Chapter 1

Measurement of Luciferase Rhythms

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Abstract

Firefly luciferase (LUC) is a sensitive and versatile reporter for the analysis of gene expression. Transgenic plants carrying *CLOCK GENE promoter:LUC* fusions can be assayed with high temporal resolution. LUC measurement is sensitive, noninvasive, and nondestructive and can be readily automated, greatly facilitating genetic studies. For these reasons, LUC fusion analysis is a mainstay in the study of plant circadian clocks.

Key words *Arabidopsis thaliana*, Biological clocks, Circadian clock, Circadian rhythms, Firefly luciferase, Reporter gene, Transcriptional regulation

1 Introduction

The circadian clock is an endogenous timekeeping mechanism that enables organisms to measure and use time to coordinate their biology with the environment. As photoautotrophs dependent on the sun for energy, plants are richly rhythmic. In plants, the circadian clock regulates many aspects of biology, including basic metabolism, and serves as a key player in the coordination of metabolic and signaling pathways [1–5]. It has also become clear that the circadian clock modulates responses to biotic and abiotic stress [6, 7]. One means by which the clock coordinates so many processes is pervasive control of gene expression at the levels of transcription, transcript processing, and transcript abundance [8–11]. Therefore, analyses of gene expression have been central to the elucidation of the timekeeping mechanism [4, 5, 7, 12].

Early studies of the role of the plant circadian clock in regulating gene expression focused on the analysis of steady-state mRNA abundance [13–18]. Time series experiments, so dear to the clock researcher, were exercises in sleep deprivation for the experimenter (for example, note the 132-h time course in Figs. 5 and 6 of Pilgrim and McClung [19]), and subsequent analysis by northern RNA blot hybridization was laborious and time consuming and

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required the destruction of the experimental material. It became abundantly clear that a facile and noninvasive assay for promoter function would enhance molecular genetic analysis of clock function, permit forward genetic analyses, and greatly enhance the recruitment of graduate and postgraduate researchers to the study of plant clocks. Firefly (*Photinus pyralis*) luciferase [20] was demonstrated to be an effective reporter of plant promoter activity [21]. A fusion construct in which the promoter of the *CHLOROPHYLL a/b BINDING PROTEIN2* (*CAB2*, also known as *LIGHT HARVESTING CHLOROPHYLL a/b BINDING1*1* or *LHCB1*1*) gene fused to the firefly *LUCIFERASE* (*LUC*)-coding sequence effectively recapitulated the circadian rhythm in gene expression with LUC activity (light production) in transgenic Arabidopsis seedlings [22]. This breakthrough vastly accelerated the subsequent elucidation of the plant circadian clock network [7, 12].

Luciferase is the generic term for a class of enzymes that oxidize a substrate with the concomitant release of a photon. Luciferases have been found in a broad range of taxa, including bacteria, dinoflagellates, copepods, fireflies and click beetles, and the colonial marine cnidarians, sea pansies (*Renilla* spp.). Emission spectra of these diverse luciferases range from 400 to 620 nm [23]; firefly luciferase has maximal emission at 560 nm [24]. Beetle luciferases, including firefly and click beetle luciferases, mediate the oxidation of their substrate, D-luciferin, in the presence of ATP, Mg^{2+}, and O_2, with concomitant light emission. The firefly luciferase reaction occurs in two steps: first luciferin (D-LH_2) reacts with ATP to yield luciferyl-adenylate (LH_2-AMP), which is oxidized by molecular oxygen to form oxyluciferin (OxyLH_2), CO_2, and AMP [25]. Other luciferases use different substrates. For example, some luciferases from deep-sea organisms oxidize coelenterazine. Uniquely, bacterial luciferases (lux) do not require exogenous substrates for light emissions, making the lux system attractive as a reporter. However, the bacterial lux system is encoded with an operon of five genes [26], in which *luxA* and *luxB* encode luciferase. *luxC*, *luxD*, and *luxE* encode a reductase, transferase, and synthase, respectively, that form a complex and generate an endogenous aldehyde substrate for the bioluminescent reaction [27]. Decanal can serve as an exogenous substrate for the luxA/luxB luciferase, but the decanal level necessary for maximal lux activity damages Arabidopsis seedlings [28]. In the cyanobacterium, *Synechococcus elongatus* PCC 7942, the *luxAB*-encoded luciferase from *Vibrio harveyi*, has proven effective [29, 30]; lethality associated with exogenous substrate addition has been overcome with the introduction of the other genes necessary for the synthesis of the aldehyde substrate into the *S. elongatus* chromosome.

The stability of firefly luciferase (LUC) mRNA, protein, and enzymatic activity is of particular relevance to the use of LUC as a reporter. *LUC* mRNA is relatively unstable. Curiously, the LUC protein itself appears to be rather stable in the absence of its luciferin substrate. Happily, LUC activity is unstable and becomes dependent on de novo translation and, hence, mRNA abundance, which in turn tracks de novo transcription of the *LUC* gene [22, 28]. Over the two decades since its introduction, firefly luciferase has become the preferred reporter for use in circadian studies in plants, and its use has been extended to include fungi, invertebrates, and vertebrates in both tissue culture and transgenic animals [31–37].

2 Materials

95 % Ethanol.

Bleach.

HCl concentrated (37 %).

1. One-half strength Murashige and Skoog Medium [38] (*see* **Note 1**): 1,650 mg/L NH_4NO_3, 440 mg/L $CaCl_2{\cdot}2H_2O$, 370 mg/L $MgSO_4{\cdot}7H_2O$, 170 mg/L KH_2PO_4, 1,900 mg/L KNO_3, 6.2 mg/L H_3BO_3, 0.025 mg/L $CoCl_2{\cdot}6H_2O$, 0.025 mg/L $CuSO_4{\cdot}5H_2O$, 27.8 mg/L $FeSO_4{\cdot}7H_2O$, 22.3 mg/L $MnSO_4{\cdot}4H_2O$, 0.83 mg/L KI, 0.25 mg/L $Na_2MoO_4{\cdot}2H_2O$, 8.6 mg/L $ZnSO_4{\cdot}7H_2O$, 37.2 mg/L $Na_2EDTA{\cdot}2H_2O$ plus 0.5 g/L MES free acid. The pH is adjusted to 5.8, and the medium is solidified with 7 g/L agar. After autoclaving, appropriate antibiotics (filter-sterilized) are added to select for the plasmid. We also add 500 μg/mL carbenicillin to select against residual *Agrobacterium*.
2. D-luciferin (potassium salt): 2.5 mM in water. We typically make a 100 mM stock solution which we store frozen and dilute 40-fold for use.
3. Hamamatsu ORCA II ER CCD camera (C4742-98 ERG; Hamamatsu Photonics, Hamamatsu City, Japan, http://www.hamamatsu.com) (*see* **Note 2**).
4. Topcount™ Microplate Scintillation Counter (Perkin Elmer) (*see* **Note 2**).
5. White or black 96-well microtiter plates (we typically use white Optiplates; Perkin-Elmer) and also clear 96-well microtiter plates (*see* **Note 2**).
6. TopSeal (Perkin-Elmer), a clear adhesive plastic sealant for the 96-well microtiter plates.

3 Methods

1. Two alternative methods to sterilize Arabidopsis seeds can be used.
   (a) Surface-sterilize seeds:
   Soak seeds in sterile water for 30 min. Decant. Soak in 95 % ethanol for 10 min. Decant. Soak in 10 % bleach for 10 min. Decant. Rinse five times (5 min per rinse) in sterile water.
   (b) Alternatively, surface-sterilize seeds by chlorine vapor [39]. Place seeds in a microcentrifuge tube in a rack within a desiccator in a fume hood. Place a 250 mL beaker containing 100 mL bleach into the desiccator jar, carefully add 3 mL concentrated HCl to the bleach, and immediately seal the jar. Allow sterilization by chlorine fumes to proceed for 3–16 h (we typically allow sterilization to continue overnight). Open container in a fume hood, seal microfuge tubes or other seed containers, and remove surface-sterilized seed for use (*see* **Note 3**).
2. Plate seeds on one-half-strength MS medium, which can be supplemented with 1–2 % sucrose (*see* **Note 4**). For imaging with a CCD camera, seeds can simply be spread at the desired density or can be arrayed (square petri dishes are useful for this, and we have also placed individual seedlings in the wells of 96-well microtiter plate containing 200 μL of one-half-strength MS medium). For analysis by luminometer, plate seeds on one-half-strength MS medium, which can be supplemented with 1–2 % sucrose and solidified with 0.7 % agar to facilitate the removal of seedlings with their roots intact.
3. Stratify for 3–4 days at 4 °C to synchronize germination.
4. Release seedlings into entraining conditions (light–dark cycles or temperature cycles) for 7–10 days or until primary leaves are emerging.
5. Prior to imaging with a CCD camera, D-luciferin must be applied (one can spray the seedlings with a 2.5 mM solution, or one can pipette luciferin onto the surface of the medium at ~30 μL per seedling) (*see* **Note 5**). Transfer seedlings into a growth chamber in continuous dark for imaging with a CCD camera supported by Metamorph software. We typically image for 30 min at 1–2-h intervals, although this can be varied according to reporter signal strength (*see* **Note 6**). Figure 1 shows examples of *CCA1* and *TOC1* promoter activity as measured with *promoter:LUC* fusions, imaging seedlings grown in 96-well microtiter plates. Rhythmic parameters (period and phase, as well as relative amplitude error, a measure of the

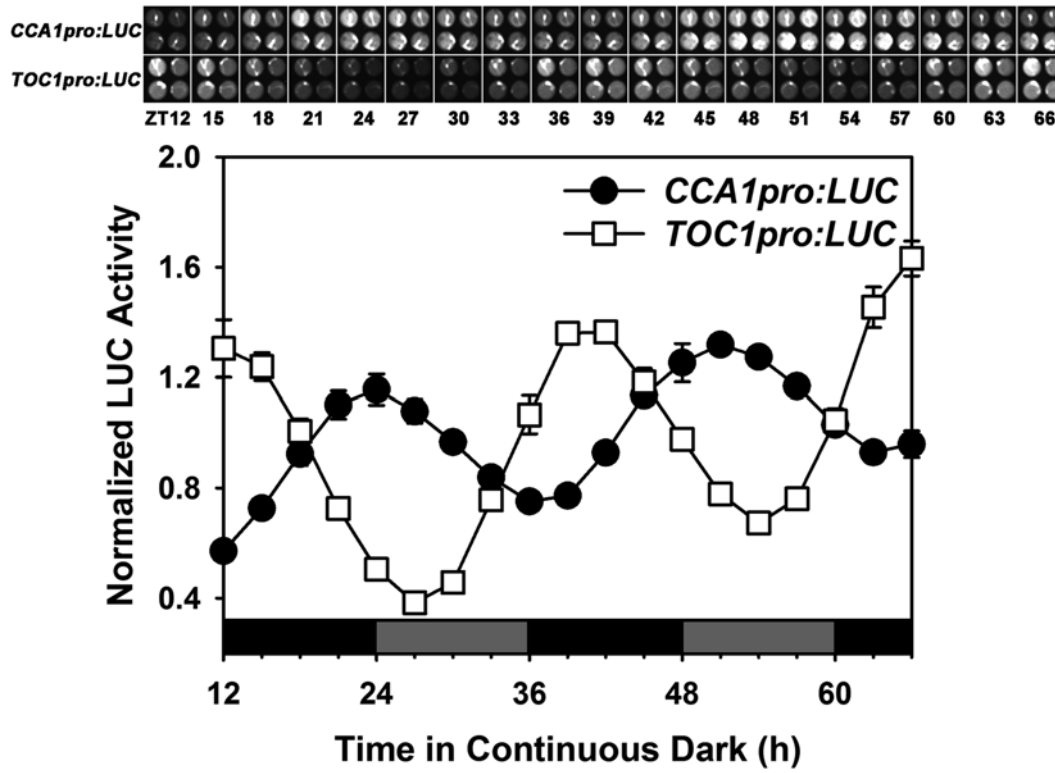

Fig. 1 Time series of images of Arabidopsis seedlings taken with a CCD camera. Seedlings (one seedling per well in a 96-well microtiter plate) were entrained to a 12-h light/12-h dark cycle and released into continuous dark for imaging. *Upper panel* shows a time series of images of transgenic Arabidopsis seedlings carrying either *CCA1pro:LUC* or *TOC1pro:LUC* (four seedlings per transgene). The *lower panel* shows quantification of *promoter:LUC* activity. Data are presented as mean ± SEM ($n = 24$). *Filled circles* indicate *CCA1pro:LUC*, and *open squares* indicate *TOC1pro:LUC. White bars* indicate subjective light, and *gray bars* indicate subjective dark

robustness of rhythmicity) are extracted from time series data by fast Fourier transform nonlinear least squares (FFT-NLLS) [40] using the BRASS software package (http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm). BRASS is a Microsoft Excel workbook for the analysis and display of rhythmic data series. BRASS provides a convenient user interface, allowing the user to import data from commonly used data acquisition packages (e.g., Metamorph, Night Owl, TopCount), automatically run FFT-NLLS, and export the output in MS Excel format.

For analysis with a TopCount, following entrainment seedlings are transferred to 96-well microtiter plates (opaque, white, or black, to prevent light contamination between wells; we use white plates, Optiplate-96, Perkin Elmer), containing 200 μL of the same medium used for seedling growth during entrainment plus 30 μL of 2.5 mM D-luciferin. Plates are then

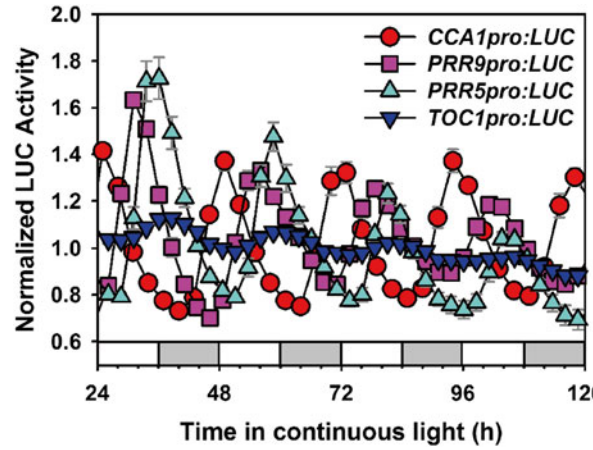

Fig. 2 Quantification of *CLOCK GENE promoter:LUC* activity in continuous light measured with a TopCount. *CCA1* (*CCA1pro:LUC*; *red circles*), *PRR9* (*PRR9pro:LUC*; *magenta squares*), *PRR5* (*PRR5pro:LUC*; *cyan triangles*), and *TOC1* (*TOC1pro:LUC*; *blue triangles*) promoter activity as measured with *promoter:LUC* fusions in transgenic Arabidopsis seedlings. For each trace, data were normalized to the mean value for that promoter over the time series, which facilitates the comparison of promoters of different strengths. Data are presented as mean ± SEM for 24 seedlings (12 for *TOC1pro:LUC*). *White bars* indicate subjective light, and *gray bars* indicate subjective dark

sealed with a clear adhesive sealant (TopSeal; Perkin Elmer); 2–3 holes should be made in the sealant above each plant with a needle to allow gas exchange. The plates are fed into the TopCount sampling chamber from "stackers" with 20- or 40-plate capacity. The TopCount is completely automated (*see* **Note 7**). A "stop plate" must be placed after the last sample plate: this plate should have two barcode stickers on the right side to be recognized by the TopCount as the last of a stack of plates. This then triggers the restacking of the plates into the feeding stacker for a second sampling (*see* **Note 8**). Figure 2 shows examples of *CCA1*, *PRR9*, *PRR5*, and *TOC1* promoter activity as measured with *promoter:LUC* fusions (*see* **Note 9**).

4 Notes

1. We buy our MS medium premixed from Phytotechnology Laboratories.
2. Luciferase activity measurement: There are chiefly two routes to high-throughput measurement of LUC activity: imaging via a low-light charge-coupled device (CCD) camera or activity measurements with a luminometer. In our lab we have relied on imaging with a Hamamatsu ORCA II ER CCD camera (C4742-98 ERG; Hamamatsu Photonics, Hamamatsu City,

Japan, http://www.hamamatsu.com) or with a Topcount™ Microplate Scintillation Counter (Perkin Elmer) with six detectors. In general, the TopCount luminometer assay permits higher throughput analyses and so is attractive for forward genetic screens [41], although the TopCount does not image and so there is a loss of spatial resolution. Light from seedlings expressing high LUC activity can "contaminate" readings from adjacent wells containing seedlings expressing lower LUC activity. Accordingly, we use opaque (white or black) 96-well microtiter plates for our samples. For experiments in continuous light or in light–dark cycles, we interleave the sample plates with three clear microtiter plates to permit light penetration to the central wells of the sample plates (*see* **Note 7**).

3. Although we describe experiments with transgenic Arabidopsis seedlings, these protocols are readily adapted for use with protoplasts [42] or with tissue culture from other species, such as *Brassica rapa* [43].
4. For experiments in continuous dark it is useful to add 1–2 % sucrose to the one-half-strength MS medium as this permits sustained rhythmicity. However, it is important to note that sucrose (3 %, or ~90 mM) affects circadian period in both continuous light and continuous dark as well as amplitude and rhythmic persistence in continuous dark [44].
5. Because LUC protein is quite stable, it accumulates prior to introduction of the substrate luciferin and the introduction of luciferin results in a transient pulse of anomalously high light production that should be allowed to dissipate prior to measurement of de novo activity. We therefore initiate imaging typically 12–24 h after luciferin addition. With the TopCount we routinely entrain seedlings within 96-well plates to which luciferin has been added for one more entraining cycle before release into continuous conditions.
6. CCD cameras can be housed in growth chambers containing lights (LEDs are best) such that continuous light or light–dark cycles can be imposed. If the lights are computer or timer controlled, one can turn off the lights prior to imaging for automated operation. Alternatively, seedlings can be grown in a growth chamber under any desired light conditions and can be manually transferred into the dark camera chamber for imaging. Of course, this type of experiment is clearly *not* automated.
7. We typically entrain seedlings to a light–dark cycle imposed by fluorescent or LED lights affixed to the TopCount and controlled by a timer allowing imposition of appropriate light–dark cycles and continuous light or dark, and the TopCount is programmed to load each successive plate into the sample chamber for luminescence measurement. To permit light to reach the plates when loaded into the stacker, we have cut

holes on all sides of the stacker without compromising its physical integrity. Between each sample plate, we interleave three clear 96-well plates to allow light to reach the seedlings; these clear plates must cycle through the sampling chamber but need not be read, which is signaled by not bar-coding them. Of course, interleaving with three clear plates reduces the capacity of the large 40-plate stacker to 11 sample plates containing 1,056 seedlings. A second potential limitation to this system is that light is not uniform across the plate, but in our experience this has only minimal effect on rhythmicity.

8. TopCounts may be purchased with 2 to 12 detectors—more detectors speed sampling proportionally. For a TopCount with 6 detectors, with a reading time for each well set to 5 s (we typically read 5 s per well, but have sampled from 1 to 10 s, depending on promoter strength), one full 96-well plate can be read in about 2 min, and a stack of 11 plates in constant light can be read in 90 min. Higher throughput, although with lower temporal resolution, can be achieved in continuous dark because it is not necessary to interleave the sample plates; many clock-regulated promoters, such as that of *CAB2*, are expressed at lower levels in the dark [22, 45], but there are notable exceptions, including *COLD AND CIRCADIAN REGULATED 2* (*CCR2*, also known as *GLYCINE-RICH RNA-BINDING PROTEIN 7*, *GRP7*) [46] and *CATALASE 3* (*CAT3*) [47]. Sucrose (1–2 %) is typically added to the medium for experiments in continuous dark because it increases amplitude and persistence of the rhythms in many genes, but *see* **Note 4**.

   If the LUC transgene is expressed at low levels, one can reduce background and enhance signal detection by extending the delay after the plate enters the sampling chamber before counting is initiated. This delay permits the background of seedling-delayed fluorescence (light emission following cessation of illumination) to diminish before initiating sampling. We typically use a delay of 1 min but have delayed for 3 min when measuring particularly weak promoters. This obviously extends sampling time and reduces sampling frequency. It should be noted that delayed fluorescence itself cycles with a circadian rhythm and has been developed as a useful non-transgenic assay to measure circadian rhythms [48].

9. *CLOCK GENE promoter:LUC* fusion constructs: LUC+ (Promega, Madison, WI) is a modified cytoplasmic form of firefly luciferase that is 5–20-fold brighter than the native peroxisomal form initially used for plant clock research [22, 28, 41]. We cloned a promoterless *LUC+* gene into pENTR1 (Invitrogen) to generate pENTR1A_luc+ (Fig. 3a). We then clone *CLOCK GENE* promoters into the multiple cloning site of pENTR1A_luc+ and then recombine these *CLOCK GENE*

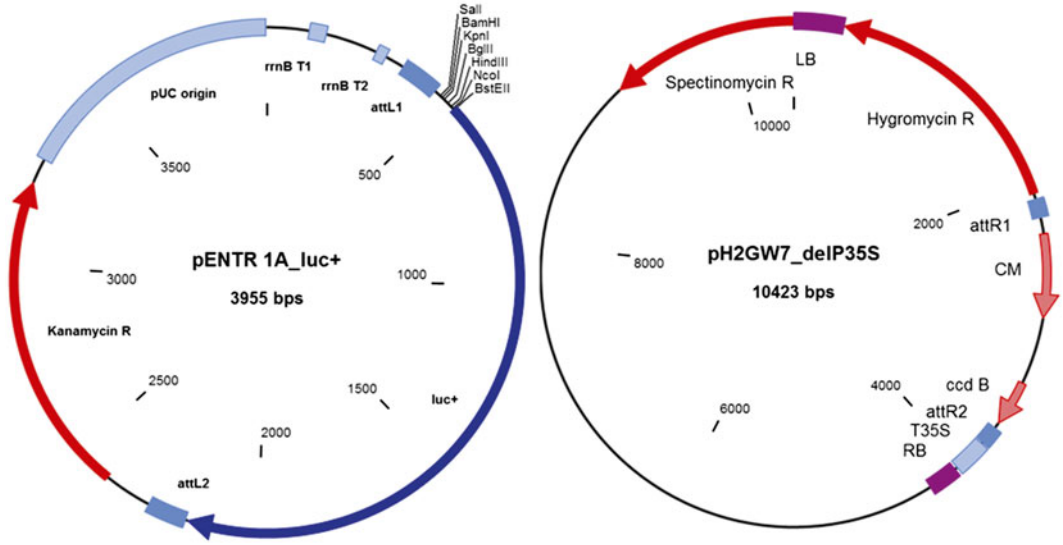

Fig. 3 Generating *CLOCK GENE promoter:LUC* fusion constructs. Promoters from the clock genes *PRR9, PRR5,* and *TOC1*were subcloned into a modified pENTR1 (Invitrogen) entry vector, pENTR1A_luc+ (*left*), and recombined into the binary vector pH2GW7 [49] from which the CaMV 35S promoter had been deleted (*right*) by LR recombination

promoter:LUC fusions into the binary vector pH2GW7 [49] from which the CaMV 35S promoter has been deleted (Fig. 3b). The resultant binary plasmids carrying the *CLOCK GENE promoter:LUC* fusions are transformed into Arabidopsis by floral dip [39] using *Agrobacterium tumefaciens* GV3101.

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References

1. Fukushima A, Kusano M, Nakamichi N et al (2009) Impact of clock-associated *Arabidopsis* pseudo-response regulators in metabolic coordination. Proc Natl Acad Sci U S A 106: 7251–7256
2. Robertson F, Skeffington A, Gardner M et al (2009) Interactions between circadian and hormonal signalling in plants. Plant Mol Biol 69:419–427
3. Graf A, Schlereth A, Stitt M et al (2010) Circadian control of carbohydrate availability for growth in *Arabidopsis* plants at night. Proc Natl Acad Sci U S A 107:9458–9463
4. Pruneda-Paz JL, Kay SA (2010) An expanding universe of circadian networks in higher plants. Trends Plant Sci 15:259–265
5. McClung CR, Gutiérrez RA (2010) Network news: prime time for systems biology of the plant circadian clock. Curr Opin Genet Dev 20:588–598
6. Hotta CT, Gardner MJ, Hubbard KE et al (2007) Modulation of environmental

responses of plants by circadian clocks. Plant Cell Environ 30:333–349

7. McClung CR (2011) The genetics of plant clocks. Adv Genet 74:105–138
8. Covington MF, Maloof JN, Straume M et al (2008) Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biol 9:R130
9. Doherty CJ, Kay SA (2010) Circadian control of global gene expression patterns. Annu Rev Genet 44:419–444
10. Filichkin SA, Priest HD, Givan SA et al (2010) Genome-wide mapping of alternative splicing in *Arabidopsis thaliana*. Genome Res 20:45–58
11. Sanchez SE, Petrillo E, Beckwith EJ et al (2010) A methyl transferase links the circadian clock to the regulation of alternative splicing. Nature 468:112–116
12. Nagel DH, Kay SA (2012) Complexity in the wiring and regulation of plant circadian networks. Curr Biol 22:R648–R657
13. Kloppstech K (1985) Diurnal and circadian rhythmicity in the expression of light-induced nuclear messenger RNAs. Planta 165: 502–506
14. Nagy F, Kay SA, Chua N-H (1988) A circadian clock regulates transcription of the wheat *Cab-1* gene. Genes Dev 2:376–382
15. Paulsen H, Bogorad L (1988) Diurnal and circadian rhythms in the accumulation and synthesis of mRNA for the light-harvesting chlorophyll a/b-binding protein in tobacco. Plant Physiol 88:1104–1109
16. Tavladoraki P, Kloppstech K, Argyroudi-Akoyunoglou J (1989) Circadian rhythm in the expression of the mRNA coding for the apoprotein of the light-harvesting complex of photosystem II. Plant Physiol 90:665–672
17. Fejes E, Pay A, Kanevsky I et al (1990) A 268 bp upstream sequence mediates the circadian clock-regulated transcription of the wheat *Cab-1* gene in transgenic plants. Plant Mol Biol 15:921–932
18. Millar AJ, Kay SA (1991) Circadian control of *cab* gene transcription and mRNA accumulation in *Arabidopsis*. Plant Cell 3:541–550
19. Pilgrim ML, McClung CR (1993) Differential involvement of the circadian clock in the expression of genes required for Ribulose-1,5-bisphosphate carboxylase/oxygenase synthesis, assembly, and activation in *Arabidopsis thaliana*. Plant Physiol 103:553–564
20. de Wet JR, Wood KV, DeLuca M et al (1987) Firefly luciferase gene: structure and expression in mammalian cells. Mol Cell Biol 7:725–737
21. Ow DW, Wood KV, DeLuca M et al (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science 234(4778):856–859
22. Millar AJ, Short SR, Chua N-H et al (1992) A novel circadian phenotype based on firefly luciferase expression in transgenic plants. Plant Cell 4:1075–1087
23. Ozawa T, Yoshimura H, Kim SB (2013) Advances in fluorescence and bioluminescence imaging. Anal Chem 85:590–609
24. Aflalo C (1991) Biologically localized firefly luciferase: a tool to study cellular processes. Int Rev Cytol 130:269–323
25. Vieira J, Pinto da Silva L, Esteves da Silva JCG (2012) Advances in the knowledge of light emission by firefly luciferin and oxyluciferin. J Photochem Photobiol B 117:33–39
26. Belas R, Mileham A, Cohn D et al (1982) Bacterial bioluminescence: isolation and expression of the luciferase genes from *Vibrio harveyi*. Science 218:791–793
27. Close D, Xu T, Smartt A et al (2012) The evolution of the bacterial luciferase gene cassette (*lux*) as a real-time bioreporter. Sensors 12:732–752
28. Millar AJ, Short SR, Hiratsuka K et al (1992) Firefly luciferase as a reporter of regulated gene expression in higher plants. Plant Mol Biol Rep 10:324–337
29. Liu Y, Golden SS, Kondo T et al (1995) Bacterial luciferase as a reporter of circadian gene expression in cyanobacteria. J Bacteriol 177:2080–2086
30. Kondo T, Strayer CA, Kulkarni RD et al (1993) Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. Proc Natl Acad Sci U S A 90:5672–5676
31. Stanewsky R (2007) Analysis of rhythmic gene expression in adult Drosophila using the firefly luciferase reporter gene. Methods Mol Biol 362:131–142
32. Morgan LW, Greene AV, Bell-Pedersen D (2003) Circadian and light-induced expression of luciferase in *Neurospora crassa*. Fungal Genet Biol 38:327–332
33. Gooch VD, Mehra A, Larrondo LF et al (2008) Fully codon-optimized luciferase uncovers novel temperature characteristics of the *Neurospora* clock. Eukaryot Cell 7:28–37
34. Wilsbacher LD, Yamazaki S, Herzog ED et al (2002) Photic and circadian expression of luciferase in mPeriod1-luc transgenic mice in vivo. Proc Natl Acad Sci U S A 99:489–494
35. Welsh DK, Yoo S-H, Liu AC et al (2004) Bioluminescence imaging of individual

fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Curr Biol 14:2289–2295

36. Yamaguchi S, Mitsui S, Miyake S et al (2000) The 5′ upstream region of mPer1 gene contains two promoters and is responsible for circadian oscillation. Curr Biol 10:873–876
37. Yamazaki S, Numano R, Abe M et al (2000) Resetting central and peripheral circadian oscillators in transgenic rats. Science 288:682–685
38. Murashige TR, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497
39. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743
40. Plautz JD, Straume M, Stanewsky R et al (1997) Quantitative analysis of *Drosophila period* gene transcription in living animals. J Biol Rhythms 12:204–217
41. Southern MM, Millar AJ (2005) Circadian genetics in the model higher plant *Arabidopsis thaliana*. Methods Enzymol 393:23–35
42. Kim J, Somers DE (2010) Rapid assessment of gene function in the circadian clock using artificial MicroRNA in Arabidopsis mesophyll protoplasts. Plant Physiol 154:611–621
43. Xu X, Xie Q, McClung CR (2010) Robust circadian rhythms of gene expression in *Brassica rapa* tissue culture. Plant Physiol 153:841–850
44. Dalchau N, Baek SJ, Briggs HM et al (2011) The circadian oscillator gene *GIGANTEA* mediates a long-term response of the *Arabidopsis thaliana* circadian clock to sucrose. Proc Natl Acad Sci U S A 108:5104–5109
45. Millar AJ, Kay SA (1996) Integration of circadian and phototransduction pathways in the network controlling *CAB* gene transcription in *Arabidopsis*. Proc Natl Acad Sci U S A 93:15491–15496
46. Strayer C, Oyama T, Schultz TF et al (2000) Cloning of the *Arabidopsis* clock gene *TOC1*, an autoregulatory response regulator homolog. Science 289:768–771
47. Michael TP, McClung CR (2002) Phase-specific circadian clock regulatory elements in *Arabidopsis thaliana*. Plant Physiol 130:627–638
48. Gould PD, Diaz P, Hogben C et al (2009) Delayed fluorescence as a universal tool for the measurement of circadian rhythms in higher plants. Plant J 58:893–901
49. Karimi M, Inzé D, Depicker A (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci 7:193–195