordinate feedback loops, one of which includes *AtGRP7* (Heintzen et al., 1997).

A second alternative to the single oscillator model considers that there are two or more independent, distinctly different clocks that share one or more components, one of which is *TOC1*. These may reside in different tissues or cell types and could be the reason for the 1.5-2 hour difference between the period of *cab2::luc* rhythm and leaf movement rhythm in *Arabidopsis* seedlings, and for the different periods observed for stomatal and leaf movement rhythms in bean (Hennessey and Field, 1992; Millar et al., 1995a; Millar and Kay, 1997). However, the possibility of inter-organ differences in the light input pathway, acting differently on the same clockwork, has not been addressed in either plant. Resolution of this issue will require identification of oscillator components and input coupling factors, and/or single cell resolution of clock-controlled outputs from differentiated tissue.

Conclusions

We have shown that a mutation in *TOC1* disrupts the function of a circadian clock that controls the timing of transcriptional, physiological and developmental processes throughout the life of the plant. These results, together with a previous report (Millar et al., 1995a), constitute the most complete description, in a single species, of the pervasive effects of the circadian clock on higher plant development. They provide the best evidence to date that a single type of circadian oscillator regulates most, if not all, diurnal and photoperiodic events in the plant and that *TOC1* is central to the proper function of this oscillator. Full confirmation of our conclusion awaits the cloning of *TOC1* and manipulation of its expression.

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