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Circadian dysfunction causes aberrant hypocotyl elongation patterns in Arabidopsis

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Summary

Many endogenous and environmental signals control seedling growth, including several phototransduction pathways. We demonstrate that the circadian clock controls the elongation of the Arabidopsis hypocotyl immediately upon germination. The pattern of hypocotyl elongation in constant light includes a daily growth arrest spanning subjective dawn and an interval of rapid growth at subjective dusk. Maximal hypocotyl growth coincides with the phase during which the cotyledons are raised, in the previously described rhythm of cotyledon movement. The rhythm of hypocotyl elongation was entrained by light–dark cycles applied to the imbibed seed and its period was shortened in the *toc1–1* mutant, indicating that it is controlled by a similar circadian system to other rhythmic markers. The daily growth arrest is abolished by the *early flowering 3* (*elf3*) mutation, suggesting that this defect may cause its long-hypocotyl phenotype. Mutations that affect the circadian system can therefore cause gross morphological phenotypes, not because the wild-type gene functions pleiotropically in several signalling pathways, but rather because the circadian clock exerts widespread control over plant physiology.

Introduction

The circadian system is an endogenous, biological timer that controls a wide range of rhythmic processes, all of which maintain rhythmic periods close to 24 h under constant environmental conditions (Lumsden and Millar, 1998; Sweeney, 1987). Circadian rhythms in *Arabidopsis thaliana* include rhythmic leaf movements (nyctinasty; Engelmann *et al.*, 1992), the rhythmic opening of stomata (Somers *et al.*, 1998; Webb, 1998), and the transcription of a number of genes, including chlorophyll *a/b*-binding protein (*CAB* or *LHC*) genes (reviewed in Fejes and Nagy, 1998). Light signals interact with the circadian system, such that light controls the phase and period of the rhythms in all eukaryotes and frequently affects the amplitude of circadian rhythms in higher plants (Johnson *et al.*, 1998). Many of the targets of circadian regulation in plants are also controlled by light. Stomatal opening and *CAB* gene expression are both rhythmic and light-regulated, for example, and the amplitude of their light regulation is modulated (gated) by the circadian clock (Gorton *et al.*, 1993; Millar and Kay, 1996).

The mechanism of the circadian oscillator in other species is thought to depend on a 24 h molecular cycle in the activity of a small number of gene products (reviewed in Dunlap, 1996; Young, 1998). These 'clock genes' were identified by mutations that altered the circadian period or abolished circadian rhythms. Similar mutants in Arabidopsis include *timing of CAB expression* (*toc1*), which has a 21 h period for *CAB* transcription and other rhythmic markers compared to the wild-type period of 24.5 h (Millar *et al.*, 1995a; Somers *et al.*, 1998), and lines that overexpress transcription factors, *late elongated hypocotyl* (*lhy*; Schaffer *et al.*, 1998) and *circadian clock-associated 1* (*CCA1*; Wang and Tobin, 1998). Plants that overexpress *LHY* and *CCA1* are arhythmic under conditions of constant light and constant darkness. The *early flowering 3* (*elf3*) mutation, in contrast, causes arhythmia only during and immediately after light exposure, suggesting that *ELF3* is involved in an interaction between phototransduction and the circadian system (Anderson *et al.*, 1997; Hicks *et al.*, 1996; Millar, 1998). The photoperiodic control of flowering is absent in *elf3* mutants, *LHY*- and *CCA1*-overexpressing plants (Schaffer *et al.*, 1998; Wang and Tobin, 1998; Zagotta *et al.*, 1992; Zagotta *et al.*, 1996); this is expected, because the circadian system is thought to be required for the measurement of day length (Carré, 1998; Coupland, 1998; Thomas and Vince-Prue, 1996). These lines also have an elongated hypocotyl (*hy*) phenotype under some lighting conditions (Schaffer *et al.*, 1998; Wang and Tobin, 1998; Zagotta *et al.*, 1992; Zagotta *et al.*, 1996). It was unclear whether the *hy* phenotype was directly related to the circadian defects of these mutants. *hy* phenotypes are more typically associated with mutants that are defective in phototransduction pathways, such as *hy1*, which lacks the phytochrome chromophore (Parks and Quail, 1991).

The elongation of the hypocotyl has long been recognised for its sensitivity to a very wide range of endogenous and environmental factors. Light signals received by phytochromes, cryptochromes and other photoreceptors inhibit

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Abbreviations: *glabrous 1, gl1*; *timing of CAB expression 1, toc1*; *early flowering3, elf3*; *long hypocotyl 1, hy1*; *late elongated hypocotyl, lhy*; *circadian clock-associated 1, CCA1*; constant light, LL; 12 h light 12 h dark cycle, LD (12,12); fast Fourier transform non-linear least squares, FFT-NLLS; relative amplitude error, RAE.

elongation at germination, but phytochromes can later promote elongation (Cashmore, 1997; Chory, 1997; Smith and Whitelam, 1997). Gibberellins, auxin and brassinosteroids are particularly associated with increased elongation, whereas cytokinins, abscisic acid and ethylene usually reduce elongation (Chory *et al.*, 1994; Creelman and Mullet, 1997; Kende and Zeevaart, 1997; McGrath and Ecker, 1998). Mutations that specifically affect these responses have been recovered in numerous genetic screens; these screens have also identified mutations in potentially novel pathways (Desnos *et al.*, 1996; Halliday *et al.*, 1996). Hypocotyl elongation appears to be controlled by a signalling network in which the response to any single factor depends critically upon the interactions among the constituent regulators (Cary *et al.*, 1995; Chory and Li, 1997; Jensen *et al.*, 1998; Su and Howell, 1995; Trewavas and Malho, 1997).

The long-term dynamics of elongation have been studied in the much larger stem internodes. Stem growth in plants under light:dark cycles typically exhibits several peaks per cycle, with the greatest elongation rates at the end of the day and in the mid- to late night (Bertram and Karlsen, 1994; Bertram and Lercari, 1997; Kerckhoffs *et al.*, 1997). Strong responses to the light–dark transitions (Parks *et al.*, 1996; 1997) tend to obscure any circadian regulation that may be present. Circadian rhythms of stem elongation have been observed under constant light in classical studies (Baranetzky, 1879) was cited in Fernandez and Wagner (1994) and more recently in several species (Assaad Ibrahim *et al.*, 1981; Erwin and Heins, 1988; Fernandez and Wagner, 1994; Lecharny and Wagner, 1984; Tutty *et al.*, 1994), including the Arabidopsis inflorescence stem (Agosti *et al.*, 1997). The various optical, mechanical and electronic techniques employed, however, are unsuitable for studies of the diminutive Arabidopsis hypocotyl.

We have used an automated video imaging system to show that hypocotyl elongation in Arabidopsis is controlled by the circadian clock from the very earliest stages of seedling development. The circadian rhythm of hypocotyl elongation was affected by previously described clock mutations, suggesting that the *hy* phenotype of some clock mutants is a specific consequence of aberrant circadian regulation, rather than a pleiotropic effect of the mutations on other signalling pathways.

Results

Video imaging of Arabidopsis seedling growth

Seed of *Arabidopsis thaliana* (Columbia *gl1*) were germinated for two days in sterile culture under 12 h light, 12 h dark cycles [LD (12,12)]. The seeds were then transferred to constant, dim white light (LL); root radicals began to emerge from the testa at approximately this time. The growth of the seedlings was recorded for several days by the Kujata digital imaging system (Figure 1a). Simple image processing allowed us to trace the position of the cotyledon tips and the lowest pigmented part of the seedling, at the top of the hypocotyl (Figure 1a). The distance between the latter marker and the agar surface represents the length of the hypocotyl.

The hypocotyls of wild-type seedlings grow for several days under these lighting conditions. Their elongation is not uniform, however: growth slows or stops for 2–6 h intervals, approximately once every 24 h close to predicted dawn (Figure 1b). Ultradian (short-period) rhythms of circumnutation (the corkscrew pattern of elongation) have previously been described in Arabidopsis hypocotyls (Schuster and Engelmann, 1997). This motion is largely in the horizontal plane; it was occasionally detectable in our images (small changes in cotyledon angles, around 36 h in Figure 1a), but rarely in the vertical elongation data.

The positions of the cotyledon tips rise progressively as the seedling hypocotyls elongate, and their positions also rise and fall rhythmically with a period of approximately 24 h (Figure 1b). The positions of the cotyledon tips reflect the sum of the rhythms of cotyledon movement and hypocotyl elongation; cotyledon movements alone are revealed by correcting each data point for hypocotyl elongation. Elongation of the cotyledons and petioles gives the rhythm of cotyledon movement a gradual, rising trend (Figure 1b). The location of the cotyledons follows a circadian rhythm, as described previously; the cotyledon tips are highest (cotyledon angle closest to vertical) during the subjective night (Engelmann *et al.*, 1992; Millar *et al.*, 1995a; Somers *et al.*, 1998). This rhythm is generally preferable as a circadian marker because it is more robust than the rhythm of hypocotyl elongation (see below).

Light-entrained, circadian regulation of hypocotyl elongation

The timing of hypocotyl elongation coincides with the cotyledons rising, suggesting that a circadian clock also controls hypocotyl elongation. The phase of maximal elongation occurs approximately at subjective dusk, with a growth arrest at subjective dawn (Figure 1b). The restricted phase of elongation led to an apparent circadian gating of seedling emergence because distinct sets of seedlings commenced elongation on 2 successive days (Figure 2). It is possible that earlier stages of germination are also gated by the circadian clock, but these could not be reliably scored in our images.

We reversed the two initial LD (12,12) cycles to DL (12,12) cycles in order to test whether the rhythm of hypocotyl elongation could be entrained by the light:dark signals, as

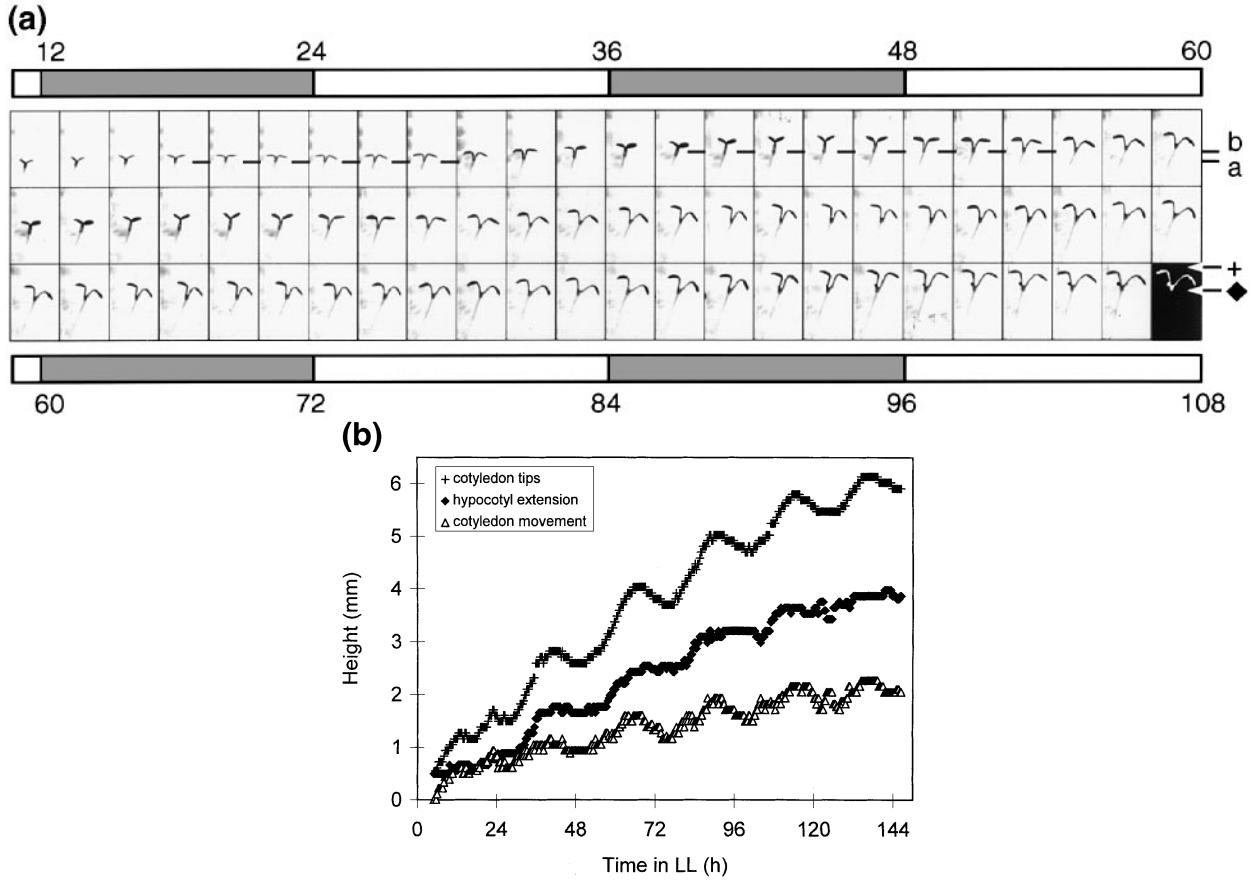

Figure 1. The circadian pattern of *A. thaliana* seedling development.
(a) Circadian rhythms of cotyledon angle and hypocotyl elongation in a sequence of images of a wild-type seedling (Columbia *gl1*) under constant dim light (LL). Time in LL is shown in hours; the images are double-plotted, so the first row depicts 12–60 h, the second row 36–84 h and the third row, 60–108 h. Open boxes, predicted day; shaded boxes, predicted night. Small horizontal bars indicate the approximate height of the hypocotyl during the first (a) and second (b) growth arrests. The last image has been inverted and thresholded. + and ◆ mark the highest and lowest pixel values, which were used in the automated monitoring of cotyledon tip movement and hypocotyl extension, respectively.
(b) Automated monitoring of rhythms in hypocotyl extension and cotyledon position in Columbia *gl1*. The position of the cotyledon tips (+) and hypocotyl apex (◆) were derived as described in (a); cotyledon movements (△) were calculated as the vertical distance between the hypocotyl apex and the cotyledon tips.

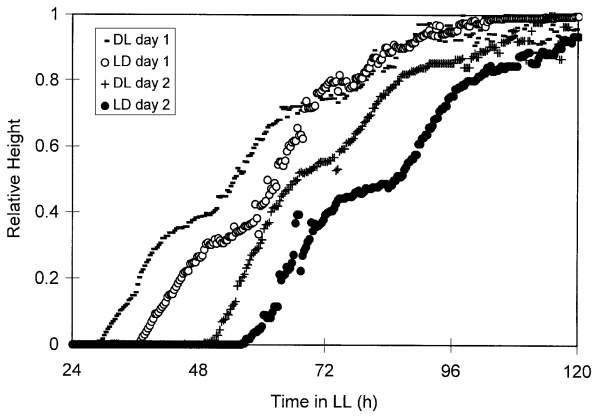

Figure 2. Entrainment of the hypocotyl elongation rhythm by light:dark cycles.
Wild-type seed (C24 parent) were entrained to two cycles of LD (12 : 12) or DL (12 : 12), before imaging as described in Figure 1. In order to compare seedlings that germinated on different days, data were normalised to the final length of each seedling. Traces are the means of three seedlings (except for LD day 2, mean of 2).

expected of a circadian rhythm. The seedlings from both conditions were assayed in adjacent wells, as described above. The seedlings commenced elongation on either of 2 successive days. On each day, the seedlings from LD cycles elongated at the opposite phase compared to those from DL cycles. The subsequent rhythms of elongation remained in antiphase for the duration of the experiment (Figure 2). The circadian system that controls this rhythm can therefore be stably entrained by light–dark signals received by the imbibed seed during germination.

Dependence of the elongation rhythm on TOC1

The *toc1–1* mutation shortens the period of *CAB* gene expression to approximately 21 h in constant light (Millar *et al.*, 1995a). We measured hypocotyl elongation in *toc1–1* and its cognate parent, a transgenic *cab2::luc* line in ecotype C24. Hypocotyl elongation was rhythmic in both lines, but the *toc1–1* mutant had a shortened circadian period

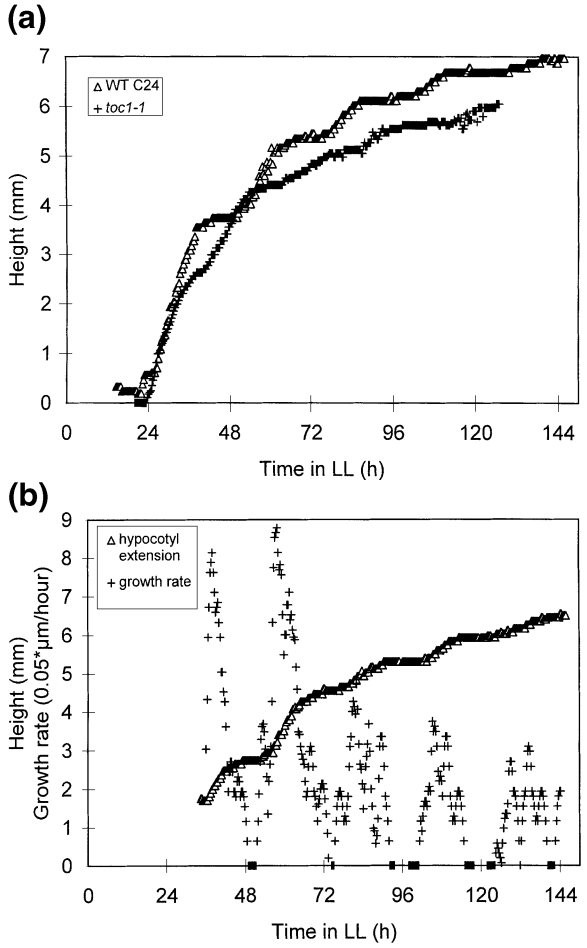

Figure 3. Hypocotyl elongation rhythms in the short period mutant, *toc1–1*.
(a) Hypocotyl elongation of C24 (△) and *toc1–1* (+) in LL.
(b) Differentiated hypocotyl elongation data for C24 (+), with the hypocotyl elongation trace from which it was derived (△).

(Figure 3a). We differentiated the raw pixel data to obtain elongation rates which were better suited to biomathematical analysis (Figure 3b). The standard fast Fourier transform non-linear least squares method (FFT-NLLS; Plautz *et al.*, 1997) was applied in order to estimate the circadian period in these lines (Table 1). The *toc1–1* mutation shortens the period of the hypocotyl elongation rhythm to approximately 21 h, as it does for *cab2::luc* expression and other rhythmic markers (Millar *et al.*, 1995a; Somers *et al.*, 1998). The circadian system controlling hypocotyl elongation therefore has a genetically related, or possibly identical, mechanism to that controlling *CAB* gene expression.

Arhythmia in elf3

Many of the phenotypes of the *elf3* mutant and *LHY*- and *CCA1*-overexpressing plants have been directly related to primary defects in the circadian system (Schaffer *et al.*, 1998; Wang and Tobin, 1998; Zagotta *et al.*, 1992; Zagotta *et al.*, 1996), with the notable exception of their long-hypocotyl trait. Our data show that the circadian clock regulates hypocotyl elongation in Arabidopsis, so a primary defect in the circadian system could also affect hypocotyl length. In particular, a mutation that abolished the daily growth arrest might be expected to have a longer hypocotyl overall. We tested the rhythm of hypocotyl elongation in *elf3* and found that elongation was uniform, with no detectable rhythmic arrests (Figure 4a).

Biomathematical analysis by FFT-NLLS detects tightly clustered periods with low relative amplitude error (RAE) in data from wild-type seedlings, which is indicative of clear circadian rhythms (Figure 4b). The more robust rhythmicity of the cotyledon tip data (Figure 4b, lower left) yields a closer clustering of periods and lower RAE compared to hypocotyl elongation data (upper left). FFT-NLLS can detect multiple rhythmic components in each data trace. Wild-type data yield some period estimates outside the circadian range (15–35 h) in addition to the principal, circadian period, in part due to short-period noise and long-period trends in the data. The data for *elf3*, in contrast, yield few periods in the circadian range and many short-period components, in the noisier, hypocotyl elongation data (Figure 4b and Table 1). Only 48% of *elf3* plants scored give any period for hypocotyl elongation in the circadian range, compared to 96% of wild-types; cotyledon movement similarly lacked robust circadian rhythms in *elf3*. The long hypocotyl of *elf3–1* is therefore correlated with the absence of the rhythmic growth arrest.

Rhythmic elongation in the long-hypocotyl mutant, hy1

The *hy1* mutant is deficient in phytochrome chromophore biosynthesis, and the resulting deficiency in phototransduction leads to a long-hypocotyl phenotype (Parks and Quail, 1991). Hypocotyl elongation was clearly rhythmic both in *hy1* and in its cognate wild-type La(*er*) (Figure 5), indicating that the uniform elongation in *elf3* is not a non-specific attribute of all long-hypocotyl mutants. The period of the elongation rhythm in *hy1* was not significantly different from La(*er*) (M.J. Doswon-Day and A.J. Millar, unpublished results), as predicted from the wild-type period of *CAB* gene expression in *hy1* under white light (Millar *et al.*, 1995b).

Discussion

Many endogenous and environmental signals regulate hypocotyl elongation; we have shown that the elongation of Arabidopsis hypocotyls is controlled by the circadian clock (Figure 1a). The circadian rhythm of hypocotyl elongation was entrained by light–dark cycles applied to the imbibed seed (Figure 2) and the *toc1–1* mutation shortened the rhythmic period (Figure 3a and Table 1). The long-hypocotyl mutant *hy1* retained rhythmic elongation

Table 1. Circadian period estimates and the proportion of arhythmic seedlings

	Data type	Period (h)	SD	SEM	Period 95% CI	Circadian periods, 15–35 h	% with no circadian period	Total periods, 5–60 h	Traces analysed	Contributing experiments
C24 WT	Hypocotyl	25.2	2.13	0.43	0.88	25	14	ND	29	4
toc1-1	Hypocotyl	21.7	1.58	0.41	0.87	15	6	ND	16	5
gl1	Hypocotyl	23.7	1.67	0.32	0.66	27	4	60	28	10
elf3	Hypocotyl	18.8	3.75	0.97	2.08	15	52	92	31	9
gl1	Cotyledon tip	25.3	1.09	0.21	0.44	26	4	48	27	10
elf3	Cotyledon tip	21.3	4.44	1.57	3.71	8	67	23	24	9

Period estimates for both hypocotyl extension and cotyledon movement were derived by the FFT NLLS program, as variance-weighted means (Period) and standard deviations (SD). The number of plants that yielded a circadian period estimate (a period in the range 15 to 35 h) is listed, together with the total number of periods (5–60 h) returned by the FFT-NLLS analysis and the percentage of plants without a circadian period estimate, as an indication of the arhythmicity of an ecotype or mutant. The total number of data traces analysed and the number of independent experiments are also shown. ND, not determined.

(Figure 5); the *elf3* mutation, which also results in a long-hypocotyl phenotype, abolished the elongation rhythm (Figure 4a).

Rhythmic growth and circadian timing

Germinating Arabidopsis seedlings exhibit circadian rhythms in hypocotyl elongation and also in cotyledon position, from the earliest stages of growth (Figure 1a). The hypocotyl elongates in distinct steps in seedlings of strains Columbia *gl1*, C24 and La(*er*) (Figures 1b, 2, 3a, 4a and 5). Maximal elongation rates occur at subjective dusk, coinciding with the raising of the cotyledons (Figure 1). The hypocotyl growth arrest occurs as the cotyledons drop to the horizontal, close to subjective dawn (Figure 1b). A similar circadian rhythm of elongation had been suggested during studies of circumnutation (Schuster and Engelmann, 1997), but the imaging method employed did not clearly distinguish rhythmic hypocotyl growth from cotyledon movements, and no phase information could be deduced.

Several characteristics of the hypocotyl elongation rhythm indicated that it was a typical circadian rhythm. First, the rhythm persisted under constant environmental conditions, with a period close to 24 h in wild-type seedlings (Table 1). Second, the rhythm was entrained by the two LD (12,12) cycles that were applied to the germinating seed, before imaging under constant light (Figure 2). Third, the period of the hypocotyl rhythm was shortened to approximately 21 h (Figure 3 and Table 1) by the *toc1–1* mutation. *toc1–1* is the best-characterised Arabidopsis mutant to be identified by its effect on circadian rhythms; circadian rhythms of gene expression and stomatal opening also have a 21 h period in the *toc1–1* mutant (Kreps and Kay, 1997; Millar *et al.*, 1995a; Somers *et al.*, 1998). The elongation rhythms were neither driven nor entrained by artefactual stimuli in our imaging system because seedlings that were assayed simultaneously exhibited elongation maxima at different phases (Figure 2) or with different periods (Figure 3). Hypocotyl elongation is therefore controlled by an endogenous, circadian timing system that shares at least one component (*TOC1*) with the system controlling *CAB* expression.

The seedling rhythms we describe occur strikingly early in development, before any other known circadian rhythm in Arabidopsis. The rhythms can be entrained by light signals immediately upon germination and before the cotyledons emerge from the seed coat, which they normally do on the third day. Circadian-regulated *CAB* gene expression has also been shown to entrain to a light signal at this stage of development in dark-grown tobacco seedlings, although the resulting rhythm was assayed several days later (Kolar *et al.*, 1998). The Arabidopsis circadian system therefore controls development throughout the life of the plant, from the earliest stage of seedling growth through to the elongation of the inflorescence stem (Agosti *et al.*, 1997).

The mechanism of circadian regulation

Circadian control may be mediated either by a direct output from the circadian clock to the immediate effectors of hypocotyl elongation, or indirectly by circadian 'gating' of another signalling pathway, which in turn controls the hypocotyl. Conceptually, signal transduction in the gated pathway proceeds at the phase when the gate is open, but is prevented at phases when the gate is closed. Circadian gating of light responses occurs together with a 'basal' circadian rhythm for both *CAB* gene expression and stomatal opening (Gorton *et al.*, 1993; Millar and Kay, 1996). Circadian gating has previously been shown to control hypocotyl elongation and hypocotyl unhooking responses

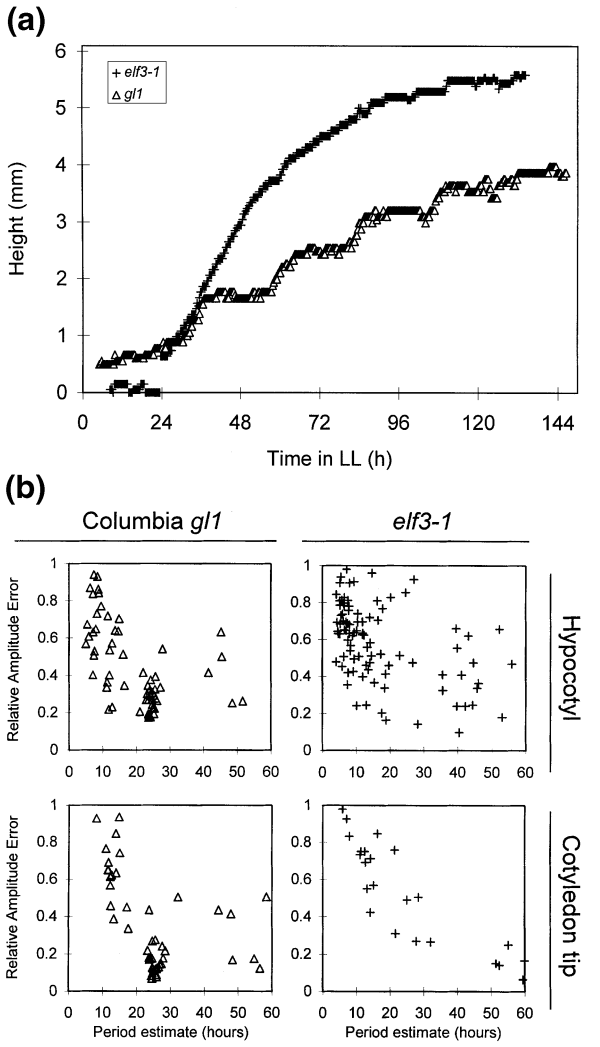

Figure 4. Arrhythmicity of hypocotyl elongation in *elf3*.
(a) Hypocotyl elongation patterns of *gl1* (△) and *elf3–1* (+) were measured in LL, as in Figure 1.
(b) Rhythmic periods in the 5–60 h range were estimated by FFT NLLS and plotted against the associated relative amplitude error (RAE) for *gl1* (△) and *elf3–1* (+). Tightly clustered periods with low RAE are indicative of robust rhythmicity.

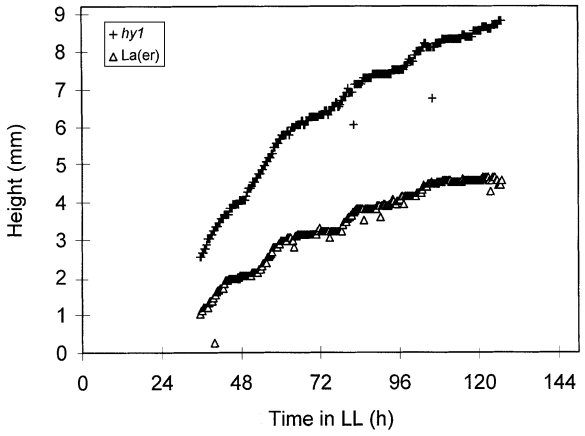

Figure 5. Circadian rhythmicity of hypocotyl extension in a long hypocotyl mutant, *hy1*.
Hypocotyl lengths for La(*er*) (△) and *hy1* (+) were measured in LL, as in Figure 1.

to phytochromes (Horwitz and Epel, 1978; King *et al.*, 1982; Wildermann *et al.*, 1978). No 'basal' circadian rhythms were evident in these studies but, under continuous light, such circadian gating could generate a circadian rhythm (Kay and Millar, 1993): slow elongation would result when the open gate allowed phototransduction to inhibit hypocotyl elongation (near subjective dawn) and rapid elongation when the gate was closed (near subjective dusk). The phase of inhibited hypocotyl elongation (close to subjective dawn) overlaps with the phase at which *CAB* gene expression becomes responsive to light activation (Millar and Kay, 1996), suggesting that shared components may participate in the circadian gating of both these light responses. The mechanism of the circadian gating interaction is not yet known, nor is it clear how widespread is the circadian modulation of other signalling pathways. The circadian or diurnal rhythms in the abundance of hormones known to control hypocotyl elongation suggest that circadian regulation may function at several levels (Finlayson *et al.*, 1998; Foster and Morgan, 1995). The identification of mutants with defective circadian gating may be necessary in order to address these questions.

Circadian-related phenotypes in the elf3 *mutant*

toc1–1 and most other *toc* mutants were identified by a period defect and have no gross morphological phenotypes (Millar *et al.*, 1995a; Somers *et al.*, 1998). The photoperiod-insensitive mutant *elf3*, in contrast, has a long-hypocotyl phenotype that is most pronounced under short photoperiods and is detectable in continuous red or blue light (Zagotta *et al.*, 1992; 1996). *elf3* mutants were arhythmic in the light but not in darkness, suggesting that the *elf3* mutation affected an interaction between phototransduction and the circadian system (Anderson *et al.*, 1997; Hicks *et al.*, 1996). It was unclear whether *ELF3* also affected additional pathway(s) that controlled hypocotyl elongation independently of the circadian clock, or whether arhythmia and the long hypocotyl were due to the same defect.

We reasoned that the long-hypocotyl phenotype was likely to be secondary to a lesion in the circadian system because our data showed that the circadian clock regulates hypocotyl elongation in the wild type. Specifically, any mutant that lacked the circadian growth arrest should have a longer hypocotyl than the wild type. The daily growth arrests were completely absent in *elf3* seedlings (Figure 4), and biomathematical analysis did not show significant circadian rhythmicity in *elf3*, either for hypocotyl elongation or for the position of the cotyledon tips (Table 1). Arhythmia is not an artefact of rapid hypocotyl elongation because

the phytochrome-deficient *hy1* mutants grew longer hypocotyls yet remained rhythmic (Figure 5). The long-hypocotyl phenotype of *elf3* is therefore probably a secondary consequence of a defect in the circadian system.

The overexpression of the transcription factors *CCA1* and *LHY* severely affects the circadian system in Arabidopsis and leads to arhythmia in light and darkness (Schaffer *et al.*, 1998; Wang and Tobin, 1998). The link between circadian defects and the long-hypocotyl trait is supported by the fact that plants overexpressing *CCA1* and *LHY* exhibit a *hy* phenotype similar to *elf3* (Schaffer *et al.*, 1998; Wang and Tobin, 1998), and similarly lack the circadian arrests of hypocotyl elongation (M.J. Dawson-Day and A.J. Millar, unpublished results). The *elf3* mutation may disrupt the circadian system by a mechanism different from the transcription factor overexpression because *elf3* is arhythmic only after light exposure, not in darkness (Anderson *et al.*, 1997; Hicks *et al.*, 1996). The identities of the photoreceptor species involved in the *elf3* phenotypes remain to be determined. Irrespective of the mechanism of arhythmia in *elf3*, our results demonstrate that the absence of circadian control in the plant regulatory network can cause gross, visible phenotypes, as well as the more subtle alterations in biological rhythms. Physiological studies have previously related circadian dysfunction to chlorosis (for example, Highkin and Hanson, 1954): the pale-green phenotype of *elf3* and of other clock mutants may be due to the loss of circadian control over chloroplast functions in addition to abnormal *CAB* gene regulation. Further analysis of clock mutants will be necessary to evaluate the full impact of circadian timing on plant physiology, and to distinguish the effects of circadian gating and basal rhythmic mechanisms.

Experimental procedures

Plant materials

We obtained *Arabidopsis thaliana* ecotype Landsberg *erecta*, La(*er*), and the mutant *hy1–1* from Maarten Koornneef (Wageningen), Columbia *gl1* from Lehle seeds (Arizona) and the mutant *elf3–1* in the *gl1* background from Ry Meeks-Wagner (Oregon). The *toc1–1* mutant and its parent, the transgenic *cab2::*Ω*-::luc* line in the C24 ecotype (referred to as C24), have been described previously (Millar *et al.*, 1992; 1995a).

Growth conditions

Seed was surface-sterilised and sown in rows on 1.0% agar containing Murashige and Skoog plant salt mixture (MS, Murashige and Skoog, 1962) and 3% sucrose. The seeds were incubated in a growth chamber under LD(12,12) of 88 μmol m^{-2} s^{-1} cool white fluorescent light, at 21.5°C ± 0.5°C. After two dark intervals, two 1.5 cm^2 agar blocks carrying up to eight seeds were transferred to a 25-well square tissue culture dish, placed vertically before a monochrome video camera. The seedlings were imaged in a growth room under LL of 10–20 μmol m^{-2} s^{-1} cool white fluorescent light at 21–22°C.

Data acquisition

The Kujata is a simple PC-based video imaging system with software control of up to 14 cameras (Mark Hyett, Virginia). This system stored inverted, thresholded, 1-bit images of up to eight seedlings per camera every 20 min and 8-bit greyscale images every 2 h (Figure 1a). The image series were analysed *post hoc* using rectangular regions of interest to follow the growth of individual seedlings. Seedlings that fell over or became entangled were not analysed.

Cotyledon movement

The movement of one cotyledon per seedling was recorded, being reflected by the highest white pixel value within a region of interest, over the time course (Figure 1). A window of between 60 and 84 h was selected for each data trace, spanning the period of most rapid hypocotyl elongation. The trace within this window was then analysed by the fast Fourier transform non-linear least squares program (FFT-NLLS; Plautz *et al.*, 1997). This fits sine waves to the data and estimates their period, phase and amplitude, with associated confidence intervals. Relative amplitude error (RAE = amplitude error/amplitude) is a measure of rhythmic robustness that varies between 0 (perfect sine wave) and 1 (rhythm not significant). One to three sine waves with different periods were estimated for each data trace. For estimation of the mean circadian period, results were restricted to periods falling within the circadian range, 15–35 h, and the number of plants to which the FFT program failed to fit a period in this range was also noted. Variance-weighted means and standard deviations were calculated (Millar *et al.*, 1995a) because these more strongly reflect the clearest traces. In addition, in order to portray the arhythmicity of an ecotype or mutant (Figure 4b), all periods within the range 5–60 h were plotted against the RAE for each estimate (Plautz *et al.*, 1997).

Hypocotyl extension

When the testa was omitted from the region of interest, the lowest white pixel reflected the position of the hypocotyl apex and its vertical movement over the time course. Absolute hypocotyl lengths (Figure 1) could be calculated from the difference in pixel positions of the testa (on the agar surface) and the hypocotyl apex; lengths in each camera were calibrated by imaging a ruler. Data presented graphically are from a single seedling of each genotype, representative of independent, replicate experiments (see Table 1). The pattern of hypocotyl extension approximates less closely to a sine wave than that for cotyledon movement (Figure 1b), so prior to period analysis, these data were differentiated by linear regression in a running, 11-point window, centred on the time of interest (Figure 3b). A 60 h window, which omitted the first growth spurt on plumule emergence, was analysed as described for cotyledon movement.

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