The metabolic sensor AKIN10 modulates the Arabidopsis circadian clock in a light-dependent manner

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Abstract

Plants generate rhythmic metabolism during the repetitive day/night cycle. The circadian clock produces internal biological rhythms to synchronize numerous metabolic processes such that they occur at the required time of day. Metabolism conversely influences clock function by controlling circadian period and phase, and the expression of core-clock genes. Here we show that AKIN10, a catalytic subunit of the evolutionarily conserved key energy sensor Snf1 (sucrose non-fermenting 1)-related kinase 1 (SnRK1) complex, plays an important role in the circadian clock. Elevated *AKIN10* expression led to delayed peak-expression of the circadian-clock evening-element *GIGANTEA* (*GI*) under diurnal conditions. Moreover, it lengthened clock period specifically under light conditions. Genetic analysis showed that the clock regulator *TIME FOR COFFEE* (*TIC*) is required for this effect of *AKIN10*. Taken together, we propose that AKIN10 conditionally works in a circadian-clock input pathway to the circadian oscillator.

Keywords

circadian clock, metabolism, light signaling, Arabidopsis, AKIN10

Introduction

It is important for plants to recognize and effectively respond to environmental changes. Rhythmic environmental stimuli caused by diurnal cycles are mostly predictable, and the circadian-clock system plays a key role to manage organism's rhythmic responses to these environmental changes. Clock activity is known to be critical for increasing fitness (Dodd *et al.*, 2005, Sanchez *et al.*, 2011). The clock consists of input pathways, a core oscillator, and output responses. Components of various input pathways recognize environmental signals, termed *zeitgebers* (time givers), as they reset the core oscillator. Light and temperature have been revealed as major input *zeitgeber* signals (Bujdoso & Davis, 2013, McClung & Davis, 2010), and metabolites have also been described as such input factors (Dalchau *et al.*, 2011, Haydon *et al.*, 2013, Haydon *et al.*, 2015). *Zeitgebers* drive the core clock to produce an approximately 24-h rhythmic periodicity, and this process is called entrainment [reviewed in (Bujdoso & Davis, 2013)]. Fully entrained plants display strong biological rhythmicity even in the absence of environmental signals.

The circadian core-oscillator has been intensively investigated using a combination of genetic approaches and computational analysis (Bujdoso & Davis, 2013, Shin & Davis, 2010). The current model is established with multiple interlocking transcriptional feedback loops. Briefly, the morning-acting elements LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) repress the transcription of the evening factor *TIMING OF CAB EXPRESSION 1* (*TOC1*) (Alabadi *et al.*, 2001). In turn, TOC1 inhibits the expression of *LHY* and *CCA1* to form the core feedback loop (Gendron *et al.*, 2012, Huang *et al.*, 2012). *PSEUDO-RESPONSE REGULATOR 7* (*PRR7*) and *PRR9* form another transcriptional feedback loop with *CCA1* and *LHY*, and this loop works during the morning phase (Nakamichi *et al.*, 2010). GIGANTEA (GI) and TOC1 are additionally proposed to compose an evening loop (Bujdoso & Davis, 2013). Finally, EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRYTHMO (LUX) were found to form a functional complex (Nusinow *et al.*, 2011) that constitutes another oscillator loop in the evening (Anwer *et al.*, 2014, Herrero & Davis, 2012, Herrero *et al.*, 2012, Kolmos *et al.*, 2011, Kolmos *et al.*, 2009). Genetic and molecular relationships between many clock genes have been discovered, and placing the molecular impact of circadian-input factors to these has remained as a next challenge [reviewed in (Bujdoso & Davis, 2013)].

The circadian clock temporally controls diverse physiological responses (Sanchez *et al.*, 2011). Sugar metabolism has long been considered as one of the clock-output responses; free sugar formation oscillates, as sugars are the products of photosynthesis, which is directly regulated by light and the clock (Blasing *et al.*, 2005, Eimert *et al.*, 1995). Starch formation and its breakdown products are also controlled by the clock (Graf *et al.*, 2010, Müller *et al.*, 2014). Metabolism, however, is not only restricted to clock-driven output responses, but also contributes to the clock activity (Bujdoso & Davis, 2013, Haydon *et al.*, 2013, Sanchez *et al.*, 2011). For example, both soluble sugars and cyclic adenosine diphosphate ribose (cADPR) were reported to regulate clock period and phase, as well as the expression of clock genes (Blasing *et al.*, 2005, Dodd *et al.*, 2007, Dodd *et al.*, 2009, Knight *et al.*, 2008). Sucrose has been specifically suggested as a potential *zeitgeber* in the clock input pathway that directly regulates the expression of the evening clock gene *GI* (Dalchau *et al.*, 2011). Metabolic processes thus seem to be intrinsic elements allowing proper clock function.

AKIN10 (also known as SnRK1.1) is an Arabidopsis metabolic sensor, which comprises evolutionarily conserved Snf1 (sucrose non-fermenting 1)-related kinase 1 (SnRK1) complex (Halford & Hey, 2009). SnRK1, and its yeast and mammalian homologs SNF1 and AMP-activated protein kinase (AMPK) are Ser/Thr protein kinases. In Arabidopsis, heterotrimeric SnRK1 complexes are formed by combinatorial assembly of a catalytic α (AKIN10 or 11), a regulatory β (AKINβ1, 2 or 3), and a γ (SNF4) subunit (Ghillebert *et al.*, 2011). In seedlings, AKIN10 contributes to over 90% of *in vivo* SnRK1 kinase activity among different α-subunits (Jossier *et al.*, 2009) and is broadly expressed in several plant tissues (Williams *et al.* 2014). Activity of AKIN10 is dependent of phosphorylation of its activating T-loop Thr175 residue (Crozet *et al.*, 2010). In response to starvation, SnRK1 is proposed to initiate metabolic reprogramming by altering the activity of several key enzymes in metabolism. For example, SnRK1 phosphorylates nitrate reductase (NR) and trehalose phosphate synthase (TPS), suggesting its role in controlling anabolism (Harthill *et al.*, 2006, Polge *et al.*, 2008, Sugden *et al.*, 1999). Other SnRK1 substrates include the sucrose phosphate synthase, the HMG-CoA reductase and FUSCA3 (FUS3) (Halford *et al*,. 2003, Tsai and Gazzarrini, 2012). In addition, overexpression of *AKIN10* in Arabidopsis protoplasts confers global changes in gene expression in stress-related regulatory pathways (Baena-Gonzalez *et al.*, 2007). Furthermore, a pulse of sucrose, fructose, or glucose treatment reduced the expression of *SnRK1.1*, but not of *SnRK1.2*. In contrast the expression of *SnRK1.2* is spatially restricted

within Arabidopsis, and can be induced by trehalose, but not other sugars (Williams *et al.* 2014). This indicates different roles in plant responses to energy and carbon pools. The induction of AKIN10 activity by sucrose has been reported in several studies (Bhalerao *et al.* 1998, Jossier *et al.*, 2009). Therefore, AKIN10 activity may be dependent not only on the type of sugars, but on the carbon pools, as suggested by Lunn *et al.* (2014).

In yeast SNF1 and mammalian AMPKs are involved in metabolic and stress responses triggered by either glucose starvation or high AMP/ATP ratio, respectively (Carlson, 1999, Ghillebert *et al.*, 2011, Hardie, 2007, Polge & Thomas, 2007, Rutter *et al.*, 2003, Young *et al.*, 2003). In Arabidopsis, SnRK1 also plays a key role in abscisic acid (ABA) hormone signaling (Jossier *et al.*, 2009, Lu *et al.*, 2007, Radchuk *et al.*, 2006), as well as regulates plant growth and development (Baena-Gonzalez *et al.*, 2007, Radchuk *et al.*, 2006, Tsai & Gazzarrini, 2012, Zhang *et al.*, 2001). SnRK1 thus has broad roles to ensure metabolic homeostasis, and this is critical for diverse biological processes.

In mammals, the SnRK1 orthologue AMPK has been shown to modulate clock proteins resulting in period lengthening (Lamia *et al.*, 2009, Um *et al.*, 2011). In the lower plant *Physcomitrella patens*, two SnRK1-encoding genes (*PpSNF1a* and *PpSNF1b*) are required for survival under autotrophic diurnal conditions (Thelander *et al.*, 2004). These studies together imply a conserved role of SnRK1/AMPKs in clock function in diverse organisms. Consistent with that, we show in this study that inducible overexpression of the SnRK1 α-subunit AKIN10 modulates the circadian clock by lengthening rhythmic period under light conditions. Under diurnal conditions, AKIN10 increases led to delaying the peak phase of the evening clock gene *GI*. Through genetic tests, we additionally show that *AKIN10* and the established clock regulator *TIME FOR COFFEE* (*TIC*) (Hall *et al.* 2003, Ding *et al.* 2007, Sánchez-Villarreal *et al.* 2013) genetically interact to modulate clock function. These results collectively propose that internal energy metabolism intercommunicates with the biological clock through AKIN10.

Material and Methods

Plant material and growth conditions

Arabidopsis thaliana Columbia (Col) accession is the genetic background of the wild type and transgenic lines used in this study. Plants were grown on MS media [half strength MS (Sigma), 0.9% phytoagar and 0.05% MES (Duchefa), pH 5.7] at 22°C under various light conditions. For luciferase-reporter assays, 3% sucrose was added to the media, whereas no additional sucrose, 1% sucrose containing, or 3% glucose MS media was used for other experiments. The bioluminescence assays were performed as previously described (Hanano *et al.*, 2006, Kolmos *et al.*, 2009) with indicated light provided by custom LED panels (~2 µmol m^{-2} s^{-1}). For RNA-based work, seedlings were grown at 22°C with 75 µmol m^{-2} s^{-1} cool white fluorescent light, as described (Shin *et al*. 013).

To generate *pER8::myc-AKIN10* plants, full-length *AKIN10* cDNA was amplified with gene-specific primers (see Supplemental Table 1), and the PCR product was inserted into pDONR201 with a Gateway BP kit (Invitrogen). An *AKIN10* construct was used in Gateway LR reactions in combination with the destination vector *pER8* (Zuo *et al.*, 2000). The construct was transformed into Col by *Agrobacterium tumefaciens*-mediated transformation (Davis *et al.*, 2009), and a homozygous line was selected. The *tic-2 pER8::myc-AKIN10* plants were generated by crossing the corresponding parental homozygous lines and genotyping F2 segregating progenies to select *tic-2* homozygous mutations, as previously described (Shin *et al.*, 2012). The *GI::LUC* construction is described (Anwer *et al.*, 2014).

Chemical treatment

For *AKIN10* overexpression analysis, *pER8::myc-AKIN10* or *tic-2 pER8::myc-AKIN10* seedlings grown on normal MS-agar media were transferred to 5 µM β-estradiol containing media for various days as indicated in the results. For preparation of β-estradiol stock solution, β-estradiol powder (Sigma) was dissolved into ethanol to a 10 mM concentration, and kept at -20 °C, until use.

Gene expression analysis

Total RNA was extracted from seedlings using Spectrum™ Plant Total RNA Kit (Sigma), according to the manufacturer's instructions. cDNA was synthesized from 4 µg of total RNA with Maxima™ First Strand cDNA Synthesis Kit (Fermentas). To amplify genes, 5 µL of 1/25 diluted cDNA was used as the template. Quantitative RT-PCR analysis was performed using SYBR and LightCycler™ 480 (Roche). Primer sequences for qRT-PCR are listed in Supplemental Table 1. The resulting gene expression levels were normalized with the level of *PP2A* (Czechowski *et al.*, 2005). Data analysis was performed using three technical replicates from each biological sample, and similar results were obtained in two biological replicates.

Protein extraction and western blotting

Protein extraction and immunoblot analyses were as described (Shin *et al.*, 2013). For detection of AKIN10-myc, the membrane was incubated with anti-myc antibody (Cell Signaling) or anti-phospho-AMPKα (Thr172) antibody (Cell Signaling) in PBS buffer containing 0.05% Tween-20. For detection of histone H3, the membrane was incubated in the same buffer with anti-histone H3 antibody (Agrisera). Antibodies were diluted according to manufacturer's instructions. Bands were visualized with an enhanced chemiluminescence (ELC) kit (GE Healthcare).

Results

Generation of chemically inducible *AKIN10* overexpressing plants

To start investigating the impact of energy metabolism for clock-oscillator function, we examined the role of AKIN10. *akin10* null mutants are not available, as eliminating SnRK1 catalytic subunit leads to severe developmental defects, and ultimately to seedling lethality (Baena-Gonzalez *et al.*, 2007, Radchuk *et al.*, 2006, Tsai & Gazzarrini, 2012, Zhang *et al.*, 2001). Therefore, we generated transgenic plants that overexpress *AKIN10* by a chemical-inducible system, and used these for genetic and molecular analysis. For this, *AKIN10* was placed under control of the β-estradiol inducible promoter, hereafter called *pER8::myc-AKIN10*. This chemical-inducible system allowed us to study the role of AKIN10 in plants after early seedling growth stage had been progressed. Without external β-estradiol treatment, *AKIN10* transcript levels in *pER8::myc-AKIN10* plants were comparable to the wild type (Col), and myc-AKIN10 protein was not expressed (Figure 1A, 1B). The transcript level of *AKIN10* was increased in plants being treated with β-estradiol for 2-3 days by 82–92 fold compared to non-treated control plants. However, with increasing duration of β-estradiol treatment, the expression level of *AKIN10* gradually decreased. Nevertheless, the *AKIN10* mRNA level was induced ~20 fold during a β-estradiol treatment for 6 days (Figure 1A). Based on these observations, we chose a 2-6 days time window for the β-estradiol treatment to analyze the effects of elevated *AKIN10* expression on clock function.

AKIN10 is thought to be active only if its activation T-loop threonine residue (T175) is phosphorylated (Crozet *et al.*, 2010) although the relationship between the residue phosphorylation and kinase activity has not been clearly established in plants (Crozet *et al.*, 2014). Using anti-phospho-AMPKα (T172) antibody, which specifically detects the phosphorylated Thr175 residue of AKIN10 (AKIN10 pT175) (Coello *et al.*, 2012, Shen *et al.*, 2009), we monitored the amount of the myc-AKIN10 pT175. In the wild type and non-induced *pER8::myc-AKIN10* plants, only the endogenously expressed AKIN10 pT175 was detected (Figure 1B, lower bands). In β-estradiol treated *pER8::myc-AKIN10* plants, a myc-AKIN10 pT175 form was readily detected (Figure 1B, additional upper band). To further confirm that expressed myc-AKIN10 is biologically functional, the transcript level of AKIN10-regulated genes were determined in *pER8::myc-AKIN10* plants. It is known that *DARK INDUCIBLE 6* (*DIN6*) and *SENESCENCE-ASSOCIATED PROTEIN 5* (*SEN5*) are

induced by AKIN10 (Baena-Gonzalez *et al.*, 2007). Consistent with previous reports, *DIN6* and *SEN5* transcript accumulation was highly elevated in β-estradiol-treated plants, compared to non-treated *pER8::myc-AKIN10* control plants (Figure 1C, 1D). These results collectively showed that myc-AKIN10 was expressed in a biologically active form in our estradiol-inducible system.

Overexpression of myc-AKIN10 lengthens clock period under light conditions

To test if AKIN10 contributes to circadian-clock function, we examined the rhythmic period of plants overexpressing *myc-AKIN10*. To monitor promoter activity of the clock evening gene *GI*, we introduced a construct harboring the *GI* promoter fused to luciferase (*GI::LUC*) into *pER8::myc-AKIN10* plants, and performed luciferase-reporter assays. Plants were entrained under 12-h light / 12-h dark (12L/12D) conditions for 8 days, then transferred into constant red and blue (R+B) light conditions. To induce *myc-AKIN10* expression, β-estradiol was added to plants approximately 36 h before transfer to free-running conditions. Circadian period was analyzed from a 12 h to 96 h time window under the constant-light conditions. This is 48 h – 132 h (from days 2 to 5.5) after supplying β-estradiol to plants. In wild-type plants, both 5μM β-estradiol and 0.05% EtOH (solvent control) did not alter the free-running period (28.9 ± 0.47 h ± ~~(~~SEM) (Figure 2A, 2B)~~ (Figure 2A)~~, which was a period length similar to that reported by Haydon *et al.* (2013) and Shin *et al.* (2013) under such low light conditions. *pER8::myc-AKIN10* plants displayed a similar free-running period as wild type under either control (non-treated) or EtOH-treated conditions. In contrast, the clock period of *pER8::myc-AKIN10* plants became significantly longer compared to the wild type when β-estradiol was applied; the transgenic plants displayed a 33.8 ± 0.48 h (± SEM) period, compared to the 28.9 ± 0.47 h (± SEM) in the wild type (Figure 2A, 2B). This > 4-h period delay was statistically significant (P-value: 3.64E-10, ANOVA). We confirmed the elevated *AKIN10* expression within the 6 days of β-estradiol treatment (Figure 1), and this corresponds to the time window that we analyzed the clock period in these plants. The relative amplitude error (RAE) is a measure of the sustainability and precision of rhythms, and it is considered as a robust rhythm when plants display RAE values below 0.6 (Hanano *et al.*, 2008, Knight *et al.*, 2008). We found induction of *pER8::myc-AKIN10* with β-estradiol resulted in rhythms that were as robust (RAE of the induced plants is at least as low) as in the controls ~~which did not change clock rhythms~~ (Figure 2C). These results collectively indicate that elevated *myc-*

AKIN10 expression lengthened the circadian period under constant R+B light conditions.

We further investigated the effects of AKIN10 on clock function under different light conditions. For this, we determined circadian period under constant blue light (Bc), constant red light (Rc), and in constant dark conditions. Consistent with constant R+B results in Figure 2, *pER8::myc-AKIN10* plants displayed a significantly longer period than wild type in response to external β-estradiol treatment under Bc and Rc conditions [P-value: 3.93E-8 (Bc), 1.8E-5 (Rc), ANOVA] (Figure 3A, 3B). In contrast, no period-lengthening effects were observed by elevated *myc-AKIN10* in darkness. If anything, *pER8::myc-AKIN10* plants displayed a slightly shorter period compared to the wild type when β-estradiol was applied, but this was not statistically significant (P-value: 0.11, ANOVA) (Figure 3C). This could have been because *myc-AKIN10* induction by β-estradiol treatment was restricted by darkness. To explore this possibility, we examined myc-AKIN10 protein accumulation in response to β-estradiol under Bc, Rc, and in dark conditions. myc-AKIN10 protein similarly accumulated in darkness as under Bc and Rc conditions (Figure 4). The level of phosphorylated myc-AKIN10 was also comparable regardless of light conditions (Figure 4), which implies induced myc-AKIN10 has similar kinase activity under the differing conditions of these experiments. Thus AKIN10 activity and its effects in gene expression, as Baena-González *et al.* (2007) showed for *DIN6* expression under darkness, could be equally independently of the light conditions. Therefore, the lack of period lengthening phenotype of *pER8::myc-AKIN10* plants in darkness does not appear to be caused by the failure of the β-estradiol-induced AKIN10 expression and/or light-specific post-translational modification of AKIN10.

Plants have been typically grown on 3% sucrose for luciferase reporter assays (Millar *et al.*, 1992). In previous studies, prolonged darkness, carbohydrate starvation, and induced senescence, have been shown to promote SnRK1 activity (Baena-Gonzalez *et al.*, 2007, Bhalerao *et al.*, 1999). However Jossier *et al.* (2009) described and increase in AKIN10 activity due to glucose addition. We thus examined the effects of the presence and/or type of sugars on the AKIN10-mediated regulation of the circadian period in darkness. The rhythmic period was determined from plants grown without exogenous sugar-, on 3% sucrose-, or 3% glucose-containing media. Consistent with a previous report (Knight *et al.*, 2008), we confirmed that sugar application shortens the circadian period (Figure 5). There were no differences between sucrose and glucose on the regulation of period length, as previously

described (Haydon *et al.* 2013). Moreover, elevation of *myc-AKIN10* expression after β-estradiol induction resulted in no effects on the rhythmic period in darkness regardless of the presence of sugars added in media (Figure 5). Even though the high sugar concentration could lead to an osmotic stress, this possibility was controlled for in past work, as Haydon *et al.* (2013) did not observe an effect on period with mannitol application. These results collectively suggest that the role of AKIN10 on the regulation of the clock function is specific to a light response.

AKIN10 regulates the peak expression phase of *GI* under diurnal conditions

We next determined the transcript accumulation of several clock components in *myc-AKIN10* overexpressing plants under diurnal conditions. *pER8::myc-AKIN10* plants were grown under 12L/12D conditions for 7 days, and transferred to β-estradiol-containing media for an additional 2 days. *AKIN10* mRNA was not rhythmically expressed in control plants, nor in plants treated with β-estradiol (Supplement Figure 1A, 1B). *AKIN10* was 42–153 fold elevated by β-estradiol treatment for all time points measured (Supplement Figure 1B). *LHY* (Figure 6A), *CCA1* (Figure 6B), *PRR7* (Figure 6C), *TOC1* (Figure 6E), *ELF4* (Figure 6F), *PRR9, PRR5, ELF3,* and *LUX* (Supplement Figure 2) were similarly expressed in β-estradiol-treated and non-treated plants. Therefore, under diurnal conditions, overexpressed *myc-AKIN10* did not affect the gene-expression profiles of most clock genes. Exceptionally, we found that *GI* expression peaked at ZT12 (ZT: *Zeitgeber* time, ZT12 indicates 12 h after lights on) in β-estradiol-treated plants, whereas it peaked at ZT8 in non-treated plants (Figure 6D). Under diurnal conditions, myc-AKIN10 induction appeared to specifically delay the peak expression phase of *GI*.

To examine the effect of elevated *AKIN10* under free-running conditions, we determined the rhythmic expression of clock genes under constant white light (LL) conditions. For this, plants were entrained under 12L/12D conditions for 8 days, and then released to LL. Plants were transferred to β-estradiol-containing media around 36 h before moving into LL. *AKIN10* mRNA accumulation was not oscillating in both control plants and β-estradiol induced plants under LL (Supplement Figure 1C, 1D). Therefore, *AKIN10* transcription is not under the control of the circadian clock. Consistent with the result in Figure 1A, we observed that *AKIN10* induction in response to β-estradiol gradually decreased as the days progressed (Supplement Figure 1D). Nonetheless, *myc-AKIN10* maintained at least ~38 fold induced at

the last time point that we analyzed (72h under LL). Morning clock gene *LHY* and the evening gene *GI* maintained their rhythmic expression patterns under LL in both *myc-AKIN10* induced and non-induced plants, with similar levels of transcript accumulation at their peaks and troughs (Figure 7). This indicates that *myc-AKIN10* overexpressing plants maintain a precise and robust biological rhythm. Notably, *myc-AKIN10*-induced plants displayed a longer rhythmic period than control plants, which is consistent with luciferase reporter-assay results under light conditions in Figure 2 and Figure 3. The peak-to-peak distance of *LHY* (Figure 7A) and *GI* (Figure 7B) were extended by about 4 h by overexpressing *myc-AKIN10*. Together with the luciferase-assay data, these results consistently indicate that the elevated *myc-AKIN10* expression lengthened the period of rhythmic clock gene expressions under free-running conditions.

AKIN10 genetically interacts with _TIC_ in periodicity determination

Altered clock activity in *myc-AKIN10* overexpressing plants is the opposite phenotype of plants having a mutation in the clock regulator gene *TIC*. *tic* is known to express *GI* around 4-h earlier than the wild type, has extensive developmental and metabolic phenotypes (Sánchez-Villarreal *et al.*, 2013), and displayed a shorter rhythmic period (Ding *et al.*, 2007, Hall *et al.*, 2003). These observations led us to test if there is a genetic relationship between *AKIN10* and *TIC* in period determination. We first examined *AKIN10* transcript accumulation in the *tic* mutant. *AKIN10* mRNA similarly accumulated in *tic* as in the wild type, both under diurnal and free-running conditions (Supplement Figure 3A). Therefore, *TIC* did not affect *AKIN10* expression at the transcript level. We next generated *tic-2 pER8::myc-AKIN10* plants by crossing *pER8::myc-AKIN10* into *tic-2*, then determined clock gene expression both in *AKIN10* induced and non-induced plants. We confirmed that *tic-2 pER8::myc-AKIN10* plants express *AKIN10* at similar patterns as *pER8::myc-AKIN10* in response to β-estradiol both under diurnal and free-running conditions (Supplement Figure 3B). These results indicate that the capacity of the *pER8* promoter to generate overexpressed *myc-AKIN10* is comparable in *tic-2* and the wild type. Consistent with previous reports in *tic* (Ding *et al.*, 2007), *GI* transcript accumulation reached to its maximum at ZT4 in *tic-2 pER8::myc-AKIN10* under β-estradiol non-treated conditions (Figure 8A). Notably, we found that elevation of *AKIN10* expression in the *tic* mutant no longer delayed the peak phase of *GI*. Rather, it displayed a phase advance relative to the wild type, similar to *tic* plants that had not been induced for

329 *myc-AKIN10* (Figure 8A). These results suggest that *TIC* is necessary for the action of
330 AKIN10 on clock periodicity.

331 Such a genetic interaction between *AKIN10* and *TIC* was further observed under free-running
332 conditions. As already reported (Ding *et al.*, 2007), we confirmed that *tic-2* mutants display
333 under LL a short period for both the morning and evening clock genes, *LHY* and *GI*,
334 respectively (Figure 8B, 8C). *myc-AKIN10* overexpression no longer lengthened circadian
335 period in the *tic-2* background (Figure 8B, 8C). In addition, we evaluated clock periodicity
336 with a luciferase reporter in *tic-2 pER8::myc-AKIN10 CCA1::LUC* plants under free
337 running conditions after induction with β-estradiol. Different from the longer period in
338 *pER8::myc-AKIN10* after the induction of *AKIN10*, the period length in *tic-2 pER8::myc-*
339 *AKIN10* seedlings was not increased even when *AKIN10* was over expressed after induc
340 tion (Supplemental figure 4A-C). These data collectively indicate that *tic* is genetically
341 epistatic to *AKIN10* overexpression for regulating the circadian periodicity.

342

Discussion

The circadian clock temporally regulates biological processes to occur at the proper time of day under repetitively changing environmental conditions. This ensures plants to achieve efficient growth and development (Delker *et al.*, 2014, Raschke *et al.*, 2015), which leads into increasing fitness (Dodd et al. 2005). Metabolic responses, such as photosynthesis and respiration are rhythmically regulated with oscillation every 24 h (Müller *et al.*, 2014). These pathways were classically considered as the circadian-output responses. However, a number of recent studies have started to suggest the existence of metabolism-mediated clock regulation pathways in plants (Dalchau *et al.*, 2011, Dodd *et al.*, 2007, Knight *et al.*, 2008, Sánchez-Villarreal *et al.*, 2013). Here we studied the central energy sensor SnRK1 to reveal its impact on the circadian clock. For molecular and genetic analysis, we generated transgenic plants overexpressing *myc-AKIN10* under control of the β-estradiol-inducible promoter. This approach provides the advantage to investigate the effects of *AKIN10* by elevating its expression only for several days after early development was established, and thus we could assess the kinase expression during any given particular time lapse of about 5 days (Figure 1).

AKIN10 encodes a catalytic α subunit of SnRK1, and it is reported to contribute to over 90% of SnRK1 activity *in vivo* (Jossier *et al.*, 2009). We showed here that AKIN10 is involved in the modulation of circadian-clock performance. *AKIN10* overexpression delayed the peak expression phase of the clock evening element *GI* under diurnal conditions (Figure 6D). The importance of *GI* in sugar signaling has been previously reported. For example, GI was shown to be involved in the starch-accumulation process. Therefore, *gi* mutants displayed enhanced starch accumulation in comparison with the wild type (Eimert *et al.*, 1995, Müller *et al.*, 2014). Additionally, GI was suggested to be a target molecule of sugar signaling within the clock (Dalchau *et al.*, 2011), particularly in a long term response to sucrose under darkness. Dalchau *et al.* (2011) observed a slight decrease in *GI:LUC* rhythms with sucrose under constant light. Comparatively, *AKIN10* overexpression increased period length of *GI* under diurnal or constant light conditions, suggesting different mechanisms for sensing and responding to sucrose. It will be informative to determine whether AKIN10 regulates *GI* directly or whether this is an emergent consequence of AKIN10 circadian inputs to other components of the circadian system. Our results further support the importance of GI on the signaling connection between the clock and the sugar responses, and moreover, suggest that

GI could be a target gene of a regulatory mechanism controlled either directly or indirectly by AKIN10.

AKIN10 was shown to specifically lengthen circadian period only under light conditions (Figure 2, Figure 3, and Figure 7). Although *myc-AKIN10* overexpressing plants displayed a long period under light conditions, the peak and trough transcript levels of clock genes were similar to those of control plants, and the rhythm was precisely maintained (Figure 2, Figure 6, Figure 7 and Supplemental Figure 2) albeit with a slight increase in amplitude in evening expressed genes *LUX*, *TOC1*, *ELF4*, and *ELF3*. Based on our results, AKIN10 seems to act in the circadian-input pathway rather than functioning in the core oscillator. In darkness, elevated myc-AKIN10 did not lengthen the clock period regardless of the presence and type of sugars supplied to the media (Figure 3E–3F, Figure 5). Thus *AKIN10* effect on clock period seems is not solely dependent on sucrose, but rather the kinase effect on the clock additionally requires light. Under our assay conditions, myc-AKIN10 protein levels and its phosphorylation status were not significantly changed in darkness, compared to light conditions (Figure 4). It is possible that other SnRK1 complex subunits are also involved in the regulation of the clock function, and their expression, availability, and/or activity is modulated depending on the light conditions. Indeed, it has been shown that the expression of three SnRK1 β subunits is differentially regulated according to environmental conditions, organs, and developmental stages (Polge *et al.*, 2008). Furthermore tissue expression specificity by *AKIN10* and *AKIN11* (SnRK1.1 and SnRK1.2, respectively) as well as responses to carbohydrates and developmental effects has been shown (Williams, 2014). The detailed molecular and biochemical relationships should be further investigated to reveal the underlying mechanism of the light-dependent effects of AKIN10 on the regulation of the clock.

In our luciferase-reporter assays, the control plants displayed around 27 h free-running period (Figure 2, Figure 3). This could be due to low intensity of light [~2 μE/m^2/s (red) and ~2 μE/m^2/s (blue)] used under free-running conditions, whereas these plants were entrained under higher intensity of white light (~75 μE/m^2/s). Indeed, it is well established that the circadian period becomes longer as light intensity decreases [reviewed in (Bujdoso & Davis, 2013)]. Thus period estimates from Figure 2 and Figure 3 obtained under low intensity blue and red light cannot be directly compared to periods derived from quantitative RT-PCR, as in

the later, the free-running conditions were under white light. Consistently, we noticed that clock genes were oscillating with 24 h free-running period in control plants when they were provided same quantity and quality of white light as they were under entrainment conditions (Figure 7).

We found a genetic interaction between *AKIN10* and *TIC*. Similar to AKIN10, TIC was shown to be required to lengthen the clock period and delay the peak expression phase of *GI* under diurnal conditions. Moreover, overexpression of *AKIN10* in the *tic* background did not restore the *tic* mutant phenotype. *tic-2 pER8::myc-AKIN10* plants periodicity were rather comparable to the *tic-2* mutant (Figure 8 and Supplemental Figure 4). These data consistently indicate that *tic* is genetically epistatic to *AKIN10* overexpression. Previously, we have shown that TIC is involved in stress responses (Shin *et al.*, 2013, Shin *et al.*, 2012, Sánchez-Villarreal *et al.*, 2013), and it has been also observed that TIC contributes to starch metabolism as its mutation results in a starch-excess phenotype (Sánchez-Villarreal *et al.*, 2013). It is interesting to note that *TIC* and *GI* share circadian and metabolic intersections, as they are both involved in starch metabolism and oxidative stress (Fornara *et al.*, 2015, Sánchez-Villarreal *et al.*, 2013). These studies together reinforce the genetic relationship between *AKIN10* and *TIC* with connections to *GI*. It will be interesting to test if TIC alters AKIN10 kinase activity in the regulation of the circadian clock. Another equally plausible scenario is a regulatory mechanism where TIC promotes the function of AKIN10, thereby AKIN10 physiological activity on the clock is attenuated in the *tic* mutant. These need not be mutually exclusive possibilities.

In animal systems, defects in AMPK complexes are known to trigger various disorders, such as metabolic syndrome, insulin resistance, obesity, cardiovascular diseases, and cancer (Hardie, 2015). The plant circadian-clock system is also critical to increase fitness, and promote growth and development in a metabolic-dependent manner (Dodd *et al.* 2005, Fukushima *et al.*, 2009, Lai *et al.*, 2012). Our study highlights a possible role of SnRK1 on circadian-clock function, and therefore, could affect plants performance. Furthermore the recent discovery of magnesium fluxes, both in the unicellular alga *Ostreococcus* and human cell lines, affect the cells energy balance through ATP (Feeney *et al.* 2016). This again highlights the role of energy balance in coordinating clock function. The genetic interactions between *AKIN10*, *TIC*, and *GI* could be that of a sensor of energy balance. In future studies, it

436 will be worth to define if AKIN10 is an evolutionarily conserved *zeitgeber* within eukaryotic
437 clocks, which serves conserved energy signaling using a same type of kinases of diverse
438 organisms.

439

Accession Numbers

Sequence data from this article can be found in TAIR databases under the following accession numbers: *AKIN10* (AT3G01090), *SEN5* (AT3G15450), *DIN6* (AT3G47340), *LHY* (AT1G01060), *CCA1* (AT2G46830), *PRR7* (AT5G02810), *GI* (AT1G22770), *TOC1* (AT5G61380), *ELF4* (AT2G40080), *PRR5* (AT5G24470), *ELF3* (AT2G25930), *LUX* (AT3G46640), *PP2A* (AT1G13320).

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454 **Figure legends**

455

456 **Figure 1. *pER8::myc-AKIN10* plants induce the expression of *AKIN10* in response to**
457 **exogenous β-estradiol.**

458 (A) Quantitative RT-PCR of *AKIN10* relative to *PP2A.* Col and *pER8::myc-AKIN10* plants
459 were grown with or without β-estradiol for 10 days in total, 5μM β-estradiol was applied for
460 the number of days as indicated. Maximum *AKIN10* induction was achieved after 3 days. The
461 measurements of gene expression indicate a mean of three technical replicates, and error bars
462 indicate standard deviation. (B) Immunoblot analysis of myc-AKIN10, phospho-myc-
463 AKIN10, and histone H3 protein in Col and *pER8::myc-AKIN10* plants. Open triangle
464 indicates endogenous phospho-AKIN10, and closed triangle indicates phospho-myc-AKIN10.
465 (C-D) Quantitative RT-PCR of *DIN6* (C) and *SEN5* (D) relative to *PP2A*. Seven day old
466 *pER8::myc-AKIN10* seedlings were treated or not with 5μM β-estradiol for 2 days. The
467 measurements of gene expression indicate a mean of three technical replicates, and error bars
468 indicate standard deviation.

469

470 **Figure 2. *AKIN10* induction lengthens circadian period under constant red+blue light**
471 **conditions.**

472 Col and *pER8::myc-AKIN10* plants harboring *GI::LUC* construct were entrained under
473 12L/12D conditions for 8 days, and transferred into constant light conditions. β-estradiol was
474 added to plants 36 h before releasing into free-running conditions. (A) Effect on period length
475 by AKNI10 gene expression induction. Error bars indicate standard error. (B) Normalized
476 bioluminescence of *GI::LUC* under constant R+B conditions after β-estradiol induction. (C)
477 Period versus relative amplitude error (RAE) of individual wild type and *pER8::myc-AKIN10*
478 plants treated with β-estradiol.

479

480

Figure 3. The effects of AKIN10 on lengthening the clock period is diminished under constant darkness. Circadian rhythmicity of *GI::LUC* in Col and *pER8::myc-AKIN10* plants under constant blue-light conditions (A-B), constant red-light conditions (C-D), and constant darkness (E-F). Col and *pER8::myc-AKIN10* plants harboring *GI::LUC* construct were entrained under 12L/12D conditions for 8 days, and transferred into constant light or dark conditions. β-estradiol was added to plants 36 h before releasing into free-running conditions. (A,C,E) Period versus treatment conditions and genotypes. Error bars indicate standard error. (B,D,F) Period versus relative amplitude error (RAE) of individual plants after exposure to β-estradiol.

Figure 4. AKIN10 protein accumulation is independent of light conditions. Immunoblot analysis of myc-AKIN10, phospho-myc-AKIN10 and histone H3 protein in *pER8::myc-AKIN10* plants. Plants were grown under 12L/12D conditions for 8 days, and transferred into constant blue, red, or dark conditions for 2 days. β-estradiol was added to plants 36 h before transferring into constant light or dark conditions.

Figure 5. Circadian periodicity of *pER8::myc-AKIN10* plants in darkness is similar to the wild type regardless of the exogenously supplied sugar types.

Circadian rhythmicity of *GI::LUC* in Col and *pER8::myc-AKIN10* plants in constant darkness. Col and *pER8::myc-AKIN10* plants harboring *GI::LUC* construct were entrained under 12L/12D conditions for 8 days, and transferred into constant darkness. β-estradiol was added to plants 36 h before releasing into free-running conditions. Error bars indicate standard error.

Figure 6. *AKIN10* delays the phase of the peak expression of *GI* under diurnal conditions.

Quantitative RT-PCR of *LHY* (A), *CCA1* (B), *PRR7* (C), *GI* (D), *TOC1* (E), and *ELF4* (F) relative to *PP2A* under diurnal conditions. *pER8::myc-AKIN10* plants were grown under 12L/12D for 9 days in total, and treated or not with 5μM β-estradiol for the last 2 days as

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shown in the diagram. The measurements of gene expression indicate a mean of three technical replicates, and error bars indicate standard deviation. White and black bars indicate light and dark conditions, respectively.

Figure 7. AKIN10 lengthens the rhythmic period of the transcript accumulation of core-oscillator genes under constant light.

Quantitative RT-PCR of *LHY* (A) and *GI*(B) relative to *PP2A* under free-running conditions. *pER8::myc-AKIN10* plants were grown under 12L/12D for 8 days, and transferred into constant white light (LL) conditions for 3 days. Plants were placed into 5μM β-estradiol-containing media 36 h before transfer into LL conditions. The measurements of gene expression indicate a mean of three technical replicates, and error bars indicate standard deviation. White, black, and grey bars denote day, night and subjective night conditions, respectively.

Figure 8. *tic* is genetically epistatic to *AKIN10* overexpression for regulating the circadian periodicity.

(A) Quantitative RT-PCR of *GI* relative to *PP2A* under diurnal conditions. *pER8::myc-AKIN10* and *tic-2 pER8::myc-AKIN10* plants were grown under 12L/12D for 9 days in total, and treated or not with 5μM β-estradiol for the last 2 days. (B-C) Quantitative RT-PCR of *LHY* (B) and *GI* (C) relative to *PP2A*. *pER8::myc-AKIN10* and *tic-2 pER8::myc-AKIN10* plants were grown under 12L/12D for 8 days, and transferred into LL conditions for 3 days. Plants were plaed into 5μM β-estradiol-containing media 36 h before transferring into LL conditions. The measurements of gene expression indicate a mean of three technical replicates, and error bars indicate standard deviation.

Supplement Figure 1. *AKIN10* is not rhythmically expressed under diurnal and free-running conditions. (A-B) Quantitative RT-PCR of *AKIN10* relative to *PP2A* under diurnal conditions. *pER8::myc-AKIN10* plants were grown under 12L/12D for 9 days in total, and

treated or not with β-estradiol for the last 2 days. (C-D) *pER8::myc-AKIN10* plants were grown under 12L/12D for 8 days, and transferred into constant white light (LL) conditions for 3 days. Plants were placved into 5μM β-estradiol-containing or control media 36 h before moving into LL conditions. The measurements of gene expression indicate a mean of three technical replicates, and error bars indicate standard deviation.

Supplement Figure 2. Quantitative RT-PCR of *PRR9* (A), *PRR5* (B), *ELF3* (C), and *LUX* (D) relative to *PP2A* under diurnal conditions. *pER8::myc-AKIN10* plants were grown under 12L/12D for 9 days in total, and were treated or not with 5μM β-estradiol for last 2 days. The measurements of gene expression indicate a mean of three technical replicates, and error bars indicate standard deviation.

Supplement Figure 3. TIC does not substantially alter AKIN10 transcript accumulation. (A) Quantitative RT-PCR of *AKIN10* relative to *PP2A* in Col and *tic-2*. Plants were grown under either diurnal conditions or constant light (LL) free-running conditions. (B) Quantitative RT-PCR of *AKIN10* relative to *PP2A* in *tic-2 pER8::myc-AKIN10* plants either under diurnal conditions or free-running conditions. Plants were treated or not with β-estradiol for 36 h before harvesting. The measurements of gene expression indicate a mean of three technical replicates, and error bars indicate standard deviation.

Supplement Figure 4. A functional *TIC* gene is necessary for *AKIN10* overexpression to have an effect on the circadian clock. (A and B) Normalized luminescence of *CCA::LUC* traces under free running conditions for Col-0, *pER8::myc-AKIN10*, *tic-2* and *tic-2/pER8::myc-AKIN10* without or with 5μM β-estradiol induction. Plants were grown under 12L/12D for 7 days and then transferred to media with or without ~~not~~ 5μM β-estradiol. 24 hours after plants were placed under constant B/R light. (C) Period length for Col-0, *pER8::myc-AKIN10*, *tic-2* and *tic-2/pER8::myc-AKIN10* with or without application of 5μM β-estradiol for the induction of the *AKIN10* expression.

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566 **Supplement Table 1. Primers**

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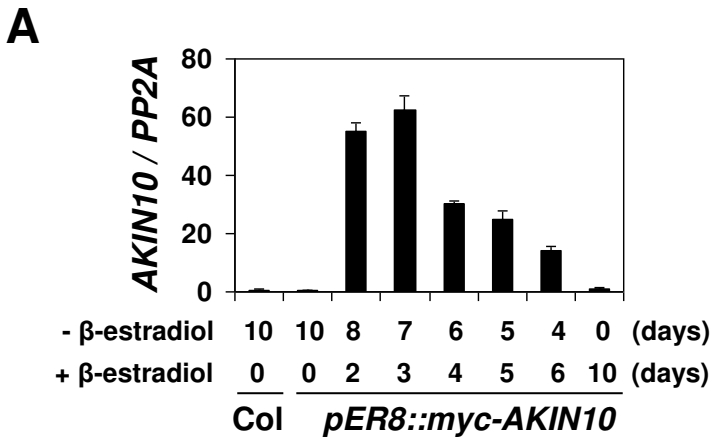
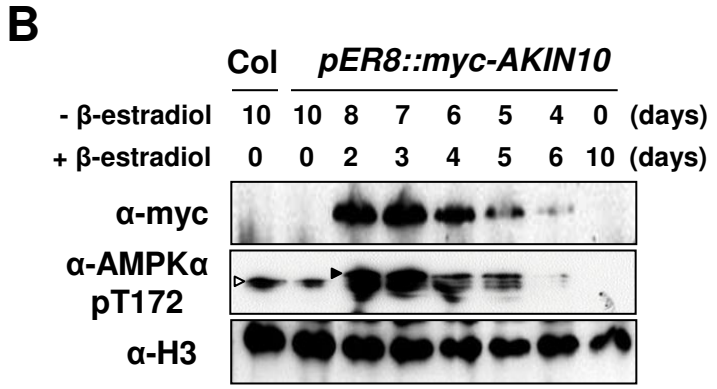
Figure 1


A
AKIN10 / PP2A
80
60
40
20
0
- β-estradiol 10 10 8 7 6 5 4 0 (days)
+ β-estradiol 0 0 2 3 4 5 6 10 (days)
Col
pER8::myc-AKIN10


B
Col
pER8::myc-AKIN10
- β-estradiol 10 10 8 7 6 5 4 0 (days)
+ β-estradiol 0 0 2 3 4 5 6 10 (days)
α-myc
α-AMPKα pT172
α-H3

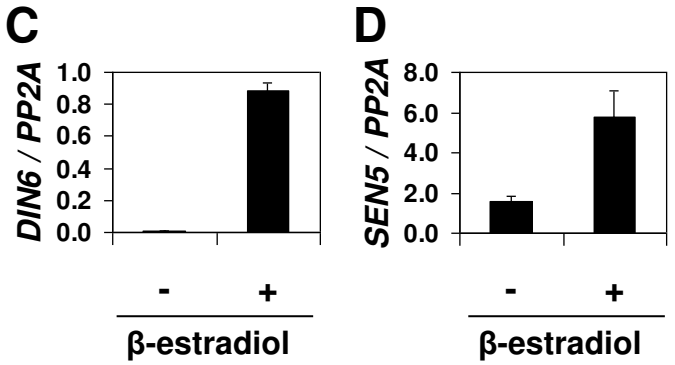
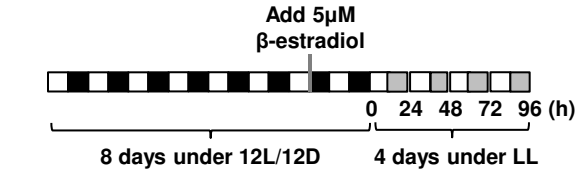

C
DIN6 / PP2A
1.0
0.8
0.6
0.4
0.2
0.0
-
+
β-estradiol
D
SEN5 / PP2A
8.0
6.0
4.0
2.0
0.0
-
+
β-estradiol

Figure 2


Add 5μM
β-estradiol
0 24 48 72 96 (h)
8 days under 12L/12D
4 days under LL

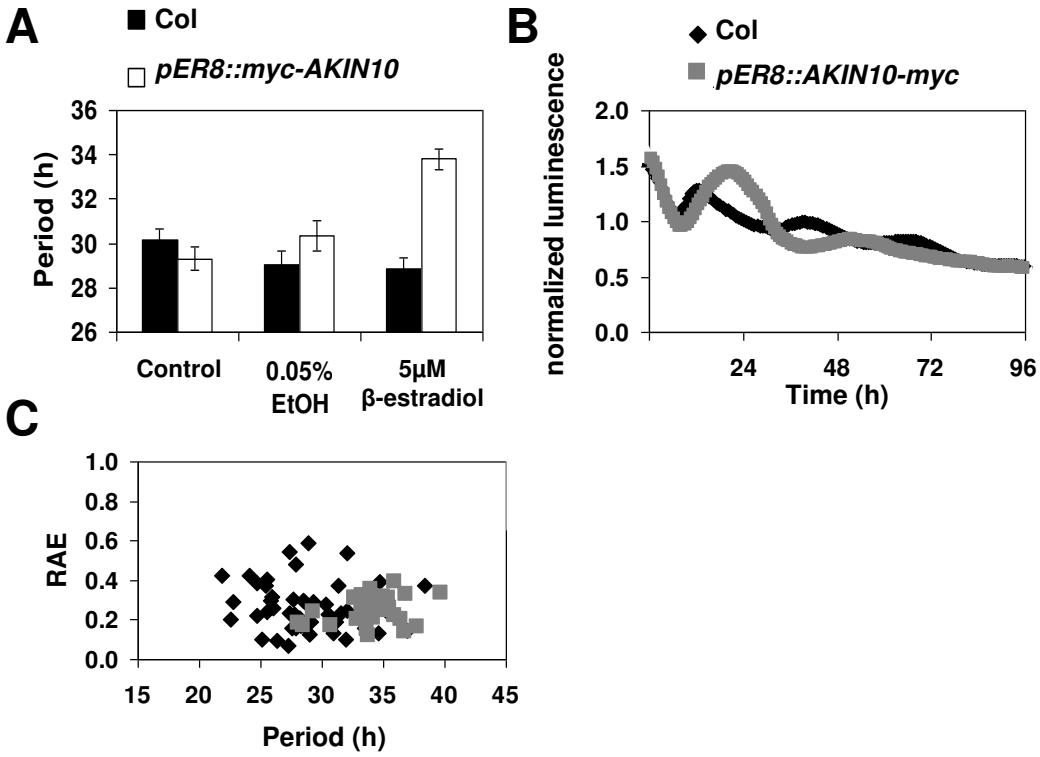

A
Col
pER8::myc-AKIN10
Period (h)
36
34
32
30
28
26
Control
0.05% EtOH
5μM β-estradiol
B
Col
pER8::AKIN10-myc
normalized luminescence
2.0
1.5
1.0
0.5
0.0
24
48
72
96
Time (h)
C
RAE
1.0
0.8
0.6
0.4
0.2
0.0
15
20
25
30
35
40
45
Period (h)

Figure 3

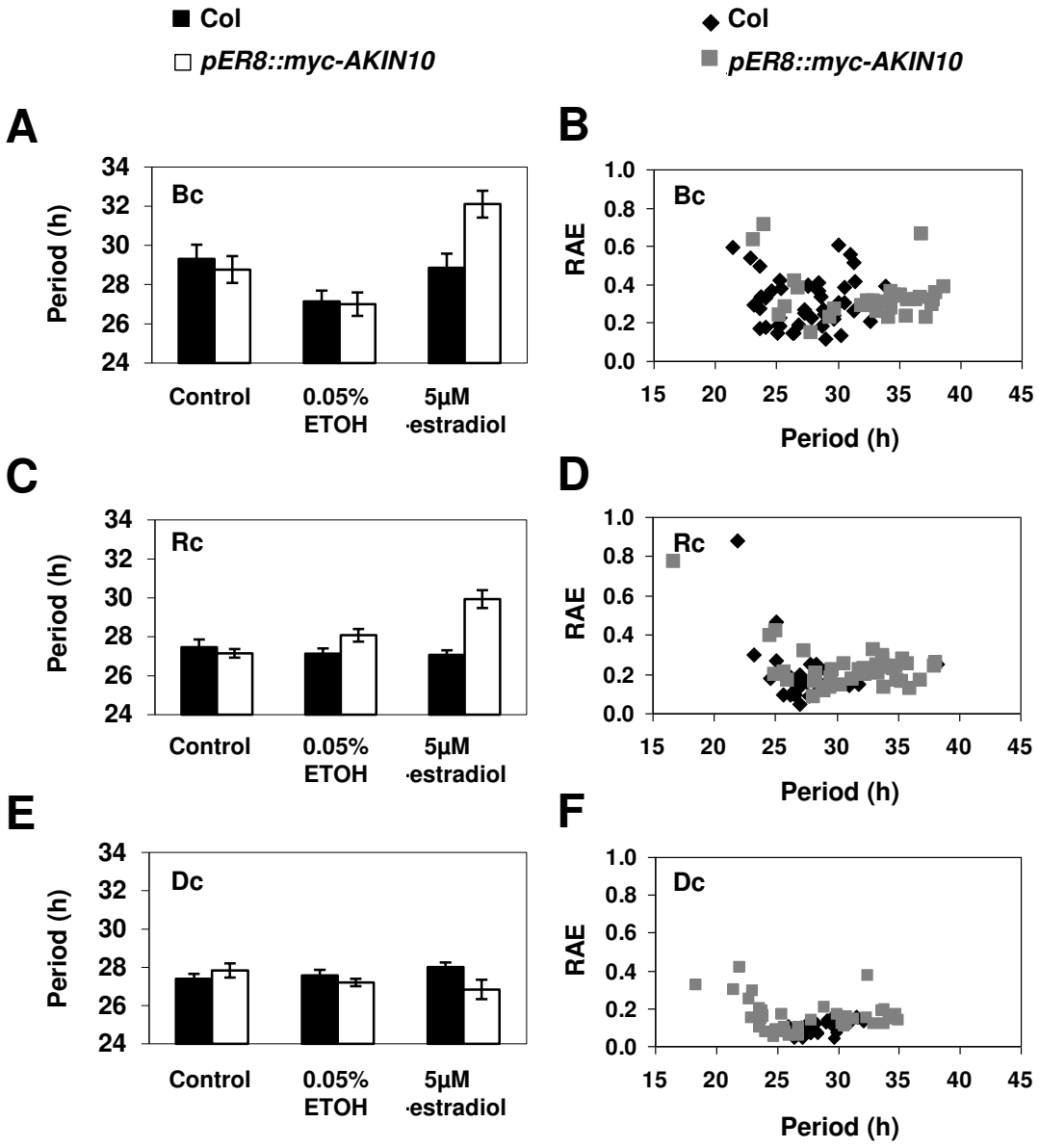

Col
pER8::myc-AKIN10
Col
pER8::myc-AKIN10
A
B
Bc
Period (h)
RAE
Control
0.05% ETOH
5μM β-estradiol
C
D
Rc
E
F
Dc

Figure 4

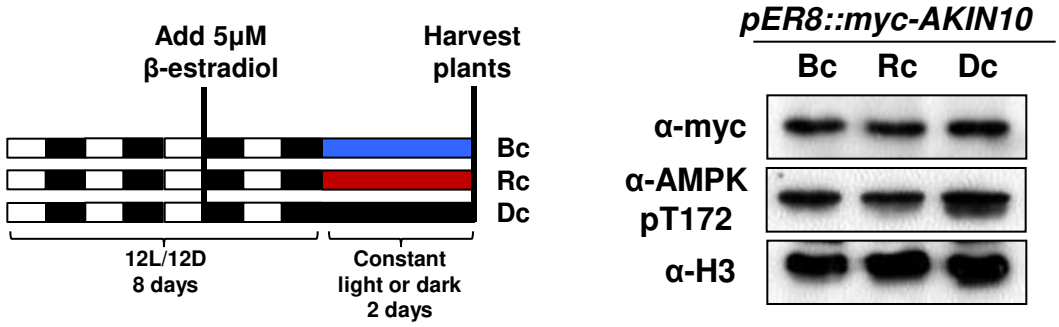

Add 5μM
β-estradiol
Harvest
plants
Bc
Rc
Dc
12L/12D
8 days
Constant
light or dark
2 days
pER8::myc-AKIN10
Bc
Rc
Dc
α-myc
α-AMPK
pT172
α-H3

Figure 5

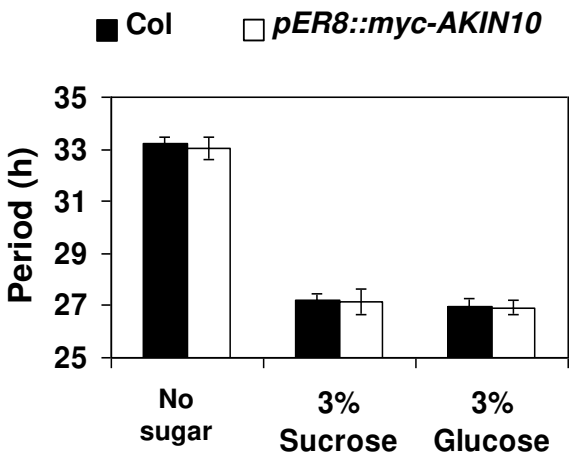
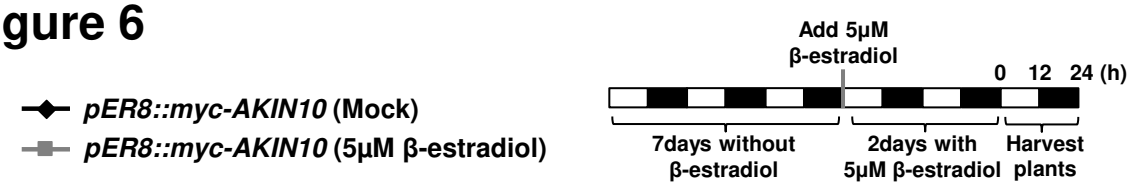

Col
pER8::myc-AKIN10
Period (h)
35
33
31
29
27
25
No sugar
3% Sucrose
3% Glucose

Figure 6


pER8::myc-AKIN10 (Mock)
pER8::myc-AKIN10 (5μM β-estradiol)
Add 5μM β-estradiol
0 12 24 (h)
7days without β-estradiol
2days with 5μM β-estradiol
Harvest plants

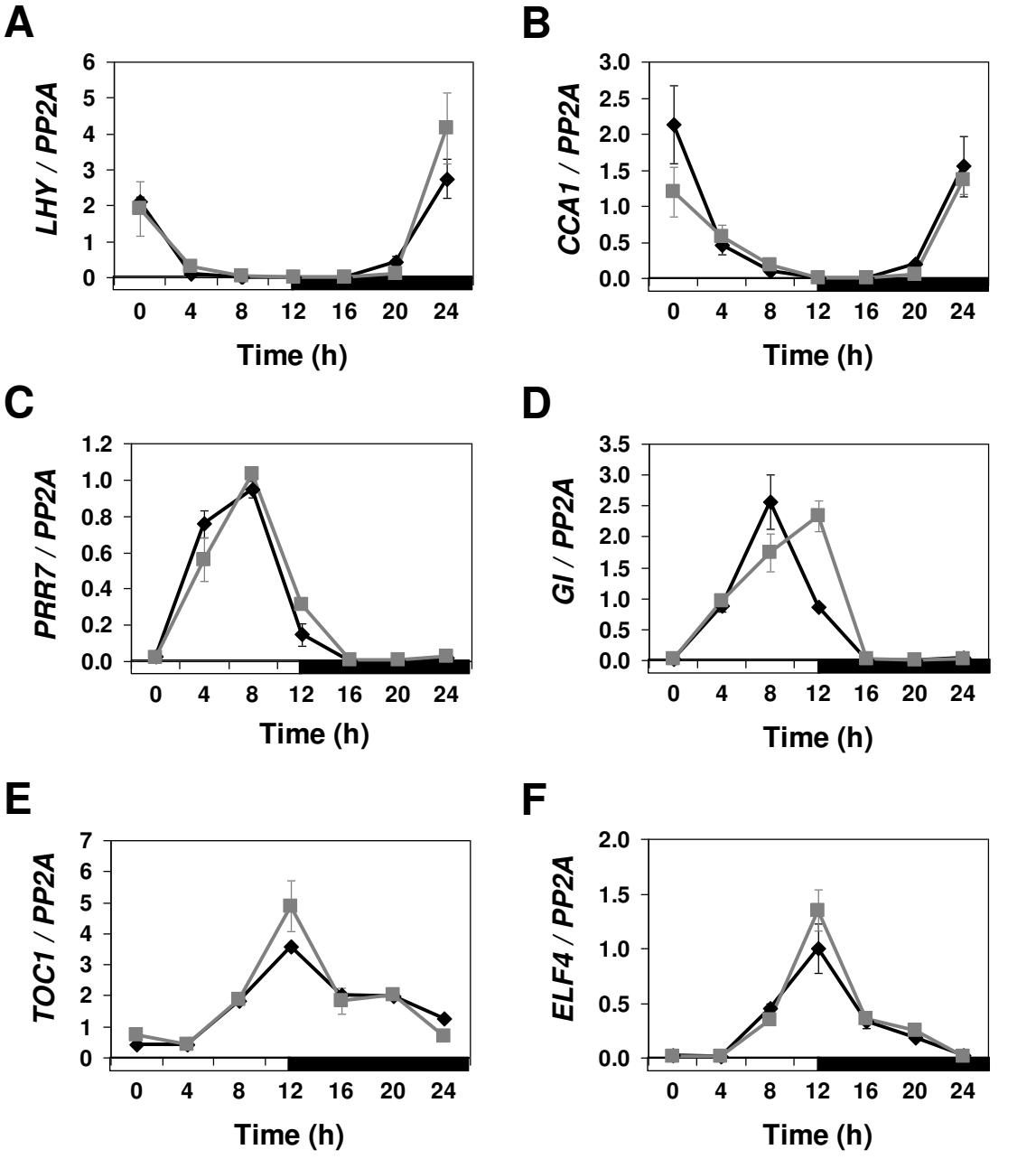

A
LHY / PP2A
B
CCA1 / PP2A
C
PRR7 / PP2A
D
GI / PP2A
E
TOC1 / PP2A
F
ELF4 / PP2A
Time (h)

Figure 7

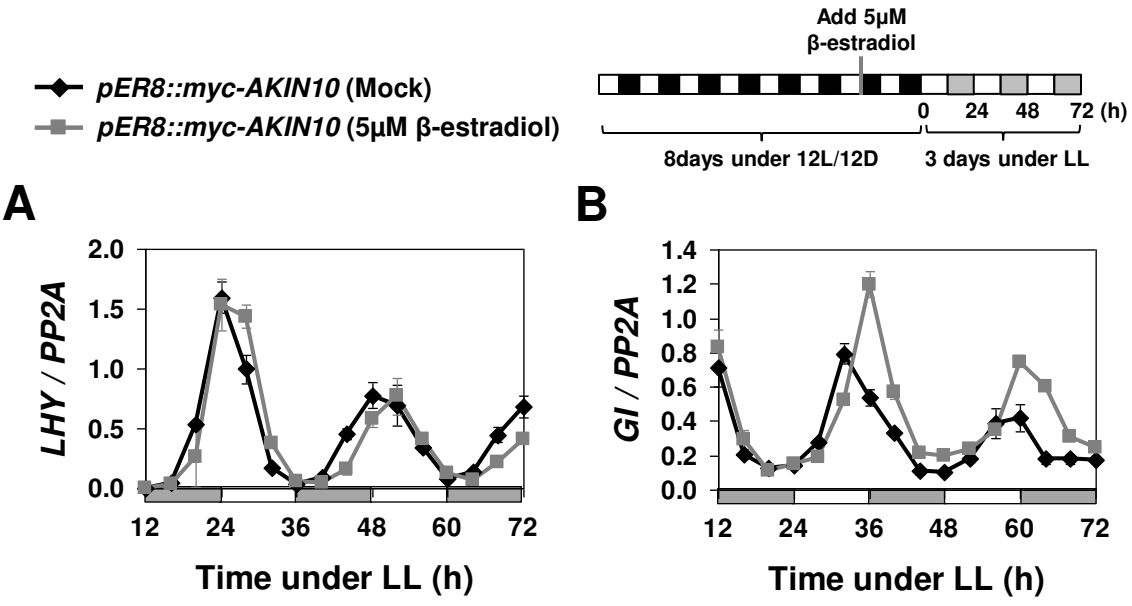
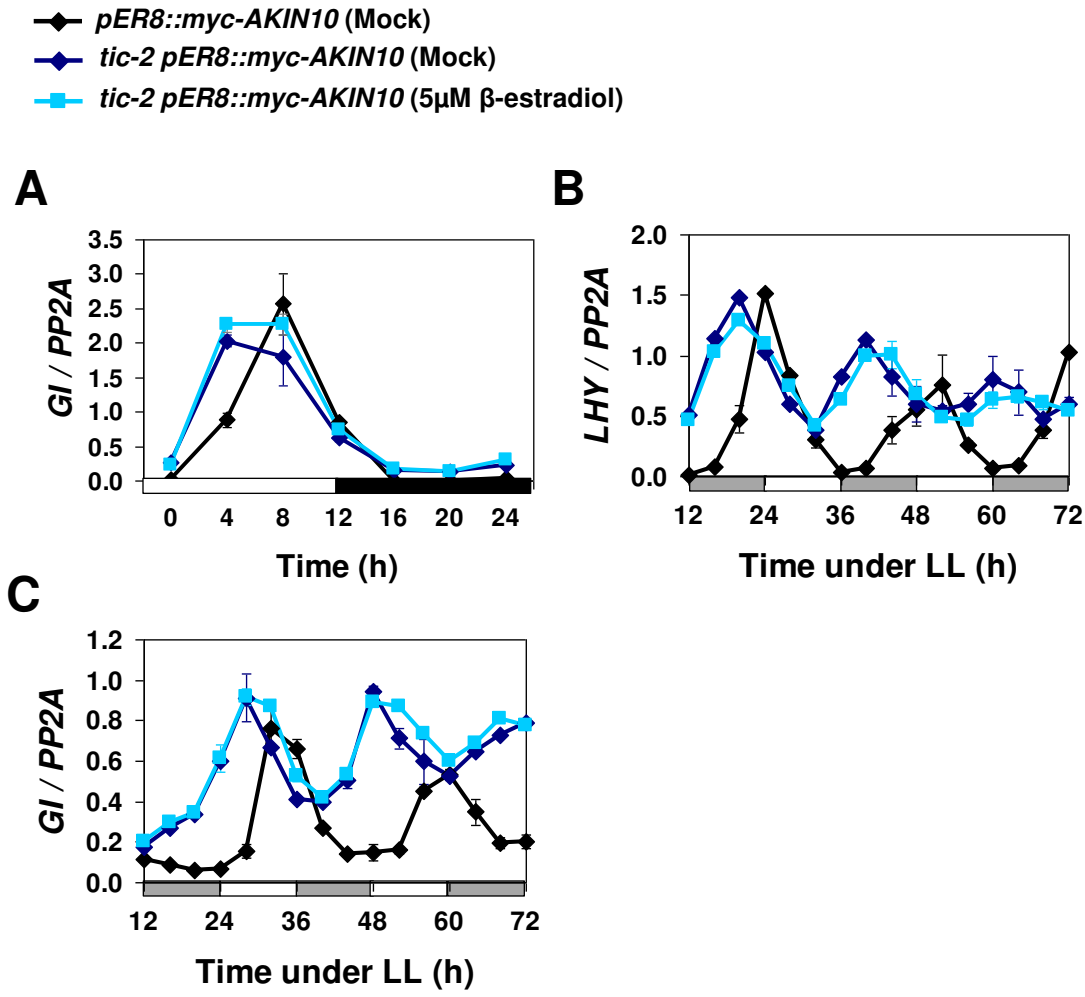
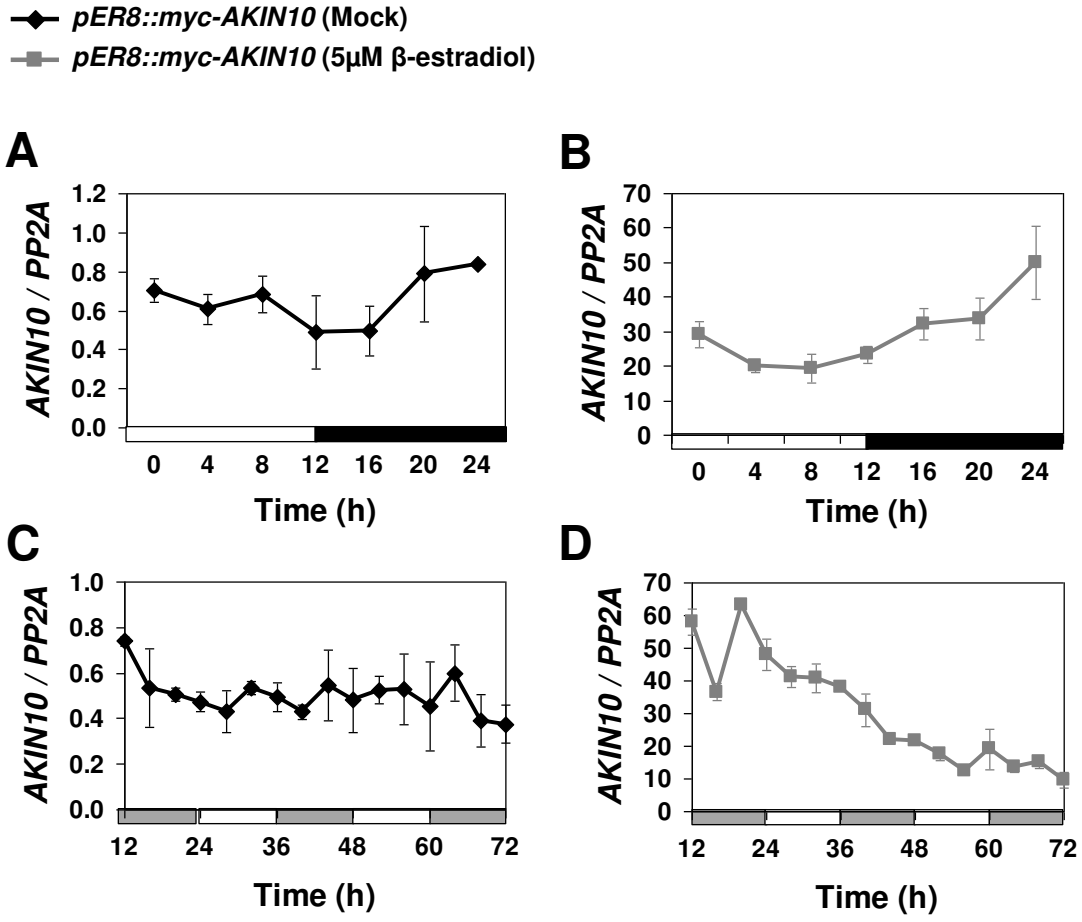

pER8::myc-AKIN10 (Mock)
pER8::myc-AKIN10 (5μM β-estradiol)
Add 5μM
β-estradiol
0 24 48 72 (h)
8days under 12L/12D
3 days under LL
A
LHY / PP2A
2.0
1.5
1.0
0.5
0.0
12 24 36 48 60 72
Time under LL (h)
B
GI / PP2A
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
12 24 36 48 60 72
Time under LL (h)

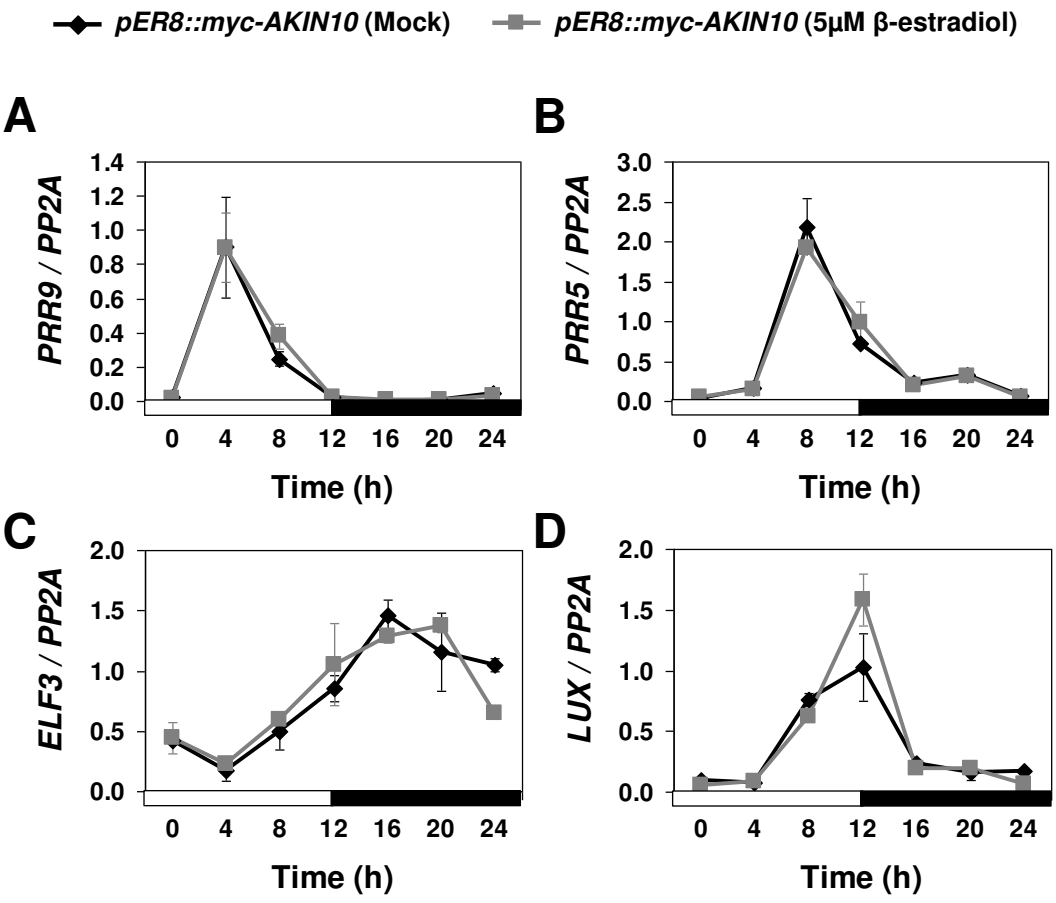
Figure 8


pER8::myc-AKIN10 (Mock)
tic-2 pER8::myc-AKIN10 (Mock)
tic-2 pER8::myc-AKIN10 (5μM β-estradiol)
A
GI / PP2A
Time (h)
B
LHY / PP2A
Time under LL (h)
C
GI / PP2A
Time under LL (h)

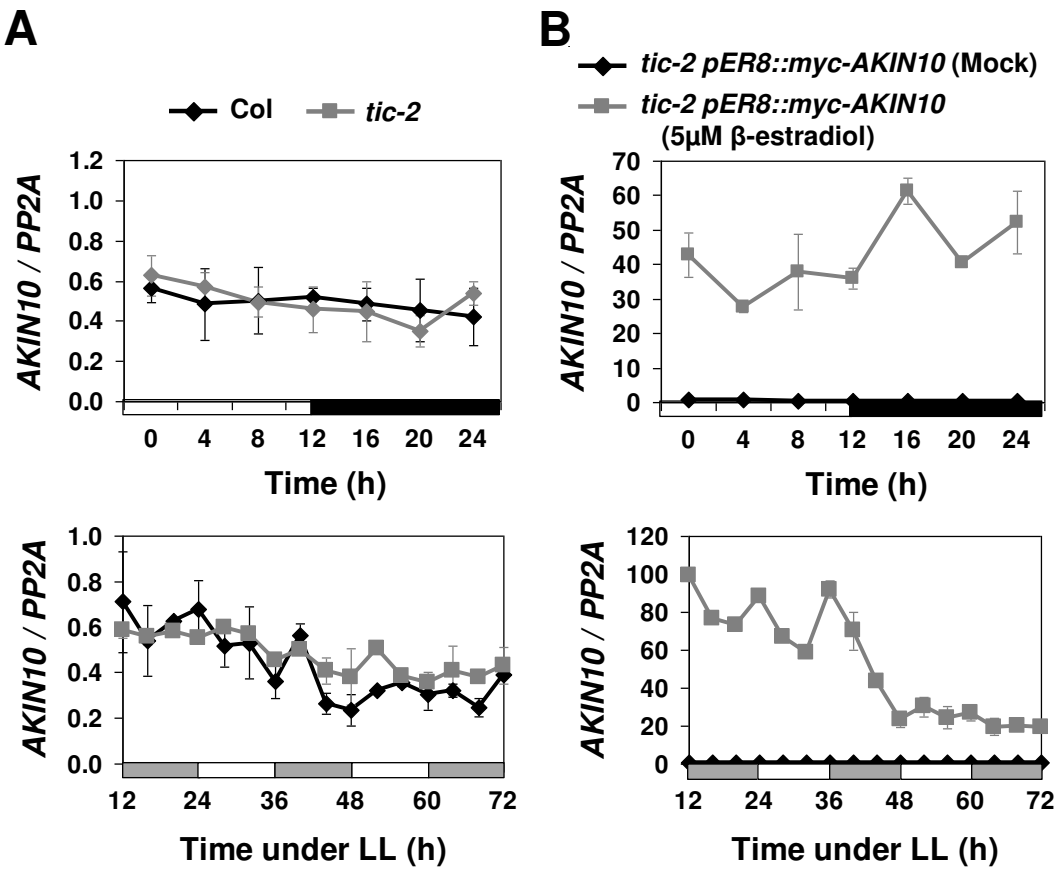
Supplement Figure 1



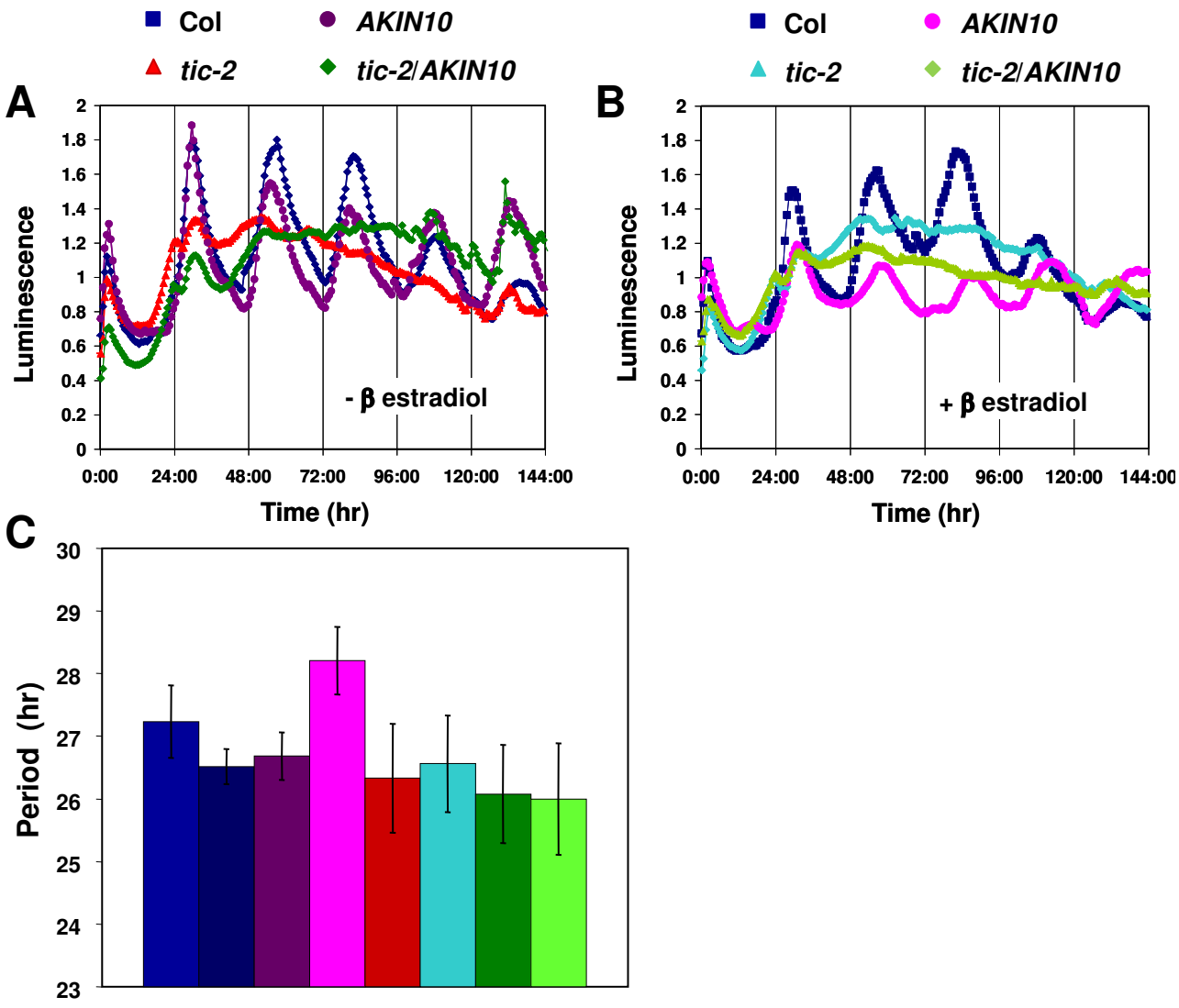
Supplement Figure 2



Supplement Figure 3



Supplement Figure 4



Supplement Table 1

GATEWAY cloning primer

AKIN10 5' primer	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAG ATAGAACCATGGATGGATCAGGCACA
AKIN10 3' primer	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAGAGGA CTCGGAGCTG

qRT-PCR primer

PP2A LP	TAT CGG ATG ACG ATT CTT CGT GCA G
PP2A RP	GCT TGG TCG ACT ATC GGA ATG AGA G
AKIN10 LP	GGG TTC CTA ACA GCA GCG CAG ATG GTA TGC
AKIN10 RP	GGA CCT TGT ACT CTC TGC AAA TCC AGT AGA
CCA1 LP2	TCTGTGTCTGACGAGGGTCGAATT
CCA1 RP2	ACTTTGCGGCAATACCTCTCTGG
LHY LP2	CAACAGCAACAACAATGCAACTAC
LHY RP2	AGAGAGCCTGAAACGCTATACGA
PRR7 LP	TGAAAGTTGGAAAAGGACCA
PRR7 RP	GTTCCACGTGCATTAGCTCT
PRR9 LP	GCACAGAGAAACCAAAGGAA
PRR9 RP	CTTTCACTCGAGGACGTTGT
GI LP	GCG GGC AAC TGA TGG AAT GCT TGT TGA TGG
GI RP	GTG CAC TTG GGT GTG AAA GGC ACC GTA TTG
TOC1 LP	CTG CTG ACT ATG ATG ACG AGG A
TOC1 RP	AAG AGC CAA CAT TGC CTT AGA G
PRR5 LP	CGT TCG TCA AGT CCA ATC CAC
PRR5 RP	AGA ACA GCT CCT GCA TCG G
ELF4 LP	CGA CAA TCA CCA ATC GAG AAT G
ELF4 RP	AAT GTT TCC GTT GAG TTC TTG AAT C
ELF3 LP	GAT GCC CAC CAT AAT GAA CC
ELF3 RP	TTG CTC GCG GAT AAG ACT TT
LUX LP	AGA TGA TGC AGA TGC CAG TT
LUX RP	TAA TTC TCA TTT GCG CTT CC
DIN6 LP	TAG GGG TCA AGA TGG TTC TCT CCG GCG AAG
DIN6 RP	GTC AAG GAA AGG AAC ACG TGC CTC TAG TCC
SEN5 LP	CCT CTC TTC GTC AAA GGT TGT TCT GTG GAC
SEN5 RP	TCA CGA AGT GTT CGA TAA GCT TCG ATC ACA