Functional independence of circadian clocks that regulate plant gene expression

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Background: Circadian clocks regulate the gene expression, metabolism and behaviour of most eukaryotes, controlling an orderly succession of physiological processes that are synchronised with the environmental day/night cycle. Central circadian pacemakers that control animal behaviour are located in the brains of insects and rodents, but the location of such a pacemaker has not been determined in plants. Peripheral plant and animal tissues also maintain circadian rhythms when isolated in culture, indicating that these tissues contain circadian clocks. The degree of autonomy that the multiple, peripheral circadian clocks have in the intact organism is unclear.

Results: We used the bioluminescent luciferase reporter gene to monitor rhythmic expression from three promoters in transgenic *Arabidopsis* and tobacco plants. The rhythmic expression of a single gene could be set at up to three phases in different anatomical locations of a single plant, by applying light/dark treatments to restricted tissue areas. The initial phases were stably maintained after the entraining treatments ended, indicating that the circadian oscillators in intact plants are autonomous. This result held for all the vegetative plant organs and for promoters expressed in all major cell types. The rhythms of one organ were unaffected by entrainment of the rest of the plant, indicating that phase-resetting signals are also autonomous.

Conclusions: Higher plants contain a spatial array of autonomous circadian clocks that regulate gene expression without a localised pacemaker. Circadian timing in plants might be less accurate but more flexible than the vertebrate circadian system.

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Background

Circadian clocks fine-tune physiology and behaviour to match the environmental day/night cycle. Circadian timing depends on a feedback loop involving the rhythmic expression of genes that have been identified in organisms ranging from cyanobacteria to mammals [1]. Input signals from daily light and temperature changes reset the circadian oscillator (Figure 1a; [2]), thus maintaining a defined phase between endogenous rhythms and external cycles, which is probably adaptively significant. Isolated plant organs and tissues support circadian rhythms, as do the specialised cells that drive rhythmic leaf and stomatal movements, indicating that these explants contain circadian clocks [3,4]. The levels of several plant hormones exhibit circadian rhythms [5,6] but it is unknown whether any such communicated, rhythmic signals can synchronise cellular clocks.

Animal species also support multiple peripheral clocks [7–13] in addition to the neural pacemakers (such as the mammalian suprachiasmatic nucleus) that regulate rhythmic behaviours. Recent results support a model in which light rapidly resets the suprachiasmatic clocks, which in turn entrain the peripheral clocks through a weak coupling pathway (Figure 1a; [14]). Within pacemaker tissues, the circadian clocks of individual cells are often closely coupled [15,16], thus increasing the precision of timing *in vivo*. A proposed function of independent or weakly coupled oscillators, in contrast, is to support greater flexibility in the entrained phases of circadian rhythms [17,18]. The architecture of the whole circadian system has not been elucidated in any complex, multicellular organism, however: it is unclear whether peripheral circadian clocks are mutually coupled and whether plants contain a localised circadian pacemaker. We have now used *in vivo* reporter gene imaging to test directly for coupling within the spatial arrays of oscillators and photoreceptors in higher plants. We found the circadian systems of organs and localised areas of tissue to be functionally independent.

Results

The promoters of the phytochrome B1 (*PHYB)*, chalcone synthase (*CHS*) and chlorophyll a/b-binding protein (*CAB* or *LHCB*) genes together mediate rhythmic transcription in almost all cells of tobacco and *Arabidopsis thaliana* seedlings. *PHYB* expression peaks 4–6 hours after lights-on and is ubiquitous in aerial tissues of tobacco [19]; *CAB* is

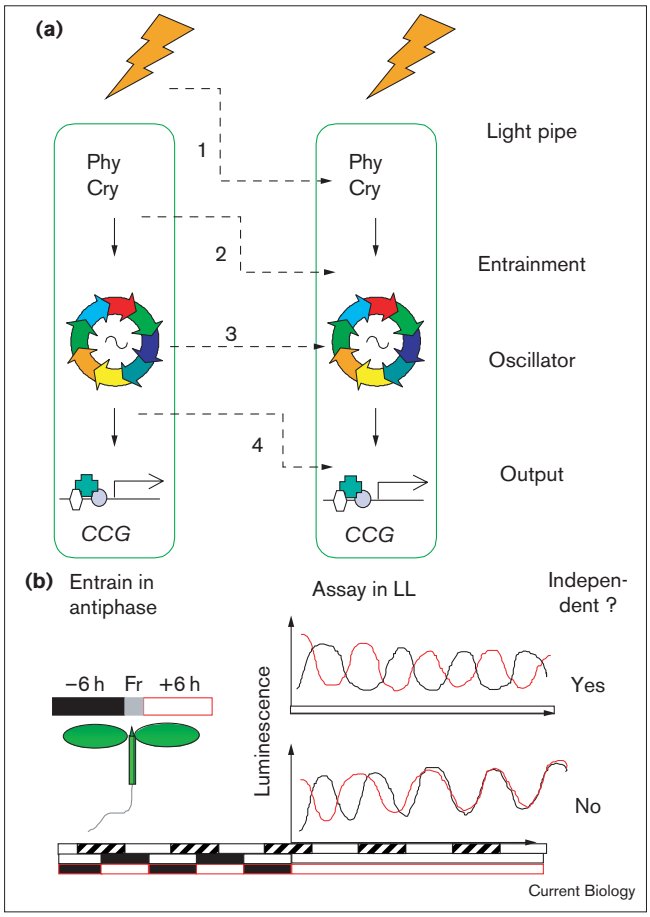


Figure 1

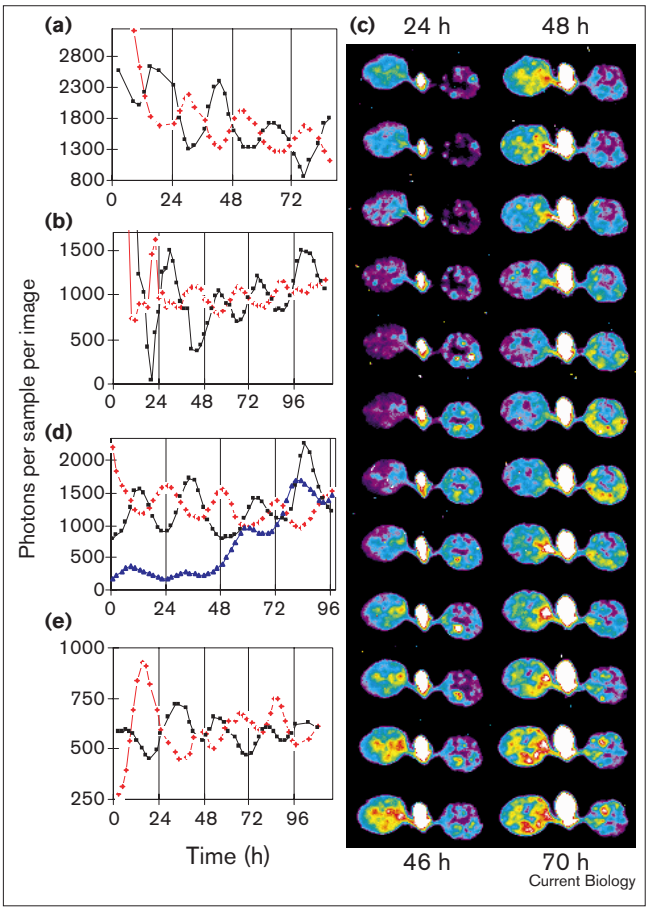
Synchronisation of circadian rhythms. **(a)** Circadian clocks are entrained by light (flash) via signalling from photoreceptors, such as phytochromes (Phy) and cryptochromes (Cry), to an oscillator and rhythmic output to target processes, such as clock-controlled gene expression (*CCG*). In nature, circadian rhythms that are expressed in different locations could potentially be synchronised: (1) by light piping, (2) by intercellular signalling in the entrainment pathway from photoreceptor to oscillator, (3) by rhythmic signals among oscillators (as in some animal pacemaker tissues), or (4) by intercellular signalling from oscillator to target rhythms. For simplicity, only unidirectional connections are shown. The rhythms of physically isolated tissue explants (Figure 2a) demonstrate that the connections are dispensable, so the isolated system is self-sustaining. Connections 1 and 2 have been demonstrated only for non-rhythmic responses in plants [21,28,29]. We have tested connections 1 and 2 (Figure 4), and 3 and 4 (Figures 2b–e and 3) and found that circadian systems in different anatomical locations are functionally independent in the intact organism. **(b)** Internal desynchronisation procedure (as in Figures 2b–e and 3). A young plant is grown under LD(12,12), before foil covers are applied to create a 6 h phase delay (–6 h) in one leaf and a 6 h phase advance (+6 h) in the other, while the apical region free-runs in LL (Fr). Luminescence rhythms in the plant are then assayed under LL (depicted in graphs), to test for independence versus mutual phase resynchronisation via connection 3 or biphasic rhythms due to connection 4. The light/dark conditions for the last days of entrainment and imaging are shown schematically below the abscissa: hatched boxes in upper row, dark intervals predicted from LD(12,12) during growth and reflected in any rhythm that has not been phase-shifted; open and filled boxes, light and dark intervals; middle row, phase-delaying treatment; red-bordered row, phase-advancing treatment.

expressed in phase with *PHYB* [20] but in the mesophyll cells; *CHS* expression peaks 2 hours before lights-on, in the epidermal cells of aerial organs and in the root (S.C.T., A.H. and A.J.M., unpublished data; see also Materials and methods). Fusions of these promoters to the firefly luciferase gene (*LUC*) allow real-time studies of circadian rhythms in single, intact transgenic plants [20]. *Arabidopsis* tissues that express the *CAB–LUC* transgene, including leaf petioles approximately 1 mm long, exhibited circadian rhythms in constant light (LL) as explants in sterile culture but the rhythmic period was variable (Figure 2a). The circadian rhythm of excised petioles was stably reset by two inverted 12 hour light:12 hour dark cycles (LD(12,12); Figure 2). Explants of cotyledons, hypocotyls, the apical region and primary leaves gave very similar results (S.C.T., A.H. and A.J.M.; unpublished data). Plant gene expression is therefore controlled by multiple copies of an entrainable, self-sustaining circadian clock in different tissues.

We tested the independence of the circadian system in the organs of intact plants, using local light treatments to desynchronise the organs (Figure 1b). Independent circadian systems would be expected to maintain the phase differences established during entrainment, after transfer to LL. Strong coupling among the circadian systems might prevent the initial desynchronisation, whereas weaker coupling would be expected to alter the relative phase under LL (Figure 1b). Tobacco seedlings expressing the *CAB–LUC* marker were grown under LD(12,12), before transfer to LL. The cotyledons were alternately covered with opaque foil to create three LD(12,12) cycles, while the rest of the seedling remained in LL. To test for independent entrainment of the clocks in opposite cotyledons, the local LD treatment was advanced by 6 hours relative to the LD growth conditions for one cotyledon and delayed 6 hours for the other, giving a 12 hour difference between cotyledons (Figure 2b). The cotyledon rhythms were entrained to opposite phases in 83–91% of plants (see Materials and methods). Foil covering and immediate removal every 12 hours had no effect on the rhythmic phase of control plants (S.C.T., A.H. and A.J.M.; unpublished data). Foil covering of entire seedlings gave very similar phases to those in desynchronised organs (S.C.T., A.H. and A.J.M.; unpublished data), indicating that organs were effectively entrained by transitions between full lighting and the dim light resulting from inevitable light leakage at the edge of the foil. All oppositely-entrained cotyledons remained in antiphase under LL (Figure 2b), indicating that their clocks were independent.

The primary leaves of tobacco plantlets expressing *PHYB–LUC* (Figure 2c,d) and *CAB–LUC* (S.C.T., A.H. and A.J.M.; unpublished data) were similarly assayed and maintained luminescence rhythms in antiphase under LL. Opposite leaves also retained 6–9 hour phase differences

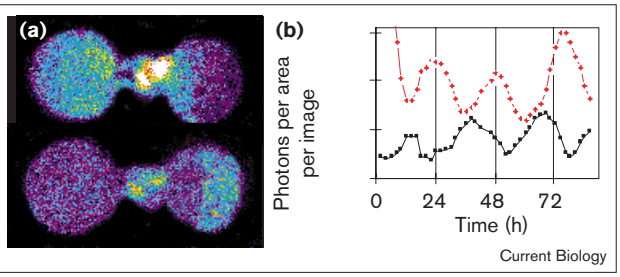
Figure 2



Independent circadian rhythms in excised organs and intact plants. **(a)** *CAB2–LUC* luminescence in isolated *Arabidopsis* petioles. Petioles of the first leaf pair were excised and treated with two LD(12,12) cycles (red crosses; lights-on at 0 h) or inverted (DL) cycles (black squares; lights-on at −12 h), before imaging in LL. The initial phases of explant rhythms matched those of intact control plants and of explants that were excised after entrainment, indicating that excision did not grossly disrupt the circadian system. The circadian period of leaf and petiole explants was more variable (from 3 h shorter to 1.5 h longer than intact seedlings; S.C.T., A.H. and A.J.M.; unpublished data). **(b–e)** The luminescence of internally desynchronised plants was imaged in LL. Foil covers were applied to the organs of intact plants to create LD treatments in antiphase (b) in the cotyledons of *CAB–LUC* tobacco seedlings, (c,d) in the leaves and apical region (blue triangles) of *PHYB–LUC* tobacco plantlets, and (e) in the aerial organs (red crosses) and roots (black squares) of *CHS–LUC Arabidopsis* seedlings. Black squares, organs 6 h phase-delayed relative to preceding LD cycle; red crosses, organs 6 h phase-advanced. (c) Median-filtered, false-coloured luminescence images obtained every 2 h from a *PHYB–LUC* plantlet with desynchronised leaves, after 24–70 h of LL. Right-hand leaf, 6 h phase-delayed; left-hand leaf, 6 h phase-advanced; blue denotes low light levels, red and white, high levels.

after suitable entrainment, so the persistence of rhythms in antiphase is not due to coupling at this unusual phase (S.C.T., A.H. and A.J.M.; unpublished data). The larger tobacco plantlets allowed us to monitor luminescence

Figure 3



Independent clocks within a single leaf. Foil covers were applied to the distal half of one leaf or to the remainder of a *PHYB–LUC* tobacco plant, in order to create LD treatments in antiphase; luminescence was imaged in LL. **(a)** False-coloured luminescence images after 24 h (upper image) and 36 h (lower image) of LL. **(b)** Quantification of the images in (a). Black squares, distal region, 6 h phase-delayed relative to preceding LD cycle; red crosses, proximal region, 6 h phase-advanced.

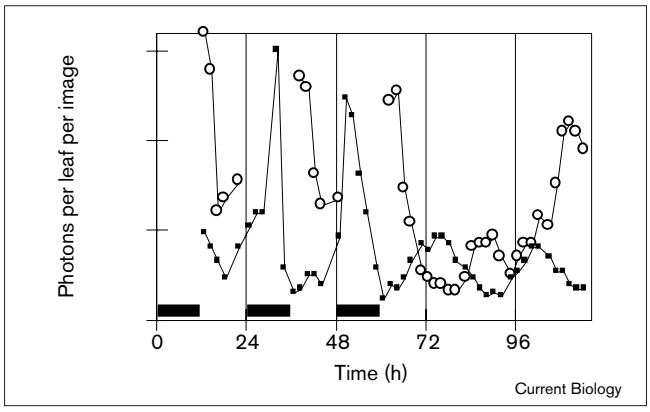
levels specifically from the apical region: luminescence increased rapidly as a new leaf expanded (after 48 hours; Figure 2d). The phase of the free-running apical region remained distinct from the phase-shifted leaves (approximately 6 hours advance or delay; Figure 2d). The initial LD cycles would likely have entrained many cells of the emerging leaf, though our data do not rule out the inheritance of phase information during cell divisions under LL.

The apical–basal axis is a major focus of hormonal communication, which might carry timing information. We assayed *Arabidopsis* seedlings carrying the *CHS–LUC* reporter, which is rhythmically expressed both in the roots and in aerial tissues. The rhythm in roots could be entrained to an opposite phase relative to the rhythm in the aerial tissues and maintained this opposite phase under LL (Figure 2e). The major organs of the vegetative plant therefore contain independent copies of the circadian systems that control the expression of three nuclear genes.

To test the independence of circadian systems in different areas of a single organ, the distal half of one leaf in each *PHYB–LUC* tobacco plant received three LD(12,12) cycles with a 6 hour phase advance, relative to the preceding LD cycles, while the remainder of the plant was treated with cycles delayed by 6 hours. The rhythms at the distal and proximal ends of the leaf were stably entrained and remained in antiphase under LL (Figure 3).

Transmissible phototransduction signals that rapidly reset distant circadian clocks to a common phase might coordinate circadian rhythms in nature (Figure 1a, connection 2), despite their independence under LL. We therefore tested whether a single leaf in LL could be reset by LD treatment of the remainder of the plant. The light scattering and piping properties of plant tissues are well documented [21]. The inference of intercellular signalling on the basis of

Figure 4



Circadian rhythms in a leaf are independent of entrainment in other organs. Foil covers were applied to *PHYB–LUC* tobacco plantlets, creating three LD treatments in antiphase to the preceding LD cycle, but leaving one leaf exposed to LL. Luminescence from the free-running (squares) and re-entrained (circles) leaves was imaged in LL. Shaded bars denote dark treatments, when signals from the re-entrained leaf were blocked by foil. Only acute activation of *PHYB–LUC* is evident after dark–light transitions [19].

localised illumination [22] therefore requires additional controls, which we obviated by using localised dark treatments. Tobacco plantlets carrying the *PHYB–LUC* reporter were imaged while foil covers were applied to create three LD(12,12) treatments in antiphase to the preceding cycles, except for one leaf that remained in LL (Figure 4). A subsequent interval of LL revealed that only the rhythms in foil-covered tissues had been reset. The test leaf in LL retained its initial phase (identical to control plants maintained under LL (S.C.T., A.H. and A.J.M.; unpublished data), indicating that its circadian oscillators were insensitive to the light/dark treatment of all other cells in the plant.

Discussion

The copies of the circadian clock that regulate the *PHYB*, *CHS* and *CAB* promoters are therefore functionally autonomous, from photoreceptors to output rhythms, within each organ of the intact plant and also within different areas of a single leaf. Autonomy is likely to be common among circadian rhythms of gene expression, because the expression patterns of the three promoters include almost all vegetative cell types and expression that peaks at different phases. Genetic mosaic experiments will now be required in order to test for short-range signals that might mutually reinforce the rhythms of neighbouring cells. Current models of the circadian oscillator mechanism in other species highlight nuclear functions [1], suggesting that rhythms of gene expression could be autonomous within each nucleus. Tissue- or cell-specific modification of the circadian system might therefore alter the properties of gene expression rhythms in a flexible manner, to meet adaptive pressures. Any very weak, resynchronising signal that could have remained undetected in our experiments would be unlikely to have physiological significance, given the daily re-entrainment of plant circadian rhythms in nature.

The circadian regulation of turgor-dependent changes in plant cell size or shape might have different constraints, because cytoplasmic connections through plasmodesmata are expected to couple the turgor rhythms of neighbouring cells, while the rigidity of shared cell walls couples their elongation. Morphological rhythms might thus be coordinated within tissues such as the pulvinus. Pulvinar cells are cytoplasmically isolated from other cell types in the leaf [23], however. The isolation of the leaf movement rhythm is underlined by the observed differences in circadian period between leaf movements and rhythms of stomatal opening and gene expression that depend on other leaf cell types [24,25]. The oscillators that confer the various periods might have the same biochemical components but with tissue-specific inputs that affect the period [4]; likewise, it is likely that the oscillators that control the three promoters tested here share the same components (A.H., S.C.T. and A.J.M.; unpublished data).

Circadian input photoreceptors function in most, if not all, plant cells including the root [26]. Entrainment is therefore likely to be intracellular, leading to the observed synchrony of rhythms among tissues by the simultaneous but independent resetting of all peripheral clocks. Consistent with this idea, the independent entrainment of single organs (Figure 4) indicates that no systemic, phototransduction signals reset the plant circadian system. It is formally possible that the LL treatment blocked the response of the free-running leaf to transmitted phototransduction signals. The entrainment of circadian rhythms by changes in light fluence rate (rather than qualitative, light/dark transitions) is well documented, however [27]: any transmitted signal therefore resets the plant circadian system much less effectively than a quantitative change in local illumination. The independent entrainment of peripheral clocks was previously suggested, on the basis of the absence of visible, cellular connections between sensory neurons of the fruit fly wing margin, which maintains persistent rhythms when isolated in culture [11], and from the entrainment of other isolated organs [8,9,13]. In contrast to the peripheral cells in *Drosophila*, phototransduction signals are transmitted among plant cells, including signals transmitted within a cotyledon that activate the *CAB* or *CHS* promoters [28,29]. The opposite entrainment of proximal and distal areas within a leaf (Figure 3) suggests that the circadian system is insensitive to any such signals in the leaf.

A range of coupling strategies is now being uncovered among taxa, from the apparent autonomy of plant cells to the strong coupling among cells in some animal pacemaker tissues. The independence of plant clocks indicates that

circadian rhythms at the level of the whole plant will be limited by the precision of individual oscillator mechanisms. Limited precision is apparently sufficient in photoautotrophic organisms with complex input photoreceptors [2] that are necessarily exposed to daily light/dark cycles. The increased precision derived from tissues of coupled oscillators could be important when exposure to entraining cues is restricted or in controlling rhythms that confer a precision-dependent advantage, possibly including rhythmic animal behaviours. Current evidence suggests that the constraints on circadian function vary among cell types. Peripheral cells with receptors for external time cues might generally have a cell-autonomous circadian system, as suggested by a recent test using transplanted insect organs [9]. Cells with little sensitivity to environmental signals, in contrast, would be expected to entrain by coupling to an endogenous pacemaker [30–32], as recent results in rodents suggest [14]. It is not yet known if the rhythms of such input-limited cells are also mutually coupled *in situ*. At an intermediate level, glands and perhaps ganglia may have environmentally entrainable clocks that are also coupled to other tissues, such as the brain [33]. Internal desynchronisation studies, similar to those reported here, will allow this diversity of circadian organisation to be tested in intact organisms with fully functional clock systems.

Conclusions

Our results demonstrate that gene expression in higher plants is controlled by autonomous circadian clocks. The circadian system in many types of isolated tissue maintains self-sustaining rhythms and is entrainable by light. The copies of this circadian system that are distributed throughout the intact plant function independently of any central pacemaker, of each other and of transmitted entraining signals. Simultaneous but independent entrainment of clocks in different anatomical locations is principally responsible for synchronising their circadian rhythms. The resulting circadian system is highly flexible but might be less accurate than the clocks of other species.

Materials and methods

Plant materials, growth and imaging conditions

All plant materials have been previously described: *Nicotiana tabacum CAB–LUC* [20] and *PHYB1–LUC* [19]; *A. thaliana CAB–LUC* [26] and *CHS–LUC* [34]; *CHS–LUC* rhythms will be described in detail elsewhere. Transgenic plants were grown and imaged on solid agar media [20] under LD(12,12). At time 0, plant tissues had been grown for 12 days (Figure 2a,b,e) or 24 days (Figures 2c,d, 3 and 4). Cotyledons were removed from some plants to avoid overlap with emerging leaves (Figures 2c,d and 3). When foil covers were required, 1% charcoal was included in the media to reduce light scattering. Luminescence signals were imaged [20,34] from groups of two to three petioles (Figure 2a) or 12 individual tobacco plantlets simultaneously. Rhythms of *CHS–LUC* activity are less robust, so *CHS–LUC* rhythms were tested in groups of 15 seedlings.

Data analysis

Luminescence data in the graphs shown were smoothed with a three-point moving average and are representative of results replicated in two (Figures 2a and 4) or three to four independent experiments. Data in Figure 2b were 'detrended' with a best-fit exponential. Scoring of peak phases in each cycle was reliable only in organs with high rhythmic amplitude. For both the desynchronised and control organs expressing *CAB–LUC* and *PHYB–LUC*, 5–20% had low initial amplitude and a further 20–27% had lost amplitude on the last cycle; 40% of *CHS–LUC* groups had an amplitude too low to score. A minority of *CAB–LUC* and *PHYB–LUC* organs with high-amplitude rhythms were not initially entrained in antiphase, because of foil displacement: Figure 2b, 1 (of 7 plants); Figure 2d and *CAB–LUC*, 5 (of 28); Figure 2e, 4 (of 17 groups); Figure 3, 1 (of 11); Figure 4, 2 (of 17).

The relative phases of organs in control plants varied by up to 2 h in each cycle and by the same amount in total after 4 days in LL (S.C.T., A.H. and A.J.M.; unpublished data). Only 4 of the 57 desynchronised plants in all experiments had altered the relative phase of their organs by more than 2 h after 4 days in LL. Phase changes relative to the phase-advanced organ averaged less than 0.6 h on the fourth cycle in all experiments. Mean unsigned (either direction) phase changes on the fourth cycle (with SD, *n*) were: Figure 2b, 1.7 h (1.5, 6); Figure 2d and *CAB–LUC*, 1.2 h (1.2, 23); Figure 2e, 1.2 h (1.5, 13); Figure 3, 1.6 h (0.8, 10). In Figure 4, the observed mean phase difference of the free-running leaf relative to the re-entrained leaf in the second cycle of LL (96–120 h) was 11.6 h (SD 1.5, $n = 15$); the predicted phase difference was 12 h.

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