A Rhythmic Gene Entrained to Midnight May Regulate Photoperiod-Controlled Flowering in Arabidopsis

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The widely held explanation for photoperiod-controlled flowering in long-day plants is largely embodied in the External Coincidence Hypothesis which posits that flowering is induced when activity of a rhythmic gene that regulates it (a putative “flowering gene”) occurs in the presence of light. Nevertheless, re-examination of the Arabidopsis flowering data from non 24-hour cycles of Roden et al. suggests that External Coincidence is not tenable if the circadian rhythm of the “flowering gene” were entrained to sunrise as commonly accepted. On the other hand, the hypothesis is supported if circadian cycling of the gene conforms to a solar rhythm, and its entrainment is to midnight on the solar clock. Data available point to flowering being induced by the gene which peaks in its expression between 16 to 19 h after midnight. In the normal 24 h cycle, that would be between 4 p.m. and 7 p.m., regardless of the photoperiod. Such timing of the “flowering gene” expression allows for variable coincidence between gene activity and light, depending on the photoperiod and cycle period. A correlation is found between earliness of flowering and the degree of coincidence of “flowering gene” expression with light (r = 0.88, p<0.01).

INTRODUCTION

To ensure that a plant’s metabolism and physiological function at the time of the day when it derives the most benefit, many of its genes undergo rhythmic activity changes in a cycle approximating twenty-four hours. In temperate region plants, such circadian rhythms can further enhance their fitness by regulating certain functions to occur at prescribed times of the year. For example, the enhancement of reproductive success through photoperiod-induced seasonal flowering is critical to the fitness of the species. This is achieved through the interactions of the circadian rhythm with environmental factors, especially the daylength [1]. In many studies conducted in environment-controlled growth chambers, the effect of the photoperiod can be isolated for detailed examination, with other factors such as ambient temperature fluctuation controlled. A model plant frequently used in such research, Arabidopsis thaliana, is a “long-day” plant that flowers when the daylength is sufficiently long. A. thaliana is facultatively long-day in that there is no critical threshold daylength that strictly determines whether it flowers or does not flower [2]. This is a useful characteristic in the understanding of photoperiod-controlled

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†Abbreviations: N-H cycle, Nanda-Hamner cycle; St, Solar time; Zt, Zeitgeber time.

Keywords: Arabidopsis, circadian rhythm, External Coincidence Hypothesis, midnight, N-H cycle, photoperiod, seasonal flowering, solar clock, solar rhythm, T cycle

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flowering since flowering outcomes in experiments are not dichotomous (“all or none”). Instead, earliness of flowering can be recorded as a continuous variable representing the propensity to flower as influenced by the photoperiod.

Circadian rhythms are by no means confined to plants, but are in fact ubiquitous in other living organisms, be they animals [3], insects [4], fungi [5], or microbes [6], although the respective controlling mechanisms may differ. What is the nature of the interaction in plants between the circadian rhythm and the photoperiod in the induction of flowering? Despite considerable progress having been achieved in recent years, the mechanism is not fully understood. The commonly accepted explanation for photoperiod-controlled flowering in Arabidopsis is embodied in the External Coincidence Hypothesis [7]. Flowering is induced when substantial activity of a putative rhythmic gene that controls it (hereafter the “flowering gene”) occurs in the presence of light. In most depictions of External Coincidence, this diurnal cycle of gene activity maintains a consistent phase when measured from “sunrise” (“lights-on” in the growth chamber), as depicted in Figures 1A-1E and 1G-1J. Nevertheless, sunrise is by no means the only possible entrainment reference that can explain photoperiod-controlled flowering. For this reason, a re-analysis of data from an earlier study is undertaken here to explore whether alternative entrainment references for the circadian rhythm, other than the light transition at “sunrise” (“lights-on”) might play a role in this respect.

Sunrise is a well-recognized plant zeitgeber (timing cue), understandably because plants can readily detect light, and therefore sense the transition from darkness to light at sunrise. In the same way that plants are adept at detecting the darkness/light transition, another obvious zeitgeber would be sunset, the transition from light to darkness. Yet, there are still other possible timing references, especially considering that sunrise and sunset have inherent liabilities in fulfilling this role. As the time of sunrise and sunset shifts with the season, genes entrained to sunrise or sunset peak earlier or later in their activities as the daylength increases or decreases over the course of the year. This variation poses a disadvantage to rhythmic genes with functions that require light, such as those linked to photosynthesis. A cyclic gene with an its peak activity consistently timed from sunrise could peak too early or too late to receive the most intense sunlight around noon at different times of the year. In this connection, Millar [8] opined in his paper that the CHLOROPHYLL A-B BINDING PROTEIN (CAB) genes would not be driven by dawn or dusk as that would obligate their phases to be at a constant time interval from sunrise or sunset. Michael et al. had earlier noted that not all plant circadian rhythms were entrained to sunrise [9]. They observed that the phase of CATALASE3 (CAT3) remained unchanged when timed from sunrise regardless of daylength. On the other hand, that of CAB2 did not maintain a constant interval from sunrise when the photoperiod was altered; instead, it peaked consistently around the mid-point of the light period (noon). From this observation, the authors proposed the existence of two clock oscillators governing plant rhythms, but they did not pursue the difference from the standpoint of different timing references.

In their microarray of rhythmic Arabidopsis genes, Michael et al. [10] showed that a large number of genes experienced a phase advance (earlier expression measured from sunrise) of 4 h when the photoperiod was reduced from 16 h light to 8 h light. Looking further afield, it becomes clear that the relationship is by no means fixed and exclusive for a 4 h phase shift against a photoperiod difference of 8 h. The phase shift is consistently half of the difference between the two photoperiods compared (examples in Appendix A). The way such a relationship could be explained is if these cyclic genes were entrained not to sunrise, but to noon or midnight. Accordingly, an alternative endogenous rhythm, the solar rhythm, has been proposed together with a possible mechanism for its operation [11,12]. Technically, any rhythm that has a cycle period approximating a 24-hour day is a circadian rhythm. As such, the solar rhythm is essentially a variant of the circadian rhythm where the time elapsed is measured from noon or midnight (Solar time, St), rather than from sunrise (Zeitgeber time, Zt). The significant characteristic of the solar clock and solar time is that, as with Greenwich Mean Time (GMT) or Coordinated Universal Time (UTC), its reference markers, noon and midnight do not shift with changes in the photoperiod. A gene on the solar rhythm that peaks in its activity around noon, for example, would continue to do so year-round independently of the season.

In all, there are hence four possible timing references against which plant rhythms might be entrained. The rhythm of a gene that regulates seasonal events such as the induction of flowering could be tethered to sunrise – as convention dictates – or to any of the other timing references, i.e. sunset, noon or midnight, the last two being non-zeitgeber timing references. In 2002, Roden et al. [2] performed experiments involving non 24-hour cycles (T cycles and Nanda-Hamner cycles [13]) of varying cycle periods to characterize photoperiod-controlled flowering in Arabidopsis. The present study re-examines the data obtained from that earlier investigation to gain further insight into the control of seasonal flowering as influenced by the daylength. For the purpose of the present analysis, the External Coincidence Hypothesis is used in arguments to examine how earliness of flowering might be controlled.

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ANALYSIS

The data for this analysis have been extracted mainly from the Arabidopsis T cycle and Nanda-Hamner (N-H) cycle results of Roden et al.; full experimental details are found in their report [2]. Briefly, the light to dark period ratio was maintained at 1:2 for varying cycle periods between 16 and 32 h in five T cycles. In four N-H cycles, the light period was fixed at 8 h for the cycle periods of 16 h (G), 20 h (H), 24 h (I) and 28 h (J). The timings of sunrise, noon and sunset are hence identical for all N-H cycles. It is only the timing of midnight that varies according to the length of the cycle period. Earliness of flowering in plants subjected to the various T cycles is shown in (F). In the N-H cycles, the light period is fixed at 8 h for the cycle periods of 16 h (G), 20 h (H), 24 h (I) and 28 h (J). The light and dark periods respectively. Curves show an example of a possible gene peak occurring in the light on long days, but not on short days. Histograms in (F) and (K) reproduced from Fig. 4 of Roden et al., Copyright (2002) National Academy of Sciences, USA.

Figure 1. Earliness of flowering in plants subjected to the various T cycles and Nanda-Hamner (N-H) cycles. With the exception of (G) where light and dark periods are equal in duration, all other diagrams depict short days in which the dark periods exceed the light periods. In the T cycles, the light to dark period ratio is maintained at 1:2 for cycle periods of 16 h (A), 20 h (B), 24 h (C), 28 h (D) and 32 h (E). Earliness of flowering in plants subjected to the various T cycles is shown in (F). In the N-H cycles, the light period is fixed at 8 h for the cycle periods of 16 h (G), 20 h (H), 24 h (I) and 28 h (J). The timings of sunrise, noon and sunset are hence identical for all N-H cycles. It is only the timing of midnight that varies according to the length of the cycle period. Earliness of flowering in plants subjected to the various N-H cycles is shown in (K). Unshaded and shaded backgrounds denote light and dark periods respectively. Curves show an example of a possible gene peak occurring in the light on long days, but not on short days. Histograms in (F) and (K) reproduced from Fig. 4 of Roden et al., Copyright (2002) National Academy of Sciences, USA.
ity of the putative “flowering gene” was expressed in the presence of light. For T cycles of 16, 20, 24, 28 and 32 h, the light period spanning the first one third of the cycle period was respectively 5.3, 6.7, 8.0, 9.3 and 10.7 h. The increasing trend in earliness of flowering could hence be explained by External Coincidence.

**Flowering in Arabidopsis Subjected to N-H cycles**

In the series of N-H cycles, the cycle periods ranged from 15 h to 28 h. Unlike the T cycles, all the N-H treatments experienced identical durations of light supplied over 8 h, starting from sunrise. The difference in cycle periods were due to varying durations of the dark period from sunset to the following sunrise (Figures 1G to 1J). Yet, compared with the 24 h cycle period (N-H 24) that is taken as a reference, both the shorter cycle period in N-H 16 and the longer period in N-H 28 showed advanced (earlier) flowering (Figure 1K). How might that be explained?

Let us first consider the case of N-H 16. Because the cycle period was shortened, the gene rhythm programmed for 24 hours could have over-shot the end of the 16-hour cycle period and carried over into the light period of the next cycle. (Figure 1G). Consequently, substantial gene activity during this overhang occurred in the presence of light.

There is also another explanation. Whereas the light period of 8 hours per cycle was maintained for all N-H treatments, individual cycles cannot be considered in isolation in relation to the induction of flowering. Instead, the effect of light on flowering would have come from a sequence of consecutive cycles. Over any specified time interval – say, 10 calendar days, for instance – more short cycles than long cycles are completed. Thus, plants in the N-H 24 treatment would have completed 10 cycles over 10 calendar days and would therefore have accumulated a total of $10 \times 8 = 80$ hours in the light. On the other hand, plants in the shorter N-H 16 periods would have experienced 15 cycles and a total of $15 \times 8 = 120$ hours in the light; that’s 50 percent more light than in the N-H 24 treatment. Could earlier flowering have resulted from increased cumulative light in N-H 16? While this explanation might work for N-H 16, it is not applicable to N-H 20 and N-H 28 as discussed below.

Like N-H 16 plants, N-H 20 plants would also have completed more cycles than N-H 24 plants for any given time period. But unlike the case of N-H 16, plants subjected to N-H 20 did not flower earlier than those in N-H 24. The N-H 20 plants were in fact delayed in flowering (Figure 1K). There is no obvious explanation for this from the standpoint of the External Coincidence Hypothesis. The case of N-H 28 is no less puzzling. When the cycle period was increased from 24 h to 28 h, flowering was advanced (Figure 1K). But why would this have happened? N-H 24 and N-H 28 had the same 8 hours of light per cycle, and the longer cycles of 28 h in fact amassed less cumulative light than the 24 h cycle. It is worth reiterating that the 4 additional hours tagged on to the N-H 28 cycle (as compared with N-H 24) were not an extra 4 hours of light, but 4 hours of darkness. How does increasing the period of darkness improve coincidence with light to promote earlier flowering? There is no obvious explanation from the External Coincidence Hypothesis.

“Displacing Gene Expression into the Day”

To explain earlier flowering in N-H 28, the statement of Roden et al. [2] that “any treatment that displaces (the ‘flowering gene’) expression into the day should mimic the effects of longer days and accelerate flowering” is examined. In the case of N-H 28, the question is how gene expression might have been “displaced into the day” (into the light). Extending the duration of the dark period does not alter the gene rhythm. Plants subjected to light/dark cycles and then transferred to continuous darkness, for example, continue to display their diurnal rhythms in the absence of light, this being among the criteria that define a circadian rhythm. The gene phase is entrained to a timing reference to maintain cyclic precision, and expression of a gene hence gets “displaced” when its timing reference is altered. In the particular case of N-H 28, it would have involved the timing reference to be re-set when a further 4 h of darkness was tagged on to the 16 h dark period of the N-H 24 reference. Conventional thinking dictates that the timing reference of circadian cycles is sunrise. But as mentioned, sunset, noon, and midnight are other possibilities that should also be considered. An examination of the N-H cycles in the study narrows down the possibilities to one.

Sunrise, noon, and sunset are all events that are defined by the light period of the photoperiod, being respectively the start, mid-point, and end of the light period. Since all N-H cycles in Roden’s study had a fixed light period of 8 h, the timings of sunrise, noon, and sunset were identical for all the cycles, regardless of the cycle period, i.e. sunrise at Zt 0, noon at Zt 4 and sunset at Zt 8. Changing the duration of the dark period of the photoperiod would have had no bearing on when sunrise, noon, or sunset occurred. Adding 4 hours of darkness to N-H 24 to arrive at N-H 28, for example, should have done nothing to the rhythm of the “flowering gene” other than embed its activity peak deeper into an extended period of darkness and delaying the advent of the next period of light. If extended darkness did not affect a gene rhythm entrained to sunrise, or to noon, or to sunset, that leaves only the timing of midnight that that could have been manipulated by changing the length of the dark period (Figure 1G to 1J). For midnight to be validated as a timing reference for the flowering gene, it must explain quantitatively the
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is present only on long-days, but not on short-days. Peak activity would not be expected to occur on long days later than Zt 16 because it would then be taking place in darkness (Figure 2A). It would not be earlier than Zt 8 either because light is present from sunrise to Zt 8 on both long days and short days; it is the presence of light between Zt 8 and Zt 16, exclusive to long days, that differentiates the long days from short days. Accordingly, expression of the “flowering gene” would be expected to fall somewhere within the 8 h window of light between Zt 8 and Zt 16 (Figure 2A). That argument holds if its circadian rhythm were entrained to sunrise, as generally accepted. However, should the rhythm of the “flowering gene” be entrained not to sunrise but to midnight, the window of coincidence between the “flowering gene” and light would no longer be from Zt 8 to Zt 16.

Let us first consider when coincidence would not occur when the timing reference of the “flowering gene” were midnight. Its peak under long-day conditions would not be expected to occur within the 8 h interval of light from Zt 4 to Zt 12 because light is present on both long days.

Figure 2. Windows of light experienced only on long days, but not on short days, where the circadian rhythm of the “flowering gene” is entrained either to sunrise or to midnight. In A, a twenty-four hour cycle of light and darkness is shown where the circadian rhythm is entrained to sunrise on short days (8 h light, upper panel), or on long days (16 h light, lower panel). In B, a twenty-four hour cycle of light and darkness is shown where the circadian rhythm is entrained to midnight on short days (8 h light, upper panel), or on long days (16 h light, lower panel). Peak expression of the “flowering gene” occurs at a constant interval from the entrainment reference (sunrise in A, or midnight in B) regardless of the photoperiod. Curves show examples of possible gene peaks occurring in the light on long days, but not on short days: 11 h from sunrise in A and 18 h from midnight in B. Double headed arrows denote the windows of light unique to long days. Unshaded and shaded backgrounds denote light and dark periods respectively.

earliness of flowering in all the T cycles and all the N-H cycles of Roden et al.

Midnight as the Timing Reference for the Rhythmic Gene that Regulates Photoperiod-controlled Flowering

External Coincidence entails a meeting between “flowering gene” expression and light. To know when that might occur, it is necessary to know both the timing of the light period and the timing of gene expression. The exact occurrence of light is known for all the T cycles and N-H cycles that were set for the study. But where in its circadian rhythm might peak activity of the “flowering gene” arise? Here, useful clues can be found by falling back on the oft-repeated comparisons between the 8 h photoperiod of short 24-h days when Arabidopsis flowering is known to be delayed, and the 16 h photoperiods of long days when flowering is accelerated. To conform with the expectations of External Coincidence, the “flowering gene” would be expected to be active at a time when light
Quantitatively. It is reasonable to expect the peak “flowering gene” activity to occur in darkness for many, if not most, of the T and N-H cycles in this study since they were short days (with the dark period exceeding the light period, excepting N-H 16 where the light and dark periods were equal). Nevertheless, gene expression is not a snapshot event, but builds up to a maximum and then subsides. So long as gene expression is positioned close to the light/dark transition, at least a part of the gene activity, even if not its maximal activity, would still have occurred in the presence of light. (See illustrations in Figure 3A). Such partial coincidence serves as a measurable variable for the degree of coincidence which can accordingly be quantified by calculating the proximity of gene expression to the nearest light/dark transition. If, for example, the gene peak occurred exactly at the light/dark transition,
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dark under this 8 h photoperiod. If the presence of light were critical to flower induction in accordance with the External Coincidence Hypothesis, then delayed flowering can be expected. Under the 16 h photoperiod of the long day, on the other hand, peak expression of the gene occurs squarely in the light, 2 h before sunset (Figure 2B, bottom panel). Early flowering can hence be expected for a long-day plant like Arabidopsis, as is the case in numerous reports.

Another approach to analysis is shown in Figure 3B which depicts an example of midnight entrainment accounting quantitatively for the earliness of flowering using a scale diagram. In the illustration, the gene activity peak is 17 h from midnight, and the gene is deemed in this example to remain active about 4 h before and after peak activity. Here, coincidence of gene expression with light is quantified based on actual measurements taken from the diagram; it serves as validation for the calculated measurements in Figure 3A. In most of the T and N-H cycles, peak activity of the gene would be closer to the light at dawn of the subsequent cycle. Here again, relative proximity to the light/darkness transition serves effectively as a variable that reflects quantitatively the coincidence of gene expression with light which is found to be correlated with earliness of flowering (r = -0.882, p<0.01) (Figure 3B, lower panel).

then (assuming a bell-shaped curve of activity rate), the gene would be expressed equally in light and in darkness. If the gene peaked in darkness but close to the light/dark transition, then its activity would have occurred less in the light and more in darkness. The converse would be true for gene peak expression occurring in the light, on the other side of the light/darkness transition. In Figure 3A, 0 on the x-axis represents the transition between light and darkness. Increasing positive values represent time further into the dark period from the transition (and therefore lesser coincidence with light) while increasing negative values represent time further into the light period from the transition (and therefore greater coincidence with light).

After the degrees of coincidence between gene expression and light are determined, correlations are carried out between this variable and earliness of flowering. No significant relationships are found in the light window 4 to 8 h from midnight. In fact, the negative (non-significant) correlations point to a trend in the “wrong” direction, with increasing coincidence associated with delayed flowering (Table 1). On the other hand, positive correlations (p<0.05 and p<0.01) are obtained in the light window between 16 and 20 h after midnight. The regression of early flowering with the degree of light coincidence in all T cycles and N-H cycles for a gene peak of activity occurring 18 h from midnight (r = 0.876, p<0.01) is shown as an example in Figure 3A.

The 24 h cycle is of particular interest in this example as maximum gene expression that takes place 18 h after midnight occurs in the dark in short days, two hours after sunset (Figure 2B, top panel). This means that the greater part of “flowering gene” expression occurs in the dark under this 8 h photoperiod. If the presence of light were critical to flower induction in accordance with the External Coincidence Hypothesis, then delayed flowering can be expected. Under the 16 h photoperiod of the long day, on the other hand, peak expression of the gene occurs squarely in the light, 2 h before sunset (Figure 2B, bottom panel). Early flowering can hence be expected for a long-day plant like Arabidopsis, as is the case in numerous reports.

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| Flower-induction light window | “flowering gene” peak expression timed from midnight (h) | Correlation coefficient, r | Statistical significance |
|------------------------------|--------------------------------------------------------|-----------------------------|--------------------------|
| 4 – 8 h from midnight        | 4                                                      | -0.608                      | NS                       |
|                              | 5                                                      | -0.608                      | NS                       |
|                              | 6                                                      | -0.608                      | NS                       |
|                              | 7                                                      | -0.608                      | NS                       |
|                              | 8                                                      | -0.608                      | NS                       |
| 16 – 20 h from midnight      | 16                                                     | 0.784                       | p<0.05                   |
|                              | 17                                                     | 0.850                       | p<0.01                   |
|                              | 18                                                     | 0.876                       | p<0.01                   |
|                              | 19                                                     | 0.809                       | p<0.05                   |
|                              | 20                                                     | 0.653                       | NS                       |

NS, not significant.
CONCLUSION AND OUTLOOK

Re-examination of the Arabidopsis flowering data from non 24-hour cycles (especially the N-H cycles) of Roden et al. [2] suggests that External Coincidence is not tenable if the rhythm of the “flowering gene” were entrained to sunrise. On the other hand, the hypothesis can be supported if the circadian cycling of this gene conforms to a solar rhythm, and its entrainment is to midnight. Flowering is induced when the gene activity coincides with a complementary external factor (a gate) which, in the present analysis, is taken to be the presence of light. The data available is consistent with flowering being induced to varying extents, depending on coincidence with light, by the gene which has its maximum activity arising between 16 to 19 h after midnight. In the normal 24 h cycle, that would be between 4 p.m. and 7 p.m. regardless of the photoperiod. As different rhythmic genes peak in expression throughout the day [14], there would be many such genes that show maximum expression within that time frame. For example, the Arabidopsis gene GIGANTEA (GI) peaks in its activity around 4 p.m. irrespective of the photoperiod (Appendix A). Rhythmic genes that cycle according to the solar rhythm (i.e. entrained to noon or midnight) are by no means a rarity. An analysis [15] of the peak activities of rhythmic Arabidopsis genes in the microarray of Michael et al. [10] found that rhythmic genes cycling on solar time are far more common than those entrained to sunrise.

While the mechanism of flower induction in this analysis is consistent with the External Coincidence Hypothesis that is used as the basis for arguments here, this does not preclude the gene activity coinciding with some other gate other than light, such as the concurrent activity of another gene as espoused by the Internal Coincidence Hypothesis [15,16]. Whatever the nature of the gate, earliness of flowering as influenced by the photoperiod appears to hinge on the calibration reference of a critical circadian rhythm to midnight. This plausible explanation proposed for Arabidopsis flowering behavior based on limited data would benefit from more thorough study for its full validation.

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APPENDIX A: THE SOLAR RHYTHM

Any endogenous rhythm where its cycle period is approximately 24 h is a circadian rhythm. When a gene on the circadian rhythm uses the zeitgeber of sunrise as its calibrating reference, its phase is then expressed at a constant interval from sunrise. As the time of sunrise shifts with the season, genes entrained to sunrise peaks earlier or later in its diurnal cycle as the daylength changes over the course of the year. This situation can be circumvented if the circadian rhythm uses not sunrise, but noon or midnight as its timing reference.

Yet, timing references at noon or midnight are problematic from the theoretical stance since there are no light cues at the mid-points of the light or dark periods that the plant can register. Nonetheless, the mid-point of a period can be determined by the plant if both its beginning and termination (the timing of sunrise and sunset) are known. It is from this standpoint that an alternative circadian rhythm – the solar rhythm – has been proposed [1,2].

When the photoperiod is altered, the phase of a gene that cycles on the solar rhythm is unaffected when measured from noon or midnight. However, when timed from sunrise, the gene phase is delayed by a duration equal to half of the increase in the photoperiod. For example, when the photoperiod is increased from 8 h to 16 h (a difference of 8 h), the gene phase is delayed 4 h. Hence, a simple way to identify a solar rhythm is to observe if the phase that is timed from sunrise changes in this manner when the photoperiod is changed. To illustrate the solar rhythm, an example of gene activity of a plant and that of an animal are presented below.

Solar rhythm of the Arabidopsis gene, GIGANTEA (GI)

Panel A in Figure S1 shows the rhythm of GI under two photoperiods, both timed from sunrise. The difference between the 8 h photoperiod and the 16 h photoperiod is 8 h. The phase of GI under the longer photoperiod is delayed 4 h as compared to that under the shorter photoperiod. Essentially, the phase shift is half the difference in the photoperiod.

In Panel B, noon and midnight of the two cycles are aligned. Measured from noon or midnight, peak gene activity occurs about 4 h from noon (4 p.m.) or 16 h from midnight, regardless of the photoperiod.

Figure S1. Diurnal cycle of GI in Arabidopsis under short and long photoperiods. The curves in (A) and (B) are identical except that the rhythms in (A) are referenced to sunrise (Zt=0 at lights-on), whereas those in (B) are referenced to noon/midnight (St=0 at the mid-point of the light period, or 12 noon). Two repeated 24 h cycles are shown. Readings for each curve are normalized by assigning a value of 50 for the highest reading. Shaded and unshaded portions of the horizontal bars denote dark and light periods respectively. Zt, zeitgeber time; St, solar time; N, noon; M, midnight. Reproduced from Fig. 1 of Yeang [1], as adapted from Fig. 5 of David et al. [3] with permission from of Oxford University Press and John Wiley & Sons respectively.
In another report appearing in the university thesis of Berns [4], the possibility of the GI rhythm being entrained to sunrise, or to sunset, or to noon/midnight is evaluated by examining its peak expression under seven photoperiods. As shown in Figure S2, GI activity peaks in the light in some photoperiods, but in darkness in others. The expression peak of GI varies considerably when measured from sunrise, ranging from about 5.5 h to 11 h after ‘lights-on’. It is hence obvious that GI is not expressed at a constant interval from sunrise. Sunset is not a timing reference here either since, as mentioned, the gene is expressed before sunset for some photoperiods, and after sunset for others. On the other hand, GI expression peaks about 4 h from noon (or 16 h from the preceding midnight), regardless of the photoperiod. The phase shift with different photoperiods is generally half the duration of the difference in the photoperiod. For example, when the photoperiod is shortened from 12 h to 6 h (a difference of 6 h), the phase is advanced by about 3 h. Such rhythmic behaviour is consistent with the GI cycle conforming to the solar cycle, using noon or midnight as the timing reference.

A gene cycling on a solar rhythm can be entrained either to noon or to midnight [2] and it is not possible to tell from the available data above if GI is entrained to which. A rhythm entrained to the solar clock is concurrently referenced both to noon and midnight since the timing of noon is ±12 hours the preceding or subsequent midnight in a 24 h cycle. To determine whether a rhythmic gene is tethered to noon or midnight, its phase could be monitored under various non 24 h cycles to observe if peak gene expression maintains a consistent interval from noon or from midnight. For example, it can be shown in this manner that the Arabidopsis circadian clock gene LHY is entrained to midnight, whereas another clock gene TOC1 is entrained to noon [2].

**Figure S2.** Timing of peak expression of GI under different photoperiods in 24 h cycles. Shaded and unshaded portions of the vertical bars denote dark and light periods respectively. Filled circles denote the time of peak gene expression for each photoperiod, and the dashed line denotes noon for the respective photoperiods. The continuous line represents the regression of the timing of GI peak expression (y) against the light period duration (x) for each photoperiod (y = 0.44x + 4.5; r = 0.96, p<0.001). The regression coefficient, 0.44, approaches 0.50, the value expected for entrainment to noon (mid-point of the photoperiod). The y-intercept at 4.5 indicates that the gene peaks about 4.5 h after mid-day, or 16.5 h from the preceding midnight.

zt, Zeitgeber time. GI expression data extracted from Figure 4.4 of Berns [4].
Solar rhythm of cortisol production in rhesus macaques

A study by Lemos et al. [5] showed that adrenal gland function in rhesus macaques (*Macaca mulatta*) is influenced by the photoperiod. When timed from sunrise, initiation of diurnal cortisol production is delayed under a long photoperiod of 16 h as compared with a short photoperiod of 8 h (Figure S3, Panel A). The delay of about 4 h is half of the difference in the duration of the two light periods. When serum cortisol is measured from noon or midnight (Figure S3, Panel B), initiation of cortisol production is similar for both photoperiods, occurring about 8 h after noon, or 20 h after midnight. This pattern is consistent with the rhythm of cortisol production being entrained to the solar rhythm.

Figure S3. Diurnal cycle of cortisol production under short and long photoperiods. The curves in (A) and (B) are identical except that the rhythms in (A) are referenced to sunrise (Zt=0 at lights-on), whereas those in (B) are referenced to noon/midnight (St=0 at the midpoint of the light period, or 12 noon). Shaded and unshaded portions of the horizontal bars denote dark and light periods respectively. Zt, zeitgeber time; St, solar time; N, noon; M, midnight. Data extracted from Fig. 1 of Lemos et al. [5]; curves smoothened using a 3-point moving averages of the readings.

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