Technical Advance

Combining GAL4 GFP enhancer trap with split luciferase to measure spatiotemporal promoter activity in *Arabidopsis*

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Running title ETSLA: Enhancer trap-compatible split luciferase assay

Summary

In multicellular organisms different types of tissues have distinct gene expression profiles associated with specific function or structure of the cell. Quantification of gene expression in whole organs or whole organisms can give misleading information about levels or dynamics of expression in specific cell types. Tissue- or cell-specific analysis of gene expression has potential to enhance our understanding of gene regulation and interactions of cell signalling networks. The Arabidopsis circadian oscillator is a gene network which orchestrates rhythmic expression across the day/night cycle. There is heterogeneity between cell and tissue types of the composition and behaviour of the oscillator. In order to better understand the spatial and temporal patterns of gene expression, flexible tools are required. By combining a Gateway®-compatible split luciferase construct with a GAL4 GFP enhancer trap system, we describe a tissue-specific split luciferase assay for non-invasive detection of spatiotemporal gene expression in Arabidopsis. We demonstrate the utility of this enhancer trap-compatible split luciferase assay (ETSLA) system to investigate tissue-specific dynamics of circadian gene expression. We confirm spatial heterogeneity of circadian gene expression in Arabidopsis leaves and describe the resources available to investigate any gene of interest.

Significance statement

Cell and tissue type resolution of gene expression is important to understand functional properties of signalling networks. We have adapted a split luciferase system for use with existing collections of enhancer trap lines to provide a versatile tool to detect dynamic tissue-specific gene expression in whole Arabidopsis seedlings and demonstrate its utility to detect spatial heterogeneity of circadian rhythms in leaves.
Introduction

In multicellular organisms different cell and tissue types have distinct gene expression profiles. Quantification of gene expression in whole organs or whole organisms can give misleading information about levels or dynamics of expression in specific cell types. Tissue- or cell-specific analyses of gene expression can enhance our understanding of transcriptional responses to environmental cues. The circadian clock orchestrates rhythmic gene expression according to daily environmental cues. The Arabidopsis core circadian oscillator is comprised of a gene network of regulatory feedback loops involving around 20 genes (Haydon et al., 2019). Although most core oscillator genes are expressed in all cells, there is spatial heterogeneity of circadian gene expression in Arabidopsis (Para et al., 2007; Xu et al., 2007; James et al., 2008; Wenden et al., 2012; Martí et al., 2013; Endo et al., 2014; Takahashi et al., 2015; Bordage et al., 2016; Kim et al., 2016; Gould et al., 2018; Greenwood et al., 2019).

Several techniques have been used to isolate specific cell populations from plant tissues for gene expression analyses but a common disadvantage of these is the destructive nature of sampling. Sampling protocols can alter gene expression, diminish intercellular signalling and limit resolution for temporal information. Laser-Capture Microdissection (LCM) was developed to isolate cell populations from sections of animal tissues for gene expression analyses (Emmert-Buck et al., 1996; Bonner et al., 1997) and has been similarly applied to plant tissues (Asano et al., 2002; Kerk et al., 2003; Nakazono et al., 2003). RNA can be extracted from LCM samples and used for quantitative transcript analyses. Using LCM in Arabidopsis, circadian rhythms have been measured in shoot apices by measuring transcripts and fluorescent proteins in dissected tissues (Takahashi et al., 2015). However, this technique is limited by accessibility of the tissue of interest and identifiable cell types. Similarly, a protocol was developed to isolate mesophyll, vasculature and epidermal tissues from Arabidopsis leaves with high purity to measure transcripts over a circadian time-course, which indicated distinct characteristics of circadian gene expression in leaf vasculature (Endo et al., 2014).

Transcriptome analyses of Fluorescence Activated Cell Sorting (FACS) of protoplasted transgenic plants expressing cell type-specific GFP markers has allowed high-resolution spatial maps of transcription in Arabidopsis roots (Birnbaum et al., 2003; Brady et al., 2007; Dinneny et al., 2008), and more recently adapted for leaves (Grønlund et al., 2012; Coker et al., 2015). However, the process of protoplasting can alter gene expression. By contrast, the INTACT method (isolation of nuclei tagged in specific cell types) uses an affinity-approach to isolate nuclei from transgenic plants expressing a cell type-specific biotinylated nuclear protein marker (Deal and Henikoff, 2011). This method has been used to determine cell type-specific nuclear transcriptomes in numerous plant species to provide spatial information about gene expression in diverse cell types (Ron et al.,...
Emerging single-cell RNA sequencing (scRNA-seq) technologies have the potential to generate high-resolution maps of gene expression networks, particularly in emerging model species where specific fluorescent markers are not available (Efroni and Birnbaum, 2016). Recent studies have performed scRNA-seq on protoplasts from *Arabidopsis* root cells using droplet-based microfluidics to provide the first gene expression maps of roots of wild-type and seedlings at single-cell resolution (Ryu et al., 2019; Denyer et al., 2019; Jean-Baptiste et al., 2019). These studies have provided high-resolution spatiotemporal maps and identified developmental waves of gene expression associated with root cell differentiation. Applications of scRNA-seq in plant systems are likely to accelerate as the technology and data analyses become more accessible.

Transgenic luciferase reporters are well-suited for measuring gene expression in planta with high temporal resolution. However, luminescence imaging systems typically have poor spatial resolution. A modified split luciferase system has been shown to be effective for measuring circadian rhythms specifically in phloem companion cells (Endo et al., 2014). The N- and C-terminal halves of luciferase were expressed from a phloem-specific and circadian gene promoter, respectively, and the reconstituted luciferase produces luminescence only in the phloem companion cells in which both transgenes are expressed. This system allows measurement of gene expression in specific cell and tissue types but depends on availability of characterised tissue-specific promoters.

Enhancer trap screens have been effective in identifying tissue-specific enhancer elements in Arabidopsis. Rather than reporting activity of a full promoter, which can be regulated by multiple endogenous and environmental signals, enhancer trap lines drive reporter activity from a specific enhancer. In this way, these lines can be advantageous over tissue-specific promoters. The GAL4 GFP enhancer trap lines carry a transgene encoding a GAL4-VP16 transcriptional activator from yeast with a minimal promoter and a modified GFP targeted to the ER (mGFP5ER) under the control of GAL4-binding upstream activation sequences (UAS). Transformants have been screened for diverse spatial patterns of GFP fluorescence, driven by enhancer elements in the vicinity of the insertion regulating GAL4-VP16 (Haseloff, 1999; Laplaze et al., 2005). Unique patterns of expression have been identified for enhancer trap lines for which there is no known gene promoter (Gardner et al., 2009). Characterised enhancer trap lines can be used to drive tissue-specific expression of any gene of interest from a UAS by introducing a second transgene by crossing or transformation. In this way, GAL4 GFP enhancer trap lines have been exploited to modify gene expression in specific cell types (Laplaze et al., 2005; Laplaze et al., 2007; Gan et al.,
or drive cell type-specific reporters (Dodd et al., 2006; Jia et al., 2007; Martí et al., 2013) by transactivation. There are approximately 250 GAL4 GFP enhancer trap lines across four sets available from stock centres and characterisation of these lines continues to increase, broadening their utility (Ckurshumova et al., 2009; Radoeva et al., 2016).

We have exploited the tissue-specific variation of the circadian oscillator to develop and test the combination of a Gateway®-compatible split luciferase construct with an established GAL4 enhancer trap system (Laplaze et al., 2005) for non-invasive detection of spatiotemporal gene expression in Arabidopsis. We demonstrate the utility of this enhancer trap split luciferase assay (ETSLA) system to investigate tissue-specific promoter activity and apply this to measure circadian gene expression. We confirm spatial heterogeneity of circadian promoter activity in Arabidopsis leaves and describe the resources available to investigate any gene of interest.
Results

Development of an enhancer trap tissue-specific split luciferase assay (ETSLA) system

To measure gene expression in specific tissues in Arabidopsis we set out to adapt a split luciferase system to be applicable with existing GAL4 GFP enhancer trap lines (Figure 1). Enhancer trap lines are available which contain a transgene including a minimal promoter driving GAL4 expression inserted adjacent to endogenous or cryptic tissue-specific enhancer elements in the Arabidopsis genome. The transgene also includes UAS elements driving GFP to localise expression of GAL4 and verify transactivation of UAS. In order to exploit these GAL4 GFP enhancer trap lines for tissue-specific luciferase, we generated two constructs. A UAS:JN construct comprising a UAS upstream of a fusion of an N-terminal half of LUCIFERASE+ (nLUC+) and the c-Jun bZIP domain of a heterodimer of the AP1 complex. A GW:AC construct comprises a Gateway® cassette upstream of a fusion of a C-terminal half of LUC+ and A-Fos, a leucine zipper domain which interacts with c-Jun (Endo et al., 2014). In principle, when both halves of LUC+ are expressed in the same cell, the stable formation of the AP1 complex will reconstitute the luciferase enzyme and emit bioluminescence in the presence of its substrate, D(+)-luciferin.

The GAL4 GFP enhancer trap system has been adapted to Arabidopsis (Haseloff, 1999) and numerous lines have been reported and characterised (Laplaze et al., 2005; Radoeva et al., 2016) and are available from seed stock centres (Table S1). As a proof of concept, we chose four Arabidopsis enhancer trap lines with distinct patterns of expression in the leaf. These lines have tissue-specific GFP expression in spongy mesophyll (JR11-2), leaf vasculature (KC274), leaf epidermis (KC464) and guard cells (E1728), as previously shown with confocal laser scanning microscopy (Gardner et al., 2009; Martí et al., 2013). To confirm that these lines can drive transactivated expression of a reporter in a second transgene in the expected tissues, we introduced a UAS:β-GLUCURONIDASE (GUS) construct by transformation. For all four GAL4 GFP lines, strong GUS activity was consistently detected in the expected tissue or cell types, (Figure 2a). Weak GUS staining was sometimes detected in neighbouring cells, which we interpret as diffusion of the reaction product. The expected GUS patterns were observed in 50/52 T1 seedlings, representing the four lines. This suggests tissue-specific transactivation is robust in these GAL4 GFP enhancer trap lines.

In order to further characterise these lines, we used Thermal Asymmetric InterLaced (TAIL) PCR (Liu and Whittier, 1995) to identify the positions of the T-DNA inserts in the genome (Figure 2b). We confirmed the T-DNA in E1728 (guard cell GAL4 GFP enhancer trap line) on chromosome 5 (position 26215578) flanked by the coding sequences of a putative chloroplast-targeted Dof zinc finger transcription factor (At5g65590; STOMATAL CARPENTER 1, SCAP1) and a L-type lectin
receptor kinase (At5g65600, **LECRK-IX.2**), as previously reported (Gardner *et al.*, 2009). The orientation of the **GAL4 GFP** transgene is in the opposite orientation to both flanking genes (Figure 2b). The T-DNA in KC464 (epidermal **GAL4 GFP** enhancer trap line) was located on chromosome 1 (position 28430296) within the second intron of the gene sequence of a C2H2-like zinc finger protein (At1g75710), 1490 bp downstream of the start codon in the same orientation as the gene. Thus, **GAL4** might be expressed similarly to the protein coding gene (Figure 2b). The T-DNA in KC274 (vascular **GAL4 GFP** enhancer trap line) is located on chromosome 3 (position 19256215), flanked by the coding sequences of a chloroplast-localised sulphate transporter (At3g51895; **SULTR3;1**) and a heat shock transcription factor (At3g51910; **HSFA7A**). The **GAL4 GFP** transgene is 538 bp upstream of At3g51895 and oriented in the same direction (Figure 2b). The T-DNA in JR11-2 (spongy mesophyll **GAL4 GFP** enhancer trap line) is on chromosome 5 (position 5217128), flanked by the coding sequences of a putative member of the pentatricopeptide repeat superfamily (At5g15980) and an NSP-interacting receptor-like kinase (At5g16000; **NIK1**). The **GAL4 GFP** transgene is in the same orientation as both flanking genes, upstream of At5g16000 (Figure 2b).

To validate the use of the selected enhancer trap lines for investigation of circadian rhythms of gene expression, we examined the rhythmic expression of the gene adjacent to each enhancer trap locus using Diurnal, a database of published microarray data sets (Mockler *et al.*, 2007; Figure 2c). The expression of At5g65590 (E1728, guard cell), At1g75710 (KC464, epidermal) or At5g16000 (JR11-2, spongy mesophyll) were not overtly rhythmic in diel (LDHC) or continuous light (LL_LDHC) conditions. Expression of At3g51895 (KC274, vascular) was rhythmic in both diel and continuous light, peaking at dawn.

Expression of the gene adjacent to the **GAL4 GFP** transgene might not be an indicative marker for the activity of the enhancer. Therefore, we directly measured expression of the **GAL4** transcript by quantitative RT-PCR (qRT-PCR) in the epidermal, vascular and spongy mesophyll lines over a 24 h diel cycle (Figure 3). Consistent with the Diurnal data (Figure 2c), diel rhythms of **GAL4** transcript level were not detected in the spongy mesophyll or epidermal lines. Transcript levels of **GAL4** peaked at zeitgeber time (ZT) 0 in the vascular line, although the amplitude was substantially lower than circadian clock genes **CIRCADIAN CLOCK ASSOCIATED 1** (**CCA1**) and **TIMING OF CAB2 1** (**TOC1**) (Figure 3). The diel oscillation of **GAL4** in this enhancer trap line might alter the rhythms of the reconstituted luciferase in these tissues. On the other hand, the relatively low amplitude rhythm of the enhancer might not be sufficient to substantively impact regulation of the UAS in the context of highly expressed circadian clock reporters. In either case, examination of rhythmic expression of **GAL4** in each enhancer line is necessary to interpret estimates of circadian rhythms with this split luciferase system.
The ETSLA system requires the introduction of two transgenes into the *GAL4 GFP* enhancer trap lines. We reasoned that best strategy was to co-transform the UAS:JN and promoter:AC construct into wild-type *A. thaliana* by floral dip and then cross the double transformants with each enhancer trap line and measure luminescence in F1 and subsequent generations. The advantage of this strategy is that differences between luciferase signal can be confidently assigned to the enhancer trap by controlling for position effects of the new transgenes. We also used an alternative strategy of transforming the UAS:JN construct into each enhancer trap line and then crossing these double transgenics to each promoter:AC transgenic. The latter strategy has the advantage of versatility of the system for new promoters of interest.

To test the application of the ETSLA system to measure circadian rhythms in Arabidopsis seedlings, we used the GW:AC vector to generate constructs for three core circadian oscillator genes with distinct phases in the morning (*CCA1*), afternoon (*PSEUDO RESPONSE REGULATOR 7; PRR7*) and evening (*TOC1*) and an evening-phased circadian output gene (*COLD, CIRCADIAN RHYTHM AND RNA BINDING 2; CCR2*). The promoter:AC and UAS:JN constructs were introduced into the enhancer trap lines by one or both of the alternative strategies described above (Table S2). We identified populations harbouring all three transgenes for the vascular and mesophyll enhancer trap lines and detected luminescence in all lines. We first confirmed that the presence of all three transgenes is both necessary and sufficient to produce luciferase luminescence. We did not detect luminescence signal above background levels in Arabidopsis seedlings containing any two of a promoter:AC, UAS:JN or a *GAL4 GFP* transgene. Clear signal was only detected when all three transgenes were present (Figure 4a). Luminescence imaging of the *TOC1* vascular ETSLA lines, carrying all three transgenes, indicated a clear vascular pattern of luminescence signal in leaves (Figure 4b). Together, these confirm that the split luciferase enzyme can be effectively reconstituted using the *GAL4 GFP* enhancer trap lines to produce tissue-specific luminescence in Arabidopsis seedlings.

**Tissue-specific features of circadian oscillator gene expression**

To measure circadian rhythms in the ETSLA lines we imaged luminescence in multiple populations (Table S2) harbouring all three transgenes in continuous light and compared them to promoter:LUC lines reporting whole-seedling promoter activity (Figure 5). Robust circadian rhythms were detected for all promoters with a period ranging from 21-28 h (mean period 22.3 ± 1.3 h; mean relative amplitude error 0.28 ± 0.15; Table S2). For the *CCA1* promoter, we could not detect a difference in circadian period or phase between the vascular or mesophyll ETSLA lines and *CCA1p:LUC* control (Figure 5a). This suggests circadian rhythms of *CCA1* expression are similar in vascular and mesophyll tissues, as previously reported (Endo et al., 2014).
PRR7p:LUC activity comprises two peaks in diel cycles (Figure 5b); a light-activated peak at dawn followed by a circadian peak in the afternoon. Rhythms of PRR7 promoter were distinct in mesophyll and vascular tissues. Circadian period was significantly longer in both tissue types compared to whole-seedling PRR7p:LUC activity, particularly in the vascular ETSLA lines. The circadian phase of the PRR7 promoter was significantly advanced by about 9 h in the vascular line compared to PRR7p:LUC, suggesting distinct oscillator behaviour between these cell types. Interestingly, the earlier peak of PRR7 in the vascular line coincides with the phase of the light-activated peak in PRR7p:LUC, so might be due to increased light-sensitivity of these cells.

The period of TOC1 promoter activity was similar in the mesophyll ETSLA lines compared to total TOC1p:LUC with a small, but significant, phase delay (1.3 h; Figure 5c). By contrast, the period of TOC1 promoter activity was significantly longer in vascular lines compared to TOC1p:LUC and the phase was dramatically advanced, similar to PRR7. Differences in circadian rhythms of TOC1 promoter have previously been reported when driven ubiquitously or in phloem companion cells from the SUCROSE-PROTON SYMPORTER 2 (SUC2) promoter (Endo et al., 2014), so we directly compared rhythms in SUC2p/TOC1p TSLA with the vascular ETSLA lines (Figure 5c). We did not detect a significant difference in circadian period or phase between the SUC2p/TOC1p TSLA lines compared to TOC1p:LUC or the TOC1 mesophyll ETSLA lines in our experiments. The differences in luciferase rhythms between the SUC2p/TOC1p TSLA and the TOC1 vascular ETSLA lines might be due to differences in tissue-specificity of these two lines. The SUC2 promoter is specifically active in phloem companion cells (Truernit and Sauer, 1995; Schulze et al., 2003), whereas we detected broad expression throughout leaf vascular bundles from the vascular enhancer (Figure 2a).

We considered whether the earlier phase in the vascular ETSLA lines for both TOC1 and PRR7 might be caused by the weak dawn phase of the vascular enhancer (Figures 2b, c). However, we think the phase reported by the ETSLA lines is a true reflection of the phase TOC1 and PRR7 promoters in this tissue because the phase of the CCR2 promoter in the vascular ETSLA lines was not shifted compared to CCR2p:LUC, which is phased in the evening similar to TOC1 (Figure 5d). Luciferase activity in the CCR2 mesophyll ETSLA lines was poorly rhythmic (Figure 5d). This might reflect expression of this circadian output in this tissue or could be due to silencing of any one of the three transgenes. The latter is a potential pitfall of introducing multiple transgenes, but this has not been a barrier for the majority of ETSLA lines. In summary, these data confirm the utility of the ETSLA system to study tissue-specific circadian rhythms and confirm distinct circadian oscillator behaviour in different tissue types.

Having observed striking phase difference of the TOC1 promoter in the vascular ETSLA lines, we further examined the expression of transcripts in these lines by qRT-PCR (Figure 6). We measured
transcripts for oscillator genes and each of the three transgenes in shoots of F2 plants of the TOC1 vascular ETSLA lines grown for 24 h in continuous light (Figure 6a). Robust rhythms of endogenous CCA1 and TOC1 expression were phased in the morning and evening, respectively, as expected. Robust, rhythmic expression was also detected for the TOC1p:AC transgene in the same phase as TOC1, confirming that the transgene is correctly expressed. Expression of GAL4 and the transactivated UAS:JN transgene were similar in these plants to that observed for GAL4 in the parental vascular enhancer trap line (Figure 3) with a low amplitude rhythm phased in the subjective morning. We also measured transcripts in these ETSLA F2 plants at ZT5 and ZT13 in a diel growth cycle (Figure 6b). We detected similar expression of the TOC1p:AC transgene to TOC1 and low levels of expression of the transactivated UAS:JN transgene. Thus, in both diel and continuous light conditions, the relative expression of the dawn-phased nLUC transcript is substantially lower than the expression of the evening-phased cLUC transcript in the TOC1 vascular ETSLA lines. This suggests that the phase shift of TOC1 promoter activity in these lines is a true reflection of the phase of TOC1 promoter activity in vascular tissue.

Tissue-specific expression of carbon starvation markers

Sugar signalling and metabolism are closely associated with the circadian clock (Blasing et al., 2005; Graf et al., 2010; Dalchau et al., 2011; Haydon et al., 2013; Haydon et al., 2017) but little is known about tissue-specificity of sugar signalling networks. We generated promoter:AC constructs for DARK INDUCIBLE 6 (DIN6) and SENESCENCE 5 (SEN5), two transcriptional markers of Snf1-related protein kinase 1 (SnRK1; Rodrigues et al., 2013), which is a signalling hub for carbon starvation (Baena-Gonzalez et al., 2007) and modulator of circadian rhythms in Arabidopsis (Frank et al., 2018). Robust circadian rhythms of luminescence were detected in both DIN6p:LUC and SEN5p:LUC transgenic lines (Figure 7), as expected (Frank et al., 2018). No significant difference in circadian period or phase was detected between DIN6p:LUC and the DIN6 vascular ETSLA line (Figure 7a). This suggests that, similar to CCR2 (Figure 5d), morning phasing of this circadian output is not apparent in vascular tissue. For the SEN5 promoter, we were able to isolate ETSLA lines for mesophyll, vasculature and guard cells (Figure 7b). Robust luciferase rhythms were detected in the SEN5 guard cell ETSLA line, suggesting this system is compatible with detecting luciferase expression in potentially very small pools of cells. No difference in circadian period was detected between any ETSLA lines and the SEN5p:LUC control, but significantly earlier phase was detected for the vascular line, similar to TOC1 and PRR7. Thus, there might be heterogeneity in distinct networks of circadian outputs.
Discussion

By building and verifying constructs for a split luciferase system that is compatible with GAL4 enhancer trap lines, we have established the tools and demonstrated utility of a versatile transgenic toolset for spatiotemporal measurement of gene expression in Arabidopsis. We have demonstrated heterogeneity of gene expression dynamics of core circadian clock gene promoters and circadian-regulated outputs. We have shown significant differences between the period and/or phase of circadian rhythms in vascular tissue compared to leaf mesophyll and whole-seedling rhythms for promoters of TOC1, PRR7 and SEN5, but not CCA1, CCR2 or DIN6. This indicates that although there is spatial heterogeneity of circadian rhythms in Arabidopsis leaves, it is not representative of all circadian oscillator components or outputs, suggesting the existence of distinct circadian networks in particular cell types.

We have used the ETSLA system to measure expression of circadian-regulated promoters because the spatial heterogeneity and organisation of circadian oscillators in plant cells is emerging as a fascinating area of research (Endo et al., 2014; Takahashi et al., 2015; Gould et al., 2018; Greenwood et al. 2019). To ensure each enhancer trap line was suitable for this purpose, we measured circadian rhythms of the GAL4 transcript, driven by the enhancer. This validation could be similarly achieved by introducing a UAS:LUC transgene. The tools we have generated are applicable to explore tissue-specific dynamics of gene expression for any stimuli of interest. The particular advantages of the ETSLA system are that detection of luminescence is non-destructive and can be measured in living plants growing in controlled conditions. Luminescence detection is not restricted by position of cell types within the tissue and has sufficient sensitivity to measure relatively small populations of cells. We have successfully detected tissue-specific gene expression using enhancer trap lines for vasculature, mesophyll and guard cells, but the system is compatible with numerous published lines (Laplaze et al., 2005; Gardner et al., 2009; Ckurshumova et al., 2009; Radoeva et al., 2016; Table S1) among hundreds available from seed stock centres. Once the lines have been generated, luminescence experiments can be easily performed. Thus, the ETSLA system is ideally suited to explore effects of environmental conditions or pharmacological treatments on gene expression. For circadian clock research, these approaches could provide insight into communication between oscillators in different cells.

The ETSLA system can be easily utilised by generating a single construct using the Gateway®-compatible GW:AC plasmid. This can be used to introduce any promoter of interest and also may be suitable for translational fusions to the A-FOS:cLUC fragment. Transformants can be crossed into any GAL4 GFP enhancer trap line carrying the UAS:JN transgene, which we have obtained for four lines in this study. This strategy of independent AC and JN transgenes allows for control of
transgene position effects. However, introducing the UAS:JN sequence into the GW:AC plasmid might be worthwhile improvement to the ETSLA system.

We did not identify epidermal ETSLA lines with detectable luminescence in this study. Notwithstanding that we could show effective tissue-specific expression of a UAS:GUS transgene (Figure 2a) and previously a UAS:AEOQRIN (Martí et al., 2013) in the epidermal enhancer trap line, the lack of signal in the ETSLA lines might be due to large vacuoles in epidermal cells affecting luciferase signal or low promoter activity in these cells of the particular genes investigated. Epidermal GAL4 GFP lines with a UAS:JN transgene identified in this study could be used to test other promoter:AC constructs.

Our observation of distinct circadian rhythms in vascular tissue is consistent with a previous study (Endo et al., 2014). A SUC2p/TOC1p TSLA line, which drives expression specifically in phloem companion cells was reported with a later phase compared to a 35Sp/TOC1p TSLA line. We did not detect a significant difference in circadian rhythms of the SUC2p/TOC1p TSLA line in our experiments compared to TOC1p:LUC or TOC1 mesophyll ETSLA seedlings. This may be due to the different control line or inclusion of sucrose in the media, which alters circadian rhythms in Arabidopsis (Haydon et al., 2013; Haydon et al., 2017) and activity of the SUC2 promoter (Truernit and Sauer, 1995). By contrast, we observed both significantly lengthened period and phase advance of TOC1 promoter activity in vascular ETSLA lines, with similar effects for PRR7 and CCR2 promoters. The very different luciferase rhythms in TOC1 vascular ETSLA lines compared to the SUC2p/TOC1p TSLA line is likely because of the different expression pattern of GAL4 and UAS:GUS in the vascular enhancer trap line (KC274), for which we detected a much broader pattern of expression throughout vascular bundles compared to SUC2 promoter activity specifically in phloem companion cells. Thus, there appears to be spatial heterogeneity of circadian rhythms even within vascular tissues.

The tissue-specific features of circadian oscillators might be important for regulating distinct physiological or developmental outputs. Expression of CCA1 from a range of tissue-specific promoters resulted in different effects on photoperiodic flowering (Shimizu et al., 2015). Expression of CCA1 in phloem companion cells from the SUC2 promoter delayed flowering in long days, but not when CCA1 was expressed from IRREGULAR XYLEM 3 (IRX3) or HOMEBOX GENE 8 (HB8) promoters which are specific to xylem and procambian, respectively (Shimizu et al., 2015). While these results are consistent with a specific role for the oscillator in companion cells for regulating flowering, they could also be explained by relatively high expression of SUC2, compared to IRX3 and HB8, or different phases of the three promoters (Mockler et al., 2007). Indeed, our characterisation of the circadian expression of GAL4 in each enhancer trap line demonstrates the importance of considering the expression dynamics of any chosen tissue-specific promoter.
The differences in gene expression dynamics of circadian clock genes, which we detected in different ETSLA lines could be due to differences in light sensitivity of particular cell types. For example, the earlier light-activated peak of *PRR7p:LUC* appeared more pronounced in the vascular ETSLA line compared to whole-seedling rhythms. It has been suggested that light-piping through vascular tissue might contribute to maintain circadian rhythms in Arabidopsis roots (Bordage *et al.*, 2016). Vascular cells might be more sensitive to light signals, or transmission of light through vasculature might be more efficient than mesophyll. The ETSLA system could be an effective tool to explore these dynamics in a wider range of vascular cell types, since several vascular *GAL4 GFP* enhancer trap lines are available (Table S1).
Conclusion

We have adapted a split luciferase system to be compatible with available collections of enhancer trap lines and Gateway®-compatible vectors to provide a versatile system for monitoring dynamic gene expression in specific tissues or cell types. We have validated a small selection of enhancer trap lines for leaf expression and confirmed their suitability to measure tissue-specific circadian rhythms. We have corroborated previous data suggesting heterogeneity in circadian behaviour between leaf mesophyll and vascular tissues and expanded the tool set to investigate this behaviour. We hope this tool will provide a flexible resource to advance research to explore spatial heterogeneity in gene expression and identify sensitivity of particular tissues to various environmental stimuli and endogenous signals in intact, living plants.
Experimental procedures

Plasmid constructs

TOC1p:AC and CCA1p:AC plasmids have been described (Endo et al., 2014). To generate CCR2p:AC, a 2024 bp promoter fragment was amplified from gDNA of Columbia-0 (Col-0) by PCR with primers in containing HindIII sites and ligated into the TOC1p:AC plasmid in place of the TOC1p sequence. The GW:AC plasmid was made by PCR amplification of a 544 bp A-Fos:nLUC (AC) fragment from TOC1p:AC using primers containing XbaI and SpeI sites and ligated into pEarlyGate301 (Earley et al., 2006). For the PRR7p:AC, SEN5p:AC and DIN6p:AC, a 1020 bp PRR7 promoter, 1666 bp SEN5 promoter and 1017 bp DIN promoter were amplified by PCR from Col-0 gDNA and cloned into pENTR/D-TOPO (Invitrogen) and recombined into the with GW:AC plasmid. The SEN5 promoter was also cloned into pEarleyGate301-LUC+, comprised of a LUC+ fragment amplified from CCR2p:LUC+ seedlings (Haydon et al., 2013) and ligated into Xbal sites of pEarleyGate301 (Earley et al., 2006).

The UAS:JN plasmid was generated by PCR amplification of a 656 bp c-Jun:cLUC (JN) fragment from gDNA of TOC1:AC / SUC2:JN seedlings (Endo et al., 2014) with primers containing BamHI and SacI restriction sites. The JN sequence was ligated into pBINYFPAEQ plasmid (Kiegle et al., 2000) in place of the YFP:AEQ sequence, downstream of the UAS. The UAS:GUS plasmid has been described previously (Møller et al. 2009).

All primers are listed in Table S3.

Plant materials

Stable transgenic lines for CCA1p:LUC+, TOC1p:LUC+, PRR7p:LUC+, CCR2p:LUC+, DIN6p:LUC+ and TOC1p:AC / SUC2p:JN are in Col-0 and have been used previously (Haydon et al., 2013; Endo et al., 2014; Frank et al., 2018). The GAL4 GFP enhancer trap lines E1728, JR11-2, KC274 and KC464 are in C24 and have been described (Gardner et al., 2009; Martí et al., 2013).

TOC1p:AC, PRR7p:AC, CCA1p:AC, CCR2p:AC, SEN5p:AC and DIN6:AC were transformed, or co-transformed with UAS:JN, into Col-0 by floral dip (Clough and Bent, 1998). UAS:JN was also transformed into each of the GAL4 GFP enhancer trap lines. Homozygous T3 populations of plants harbouring both the promoter:AC and UAS:JN transgenes were crossed to GAL4 GFP lines. As an alternative approach, UAS:JN transformants were identified for all four GAL4 GFP enhancer trap lines and T1-T2 populations of promoter:AC transformants were crossed to T1 populations of
UAS:JN transformants. Experiments were performed with F1 or F2 populations which would be heterozygous or segregating for the three dominant transgenes, respectively.

Luminescence experiments

Seeds were surface sterilised with a solution of 20 % (v/v) bleach, 0.02 % (v/v) Triton X-100 and washed three times with sterile deionised water. Seeds were sown in clusters of five to twenty on modified Hoagland media (Haydon et al., 2012), solidified with 0.8 % agar type M. Plates were chilled for 2 days at 4°C and grown in 12 h light, 12 h dark (LD) cycles at 20 °C. Light was supplied from red (660 nm), green (550 nm), blue (450 nm) and far-red (730 nm) LED array (HiPoint) at 50 μmol m⁻² s⁻¹. Ten to 14 d old seedlings were treated twice with a topical application of 1 mM D-luciferin, K⁺ salt at least 24 h prior to photon counting. Luminescence was measured for 600 s, following a 2 min delay to decay chlorophyll fluorescence (Gould et al. 2009) at 1 h intervals for 48 h in LD and 120 h of continuous light in a HRPCS2 (Photek) with light supplied from red (660 nm) and blue (470 nm) LEDs at 50 μmol m⁻² s⁻¹. Luminescence for each cluster was normalised to average counts across the time series. Circadian period and circadian phase (corrected for circadian period in free-running conditions) estimates were performed on raw luminescence data between 24 and 120 h in continuous light using fast Fourier Transform-nonlinear least squares (FFT-NLLS) analysis, using BioDare2 (https://biodare2.ed.ac.uk/) (Zielinski et al., 2014). Spatial imaging of luminescence in ETSLA lines (Figure 4) used a NightShade LB 985 Plant Imaging System (Berthold).

Thermal Asymmetric InterLaced (TAIL) PCR

The genomic locations of the GAL4 GFP T-DNAs were determined by TAIL PCR, essentially as described (Liu and Whittier, 1995), using nested specific primers complementary to the right or left T-DNA borders and a degenerate primer (Table S3), as described (Gardner et al., 2009). The products of the tertiary reaction were cloned and sequenced to identify the flanking genomic region(s) of the T-DNA.

Quantitative RT-PCR

Shoots of 14 d old seedlings were snap frozen in liquid N. Total RNA was extracted from frozen tissue using Isolate II Plant RNA Kit (Bioline) with on-column DNasel treatment. cDNA was prepared from 0.5 μg RNA with Tetro cDNA Synthesis Kit (Bioline) using oligo-dT primer. Technical replicates of gene-specific products were amplified with primers in Table S2 in 10 μL reactions using SensiFAST SYBR no-ROX kit (Bioline) on a CFX96 Thermocycler (BioRad). Transcript levels were calculated from C₅ values, incorporating PCR efficiencies calculated with LinRegPCR (Ruijter et al., 2009), relative to the geometric mean of two reference genes.
ISOPENTENYL PYROPHOSPHATE:DIMETHYLALLYL PYROPHOSPHATE ISOMERASE 2 (IPP2) and PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2AA3) (Vandesompele et al., 2002).

β-glucuronidase (GUS) stains

T1 seedlings of GAL4 GFP enhancer trap lines transformed with UAS:GUS were GUS-stained overnight as previously (Haydon et al. 2007) and imaged with a SMZ800 stereomicroscope (Nikon). For leaf sections, seedlings were fixed in a formaldehyde-acetic acid-ethanol (3.7 %-5 %-50 %) mix, dehydrated in a series of an increasingly concentrated ethanol solution and imbibed in series of histoclear. Tissue was infiltrated with wax (Paraplast plus) and subsequently sectioned (8 µm). Sections were imaged with a BX60 microscope (Olympus) using DIC optics.
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Table titles and Figure legends

Figure 1. Components of the enhancer trap tissue-specific split luciferase assay system. In the GAL4 GFP enhancer trap lines, expression of GAL4 is driven by a minimal promoter in the transgene and a tissue-specific enhancer in the genome. GAL4, a yeast transcriptional activator, binds to upstream activation sequences (UAS) to produce tissue-specific expression of GFP, which is encoded by the same transgene. The split luciferase system requires introduction of two transgenes. In one, UAS is upstream of a sequence coding a fusion product of an N-terminal region of luciferase and a c-Jun subunit of the AP1 complex. In the second, a promoter of interest is inserted between att sites by Gateway® cloning upstream of a sequence for a fusion of the C-terminal region of luciferase and an A-Fos subunit of the AP1 complex. Co-expression of all three transgenes in a cell (for example, in leaf vasculature) allows reconstitution of a functional luciferase enzyme, facilitated by the stable interaction of the AP1 complex subunits, generating tissue-specific luminescence (yellow line). Cells in which the tissue-specific enhancer is not activated express only the C-terminal half of luciferase (blue line) and do not produce luminescence.

Figure 2. Characterisation of GAL4 GFP enhancer trap lines used in this study. (a) GUS stains of 20 d old T1 transformants of UAS:GUS in GAL4 GFP enhancer trap lines in guard cells (E1728), leaf epidermis (KC464), vasculature (KC274) and spongy mesophyll (JR11-2). Leaf cross sections are shown for the epidermal, vascular and mesophyll lines (right). Bars represent 1 mm (left, middle) or 100 µm (right). (b) Genomic location and orientation of the GAL4 GFP T-DNAs. Flanking protein coding genes are shown. Arrows indicate direction of transcription. (c) Expression of transcripts adjacent to each GAL4 GFP T-DNA in diel (LDHC) or continuous light (LL_LDHC) conditions. Data were obtained from diurnal.mocklerlab.org (Mockler et al., 2007).

Figure 3. Transcript levels in GAL4 GFP enhancer trap lines in diel conditions. Transcript levels of TOC1, CCA1 and GAL4, relative to IPP2 and PP2AA3, in 10-14 d old enhancer trap line seedlings growing in light-dark cycles. Values are means ± SD, n=3.

Figure 4. Tissue-specific luminescence using ETSLA. (a) Luciferase luminescence in transgenic seedlings containing combinations of a CCA1p:AC (cLUC), TOC1p:AC and UAS:JN (nLUC) transgenes transformed into wild-type (Col-0) or GAL4 GFP enhancer trap lines. Data are represented as Min Max box plots, n=4. Asterisks indicated statistical difference from non-transformed wild-type by two-tailed t-test with Bonferroni corrections (P < 0.01). (b) Luminescence image overlayed on a bright-field image of 15 d old seedlings containing TOC1p:AC and UAS:JN transgenes in the vascular GAL4 GFP enhancer trap line (KC274). Bar represents 10 mm. False colour scale represents counts per second (cps).
**Figure 5.** Tissue-specific circadian clock promoter activity using ETSLA.

Normalised luminescence, period and phase estimates of luciferase activity in 10-14 d old seedlings in continuous light for (a) CCA1, (b) PRR7, (c) TOC1 and (d) CCR2 promoters. ETSLA lines for vascular and mesophyll expression are shown, compared to the promoter:LUC control for each gene and the SUC2-driven TSLA line for TOC1. Luminescence values are means ± SEM, n=4-8. Period and phase estimates are represented as Min Max box plots. Statistical differences from the promoter:LUC control were determined by One-way ANOVA with Tukey's post-hoc tests. Different letters indicate significant differences between samples (P < 0.05).

**Figure 6.** Transcript levels in the TOC1 vascular ETSLA line.

Transcript levels of TOC1, CCA1, GAL4, nLUC and cLUC, relative to IPP1 and PP2AA3 in 10-14 d old TOC1 vascular ETSLA line seedlings growing in (a) continuous light or (b) diel conditions. Values are means ± SD, n=3.

**Figure 7.** Tissue-specific carbon starvation promoter activity using ETSLA.

Normalised luminescence, period and phase estimates of luciferase activity in 10-14 d old seedlings in continuous light. (a) DIN6 vascular ETSLA line and (b) SEN5 vascular, mesophyll and guard cell ETSLA lines are shown, compared to the promoter:LUC control for each gene. Luminescence values are means ± SEM, n=4. Period and phase estimates are represented as Min Max box plots. Statistical differences from the promoter:LUC control were determined by One-way ANOVA with Tukey's post-hoc tests. Different letters indicate significant differences between samples (P < 0.05).

**Table S1.** List of published GAL4 enhancer trap lines compatible with the ETSLA system.

**Table S2.** Summary of transgenic lines used in this study.

**Table S3.** Sequences of primers used in this study.
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Tissue specific endogenous enhancer

Circadian promoter

Transactivation

UAS:JN

Gene promoter:AC

GAL4 GFP enhancer trap line

UAS:JN

Gene promoter:AC

Tissue specific luminescence

No luminescence
Zeitgeber time (h)

CCA1 transcript level

TOC1 transcript level

GAL4 transcript level

JR11-2 (mesophyll)  KC464 (epidermal)  KC274 (vascular)
(a)

| Treatment                  | Luminescence |
|----------------------------|--------------|
| CCA1p:cLUC                 | – + – – – – – + + + – – |
| TOC1p:cLUC                 | – – + – – – – – – + + + |
| UAS:nLUC                   | – + + + + + + + + + + + |
| mesophyll GAL4             | – – – + – + – + – + – |
| vascular GAL4              | – – – – + – + – – + – |

(b)

**TOC1p:cLUC / UAS:nLUC / vascular GAL4**

![Image of luciferase expression](image-url)
Time in continuous light (h)

(a) CCA1

(b) TOC1

GAL4

UAS:nLUC

TOC1:cLUC

Time in continuous light (h)

(b) Normalised Transcript Level

ZT 5  ZT 13

TOC1  UAS:nLUC  TOC1:cLUC
(a) Normalised luminescence (b) Normalised luminescence

- DIN6:LUC
- DIN6 SL V
- SEN5:LUC
- SEN5 SL M
- SEN5 SL G

Time in continuous light (h): 
-24 0 24 48 72 96

Period (h): 
0 4 8 12 16 20 24

Circadian phase (h): 
20 22 24 26
| Line/ABRC number | Expression Pattern | References |
|------------------|--------------------|------------|
| Root | Shoot |
| J0121/N9090 | Xylem pole pericycle cells | Young leaf primordia | Laplaze et al., 2005; Radoeva et al., 2016 |
| J0192 | Lateral root primordia | | Laplaze et al., 2005 |
| J2772 | Root cap, lateral root primordia, base of lateral roots | | Laplaze et al., 2005 |
| Q850 | Root cap, lateral root primordia | | Laplaze et al., 2005 |
| N9113 | All tissues | | Jia et al., 2007 |
| N9093 | Distal root and root-hypocotyl junction | | Jia et al., 2007 |
| N9195 | Some specific tissues of the root | Leaves | Jia et al., 2007 |
| E1728 | nd | Guard cells | Dodd et al., 2006; Gardner et al., 2009 |
| E2036 | nd | Guard cells | Gardner et al., 2009 |
| E292 | nd | Guard cells | Gardner et al., 2009 |
| E361 | Epidermis, cortex | Guard cells, epidermis, mesophyll, cortex, apical meristem | Gardner et al., 2009 |
| E551 | nd | Guard cells, trichomes, epidermis | Gardner et al., 2009 |
| E566 | nd | Guard cells, trichomes, apical meristem | Gardner et al., 2009 |
| E910 | nd | Guard cells, trichomes, epidermis | Gardner et al., 2009 |
| E994 | Epidermis, vasculature, root tip | Guard cells, apical meristem | Gardner et al., 2009 |
| Accession | Tissue 1          | Tissue 2          | Reference                      |
|-----------|-------------------|-------------------|--------------------------------|
| J1512     | Cortex            | Guard cells       | Gardner et al., 2009           |
| KS019     | Epidermis         | Guard cells, apical meristem | Gardner et al., 2009 |
| Q1621     | Epidermis, vasculature | Guard cells, apical meristem | Gardner et al., 2009 |
| Q1622     | Vasculature       | Guard cells       | Gardner et al., 2009           |
| Q2480     | Epidermis, cortex, root tip | Guard cells       | Gardner et al., 2009           |
| Q2481     | Epidermis, cortex, root tip | Guard cells       | Gardner et al., 2009           |
| J2103     | Epidermis, vasculature, root tip | Guard cells, epidermis | Gardner et al., 2009 |
| R010/11   | Epidermis         | Epidermis         | Gardner et al., 2009           |
| Q2610     | All root tissues  | nd                | Waki et al., 2013              |
| Q0990/N9217 | Vasculature       | nd                | Radoeva et al., 2016           |
| Q2500/N9135 | Ground tissue, pericycle and QC | Cotyledon junction and guard mother cell | Radoeva et al., 2016 |
| J0571/N9094 | Ground tissue, some QC cells | Guard and pavement cells | Radoeva et al., 2016 |
| J2731     | Cells above QC    | Guard and pavement cells | Radoeva et al., 2016           |
| M0148/N9303 | Pericycle cells  | Cells surrounding SAM | Radoeva et al., 2016           |
| M0164/N9307 | Pericycle        | Shoot vasculature | Radoeva et al., 2016           |
| M0167/N9308 | nd                | Cotyledon junction | Cary et al., 2002; Radoeva et al., 2016 |
| M0223/N9336 | nd                | Cotyledon junction | Cary et al., 2002; Radoeva et al., 2016 |
| J1092/N9147 | Root apical meristem, LRC | nd                | Radoeva et al., 2016           |
| Sample ID | Description                          | Result | Reference                  |
|-----------|--------------------------------------|--------|----------------------------|
| J3281/N9128 | Young Vasculature tissue, columella cells | nd     | Radoeva et al., 2016       |
| J3411/N9131 | Root apical meristem, LRC            | nd     | Radoeva et al., 2016       |
| M0028/N9274 | Columella cells                      | nd     | Radoeva et al., 2016       |
| Q0680/N9209 | Columella cells                      | nd     | Radoeva et al., 2016       |
| Q1630/N9227 | Columella cells                      | nd     | Radoeva et al., 2016       |
| Q0171/N9207 | Root cap                             | nd     | Radoeva et al., 2016       |
| RM1000     | Leaf margins                         | nd     | Radoeva et al., 2016       |
| J2501/N9121 |                                        | nd     | Radoeva et al., 2016       |
| J2661/N9187 |                                        | nd     | Radoeva et al., 2016       |
| M0136/N9302 |                                        | nd     | Radoeva et al., 2016       |
| M0171      | Suspensor cells                      |        | Radoeva et al., 2019       |

nd: not detected
Table S2. Summary of transgenic lines used in this study.

| ETS LA line | Parent 1 | Parent 2 | Period (mean ± SD) | Phase (mean ± SD) | RAE (mean ± SD) | Experiment |
|-------------|----------|----------|--------------------|-------------------|-----------------|------------|
| CCA1p:LUC   | n/a      | n/a      | 22.2 ± 1.1         | 4.8 ± 0.79        | 0.31 ± 0.07     | Fig 4a     |
| CCA1p:LUC   | n/a      | n/a      | 22.3 ± 0.77        | 3.7 ± 1.0         | 0.25 ± 0.16     | -          |
| CCA1p-V-LUC F1 | CCA1p:AC/UAS:JN T3 (A3850/52) | KC274 | 21.6 ± 0.08        | 5.6 ± 0.33        | 0.29 ± 0.02     | Fig 4a     |
| CCA1p-V-LUC F1 | CCA1p:AC T1 (A3420) | UAS:JN/KC274 T1 (A3727) | 22.2 ± 0.19 | 5.0 ± 0.92 | 0.33 ± 0.13 | -         |
| CCA1p-M-LUC F1 | CCA1p:AC/UAS:JN T3 (A3850/52) | JR11-2 | 21.6 ± 0.18        | 5.2 ± 0.63        | 0.41 ± 0.07     | Fig 4a     |
| CCA1p-M-LUC F1 | CCA1p:AC T1 (A3420) | UAS:JN/JR11-2 T1 (A3740) | 22.0 ± 0.17 | 6.7 ± 0.45* | 0.70 ± 0.15 | -         |
| PRR7p:LUC   | n/a      | n/a      | 20.5 ± 0.28        | 13.8 ± 2.2        | 0.27 ± 0.05     | Fig 4b     |
| PRR7p:LUC   | n/a      | n/a      | 21.5 ± 1.0         | 14.2 ± 2.5        | 0.13 ± 0.05     | -          |
| PRR7p-V-LUC F1 | PRR7p:AC T1 (A3922) | UAS:JN/KC274 T1 (A3727) | 22.6 ± 0.29 | 5.3 ± 0.63* | 0.24 ± 0.04 | Fig 4b     |
| PRR7p-V-LUC F1 | PRR7p:AC T2 (A3922) | UAS:JN/KC274 T1 (A3738) | 22.4 ± 0.29* | 8.5 ± 1.1* | 0.28 ± 0.12 | -         |
| PRR7p-M-LUC F1 | PRR7p:AC T1 (A3922) | UAS:JN/JR11-2 T1 (A3743) | 21.3 ± 0.34* | 12.8 ± 4.5 | 0.42 ± 0.10 | Fig 4b     |
| PRR7p-M-LUC F1 | PRR7p:AC T2 (A3922/4112) | UAS:JN/JR11-2 T1 (A3740) | 23.4 ± 1.1 | 12.9 ± 0.21 | 0.37 ± 0.17 | -         |
| TOC1p:LUC   | n/a      | n/a      | 22.8 ± 0.19        | 15.3 ±0.26        | 0.08 ± 0.01     | Fig 4c     |
| TOC1p:LUC   | n/a      | n/a      | 22.6 ± 0.38        | 17.3 ±0.85        | 0.18 ± 0.06     | -          |
| TOC1/SUC2 TSLA | n/a    | n/a      | 22.0 ± 0.14        | 15.6 ±0.86        | 0.17±0.02       | Fig 4c     |
| TOC1p-V-LUC F2 (A5073) | TOC1p:AC/UAS:JN T3 (A3452) | KC274 | 23.7 ± 0.99*        | 3.2 ± 0.58*       | 0.28 ± 0.11     | Fig 4c     |
| Construct | Control | Treatment | Control | Treatment | Control | Treatment | Control | Treatment | Control | Treatment |
|-----------|---------|-----------|---------|-----------|---------|-----------|---------|-----------|---------|-----------|
| TOC1p-V-LUC F1 | TOC1p:AC/UAS:JN T3 (A3942/49) | KC274 | 22.2 ± 0.73 | 7.8 ± 3.2* | 0.36 ± 0.10 | - |
| TOC1p-M-LUC F2 (A5078) | TOC1p:AC/UAS:JN T3 (A3452) | JR11-2 | 23.2 ± 0.26 | 16.6 ± 0.66* | 0.15 ± 0.03 | Fig 4c |
| TOC1p-M-LUC F1 | TOC1p:AC/UAS:JN T3 (A3942/49) | JR11-2 | 23.4 ± 1.2 | 14.1 ± 3.8 | 0.52 ± 0.24 | - |
| CCR2p:LUC | n/a | n/a | 22.0 ± 1.2 | 14.8 ± 2.5 | 0.28 ± 0.04 | Fig 4d |
| CCR2p:LUC | n/a | n/a | 22.9 ± 0.77 | 14.5 ± 0.99 | 0.22 ± 0.04 | - |
| CCR2p-V-LUC F1 | CCR2p:AC T1 (A3433) | UAS:JN/KC274 T1 (A3727) | 21.0 ±0.12 | 16.0 ± 0.53 | 0.44 ± 0.04 | Fig 4d |
| CCR2p-V-LUC F1 | CCR2p:AC T1 (A3433) | UAS:JN/KC274 T1 (A3739) | 22.0 ± 0.35 | 10.3 ± 0.51* | 0.22 ± 0.01 | - |
| CCR2p-M-LUC F1 | CCR2p:AC T1 (A3433) | UAS:JN/JR11-2 T1 (A3740) | 27.7 ± 0.71* | 4.8 ± 1.7* | 0.77 ± 0.21 | Fig 4d |
| CCR2p-M-LUC F1 | CCR2p:AC T1 (A3433) | UAS:JN/JR11-2 T1 (A3729/37/40) | 20.5 ± 0.12* | 20.6 ± 0.81* | 0.42 ± 0.14 | - |
| DIN6p:LUC | n/a | n/a | 23.0 ± 0.29 | 7.5 ± 0.79 | 0.35 ± 0.12 | Fig 7a |
| DIN6p:LUC | n/a | n/a | 22.0 ± 0.24 | 11.3 ± 1.3 | 0.10 ± 0.04 | - |
| DIN6p-V-LUC F1 | DIN6p:AC T2 (A3972) | UAS:JN/KC274 T1 (A3738) | 22.7 ± 0.16 | 7.5 ± 0.66 | 0.13 ± 0.05 | Fig 7a |
| DIN6p-V-LUC F1 | DIN6p:AC T2 (A3972) | UAS:JN/KC274 T1 (A3727/38) | 22.1 ± 0.13 | 9.7 ± 1.0 | 0.20 ± 0.05 | - |
| SEN5p:LUC | n/a | n/a | 22.2 ± 0.24 | 9.9 ± 0.92 | 0.10 ± 0.02 | Fig 7b |
| SEN5p-V-LUC F1 (A4856-9) | SEN5p:AC/UAS:JN T3 (A3505) | KC274 | 22.6 ± 0.28 | 5.5 ± 2.6* | 0.21 ± 0.05 | Fig 7b |
| SEN5-M-LUC F1 (A4860) | SEN5p:AC/UAS:JN T3 (A3505) | JR11-2 | 22.5 ± 0.06 | 8.1 ± 0.42 | 0.15 ± 0.02 | Fig 7b |
| SEN5p-G-LUC F1 (A4851-55) | SEN5p:AC/UAS:JN T3 (A3505) | E1728 | 22.3 ± 0.35 | 8.6 ± 1.3 | 0.32 ± 0.06 | Fig 7b |

* Period and phase estimates significantly different from control by one-way ANOVA with Tukey’s post-hoc test (P < 0.05).
Table S3. Sequences of primers used in this study.

| Primer Name         | Primer Sequence (5'-3')                                                                 | Purpose                                      |
|---------------------|---------------------------------------------------------------------------------------|----------------------------------------------|
| AC-XbaI_F           | AAATCTAGAGGATCCCCCGGTACCATG                                                           | GWp:AC construct                             |
| AC-SpeI_R           | AAACCATGTTTACACGGCGATCTTTCCG                                                          | GWp:AC construct                             |
| PRR7p_F             | CACCTGTGATATGTCGAGTGGT                                                               | PRR7:AC construct                            |
| PRR7p_R             | CACACCAACTCTGTCTCGCTGAA                                                              | PRR7:AC construct                            |
| CCR2p-HindIII_F     | AAAAAGCTTTAGACGAGCCATCTTCAAT                                                      | CCR2:AC construct                            |
| CCR2p-HindIII_R     | AAAAAGCTTTAGAAATTTGGAAAAAGATCTA                                                     | CCR2:AC construct                            |
| DIN6p_F             | CACCCTTTAATACACGCCAAGAAGAC                                                          | DIN6:AC construct                            |
| DIN6p_R             | GTTTTTTTTTAAAGAAGATGAA                                                             | DIN6:AC construct                            |
| SEN5p_F             | CACCCCGTGTCCTCTCAAGTTA                                                           | SEN5:AC construct                            |
| SEN5p_R             | CACCCCTTTTTGTTGGTTTAATCC                                                           | SEN5:AC construct                            |
| JN-BamHI_F          | AAAGGATCCCCAAAGGGTGATCTGAC                                                       | UAS:JN construct                             |
| JN-SalI_R           | AAAGAGCTTTGATCTCTGTGATCTCAAT                                                        | UAS:JN construct                             |
| LUC+-XbaI_F         | AAATCTAGAGGATGAGGACGCGGCAAAGAACAGAT                                                 | pEarleyGate301-LUC+ construct               |
| LUC+-XbaI_R         | ATATCTAGAGGATGAGGAGGCGGCAAAGAACAGAT                                                | pEarleyGate301-LUC+ construct               |
| AD1                 | (AGCT)GTGCAG(AGCT)GA(AGCT)GA                                                        | TAIL PCR                                     |
| GAL4131REV          | GACACTTGGCGCAGATCCGCGGCTGGTCC                                                      | TAIL PCR                                     |
| GAL499REV           | CACTTGAGTCTTGAGGCGGGCGAGG                                                          | TAIL PCR                                     |
| GAL445REV           | AGCTTCATTGTTGAGGATCCGGTTTCC                                                        | TAIL PCR                                     |
| LB5102FWD           | GGAACACCACGTGAACCTTCTGGG                                                          | TAIL PCR                                     |
| LB5147FWD           | GGGATTCTTGGCATTCGGAACACC                                                          | TAIL PCR                                     |
| LB5199FWD           | GCAACACCGCTGGACGCTTGCTG                                                           | TAIL PCR                                     |
| At5g55590_F         | TCACCCTGTTGTCGACTCTGGA                                                            | sequence T-DNA flanking region               |
| Genbank Accession  | T-DNA Flanking Sequence | Method          |
|-------------------|--------------------------|-----------------|
| At1g75710_F       | GTCACATGATCGTTGACCCG      | sequence T-DNA flanking region |
| At3g51910_R       | TGCAACCGTTACAAAAACCC     | sequence T-DNA flanking region |
| At5g15980_F       | GGACTGAACGAGGAGGAACA     | sequence T-DNA flanking region |
| CCA1_F            | GATGATTTGAGCGGATG        | qRT-PCR         |
| CCA1_R            | TGGTGTTAACTGAGCTGTGAAG   | qRT-PCR         |
| TOC1_F            | TCTTCGCAGAATCCCTGTGAT    | qRT-PCR         |
| TOC1_R            | GCTGCACCTAGCTTCAAGCA     | qRT-PCR         |
| GAL4_F            | GACATCTGCCGCTCAAG        | qRT-PCR         |
| GAL4_R            | GCGACACTCCAGTTGTTTCT     | qRT-PCR         |
| IPP2_F            | GTATGAGTGGTTCACGAAAG     | qRT-PCR         |
| IPP2_R            | GAGGATGGCTGCAACAGTGT     | qRT-PCR         |
| PP2AA3_F          | TAACGTGGCACAATGATGC      | qRT-PCR         |
| PP2AA3_R          | GTTCTCCACAACCGCTTGTG     | qRT-PCR         |
| UAS:nLUC_F        | GAAGAGATACGCCCTGTTCTC    | qRT-PCR         |
| UAS:nLUC_R        | CCGATAAAATAACGGCCCAA     | qRT-PCR         |
| TOC1p:cLUC_F      | TTGTCCAAACACCCCAACAT     | qRT-PCR         |
| TOC1p:cLUC_R      | TCATCGTCTTCCGTGCTCC      | qRT-PCR         |
| GAL4FP            | GACATCTGCCGCTCAAG        | in situ probe synthesis |
| GAL4RP            | GCTCGAGACGGTCAGACTG      | in situ probe synthesis |
| M13 (-40)         | GTTTTCCAGTCACGAC         | in situ probe synthesis |