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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.

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

3

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20

21 **Abstract**

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

34

35 **Keywords**

36 circadian clock, metabolism, light signaling, Arabidopsis, AKIN10

37

38 Introduction

39 It is important for plants to recognize and effectively respond to environmental changes.
40 Rhythmic environmental stimuli caused by diurnal cycles are mostly predictable, and the
41 circadian-clock system plays a key role to manage organism's rhythmic responses to these
42 environmental changes. Clock activity is known to be critical for increasing fitness (Dodd *et*
43 *al.*, 2005, Sanchez *et al.*, 2011). The clock consists of input pathways, a core oscillator, and
44 output responses. Components of various input pathways recognize environmental signals,
45 termed *zeitgebers* (time givers), as they reset the core oscillator. Light and temperature have
46 been revealed as major input *zeitgeber* signals (Bujdoso & Davis, 2013, McClung & Davis,
47 2010), and metabolites have also been described as such input factors (Dalchau *et al.*, 2011,
48 Haydon *et al.*, 2013, Haydon *et al.*, 2015). *Zeitgebers* drive the core clock to produce an
49 approximately 24-h rhythmic periodicity, and this process is called entrainment [reviewed in
50 (Bujdoso & Davis, 2013)]. Fully entrained plants display strong biological rhythmicity even
51 in the absence of environmental signals.

52 The circadian core-oscillator has been intensively investigated using a combination of genetic
53 approaches and computational analysis (Bujdoso & Davis, 2013, Shin & Davis, 2010). The
54 current model is established with multiple interlocking transcriptional feedback loops. Briefly,
55 the morning-acting elements LATE ELONGATED HYPOCOTYL (*LHY*) and CIRCADIAN
56 CLOCK ASSOCIATED 1 (*CCA1*) repress the transcription of the evening factor *TIMING OF*
57 *CAB EXPRESSION 1 (TOC1)* (Alabadi *et al.*, 2001). In turn, *TOC1* inhibits the expression of
58 *LHY* and *CCA1* to form the core feedback loop (Gendron *et al.*, 2012, Huang *et al.*, 2012).
59 *PSEUDO-RESPONSE REGULATOR 7 (PRR7)* and *PRR9* form another transcriptional
60 feedback loop with *CCA1* and *LHY*, and this loop works during the morning phase
61 (Nakamichi *et al.*, 2010). *GIGANTEA (GI)* and *TOC1* are additionally proposed to compose
62 an evening loop (Bujdoso & Davis, 2013). Finally, *EARLY FLOWERING 3 (ELF3)*, *ELF4*,
63 and *LUX ARRHYTHMO (LUX)* were found to form a functional complex (Nusinow *et al.*,
64 2011) that constitutes another oscillator loop in the evening (Anwer *et al.*, 2014, Herrero &
65 Davis, 2012, Herrero *et al.*, 2012, Kolmos *et al.*, 2011, Kolmos *et al.*, 2009). Genetic and
66 molecular relationships between many clock genes have been discovered, and placing the
67 molecular impact of circadian-input factors to these has remained as a next challenge
68 [reviewed in (Bujdoso & Davis, 2013)].

69 The circadian clock temporally controls diverse physiological responses (Sanchez *et al.*,
70 2011). Sugar metabolism has long been considered as one of the clock-output responses; free
71 sugar formation oscillates, as sugars are the products of photosynthesis, which is directly
72 regulated by light and the clock (Blasing *et al.*, 2005, Eimert *et al.*, 1995). Starch formation
73 and its breakdown products are also controlled by the clock (Graf *et al.*, 2010, Müller *et al.*,
74 2014). Metabolism, however, is not only restricted to clock-driven output responses, but also
75 contributes to the clock activity (Bujdoso & Davis, 2013, Haydon *et al.*, 2013, Sanchez *et al.*,
76 2011). For example, both soluble sugars and cyclic adenosine diphosphate ribose (cADPR)
77 were reported to regulate clock period and phase, as well as the expression of clock genes
78 (Blasing *et al.*, 2005, Dodd *et al.*, 2007, Dodd *et al.*, 2009, Knight *et al.*, 2008). Sucrose has
79 been specifically suggested as a potential *zeitgeber* in the clock input pathway that directly
80 regulates the expression of the evening clock gene *GI* (Dalchau *et al.*, 2011). Metabolic
81 processes thus seem to be intrinsic elements allowing proper clock function.

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

101 within Arabidopsis, and can be induced by trehalose, but not other sugars (Williams *et al.*
102 2014). This indicates different roles in plant responses to energy and carbon pools. The
103 induction of AKIN10 activity by sucrose has been reported in several studies (Bhalerao *et al.*
104 1998, Jossier *et al.*, 2009). Therefore, AKIN10 activity may be dependent not only on the
105 type of sugars, but on the carbon pools, as suggested by Lunn *et al.* (2014).

106 In yeast SNF1 and mammalian AMPKs are involved in metabolic and stress responses
107 triggered by either glucose starvation or high AMP/ATP ratio, respectively (Carlson, 1999,
108 Ghillebert *et al.*, 2011, Hardie, 2007, Polge & Thomas, 2007, Rutter *et al.*, 2003, Young *et al.*,
109 2003). In Arabidopsis, SnRK1 also plays a key role in abscisic acid (ABA) hormone
110 signaling (Jossier *et al.*, 2009, Lu *et al.*, 2007, Radchuk *et al.*, 2006), as well as regulates
111 plant growth and development (Baena-Gonzalez *et al.*, 2007, Radchuk *et al.*, 2006, Tsai &
112 Gazzarrini, 2012, Zhang *et al.*, 2001). SnRK1 thus has broad roles to ensure metabolic
113 homeostasis, and this is critical for diverse biological processes.

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

127

128 **Material and Methods**

129

130 **Plant material and growth conditions**

131 *Arabidopsis thaliana* Columbia (Col) accession is the genetic background of the wild type
132 and transgenic lines used in this study. Plants were grown on MS media [half strength MS
133 (Sigma), 0.9% phytoagar and 0.05% MES (Duchefa), pH 5.7] at 22°C under various light
134 conditions. For luciferase-reporter assays, 3% sucrose was added to the media, whereas no
135 additional sucrose, 1% sucrose containing, or 3% glucose MS media was used for other
136 experiments. The bioluminescence assays were performed as previously described (Hanano *et al.*,
137 2006, Kolmos *et al.*, 2009) with indicated light provided by custom LED panels ($\sim 2 \mu\text{mol}$
138 $\text{m}^{-2} \text{s}^{-1}$). For RNA-based work, seedlings were grown at 22°C with $75 \mu\text{mol m}^{-2} \text{s}^{-1}$ cool
139 white fluorescent light, as described (Shin *et al.* 013).

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

149 **Chemical treatment**

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

155

156 Gene expression analysis

157 Total RNA was extracted from seedlings using Spectrum™ Plant Total RNA Kit (Sigma),
158 according to the manufacturer's instructions. cDNA was synthesized from 4 µg of total RNA
159 with Maxima™ First Strand cDNA Synthesis Kit (Fermentas). To amplify genes, 5 µL of
160 1/25 diluted cDNA was used as the template. Quantitative RT-PCR analysis was performed
161 using SYBR and LightCycler™ 480 (Roche). Primer sequences for qRT-PCR are listed in
162 Supplemental Table 1. The resulting gene expression levels were normalized with the level of
163 *PP2A* (Czechowski *et al.*, 2005). Data analysis was performed using three technical replicates
164 from each biological sample, and similar results were obtained in two biological replicates.

165 Protein extraction and western blotting

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

173

174 Results

175 Generation of chemically inducible *AKIN10* overexpressing plants

176 To start investigating the impact of energy metabolism for clock-oscillator function, we
177 examined the role of *AKIN10*. *akin10* null mutants are not available, as eliminating SnRK1
178 catalytic subunit leads to severe developmental defects, and ultimately to seedling lethality
179 (Baena-Gonzalez *et al.*, 2007, Radchuk *et al.*, 2006, Tsai & Gazzarrini, 2012, Zhang *et al.*,
180 2001). Therefore, we generated transgenic plants that overexpress *AKIN10* by a chemical-
181 inducible system, and used these for genetic and molecular analysis. For this, *AKIN10* was
182 placed under control of the β -estradiol inducible promoter, hereafter called *pER8::myc-*
183 *AKIN10*. This chemical-inducible system allowed us to study the role of *AKIN10* in plants
184 after early seedling growth stage had been progressed. Without external β -estradiol treatment,
185 *AKIN10* transcript levels in *pER8::myc-AKIN10* plants were comparable to the wild type
186 (Col), and myc-*AKIN10* protein was not expressed (Figure 1A, 1B). The transcript level of
187 *AKIN10* was increased in plants being treated with β -estradiol for 2-3 days by 82–92 fold
188 compared to non-treated control plants. However, with increasing duration of β -estradiol
189 treatment, the expression level of *AKIN10* gradually decreased. Nevertheless, the *AKIN10*
190 mRNA level was induced ~20 fold during a β -estradiol treatment for 6 days (Figure 1A).
191 Based on these observations, we chose a 2-6 days time window for the β -estradiol treatment
192 to analyze the effects of elevated *AKIN10* expression on clock function.

193 *AKIN10* is thought to be active only if its activation T-loop threonine residue (T175) is
194 phosphorylated (Crozet *et al.*, 2010) although the relationship between the residue
195 phosphorylation and kinase activity has not been clearly established in plants (Crozet *et al.*,
196 2014). Using anti-phospho-AMPK α (T172) antibody, which specifically detects the
197 phosphorylated Thr175 residue of *AKIN10* (*AKIN10* pT175) (Coello *et al.*, 2012, Shen *et al.*,
198 2009), we monitored the amount of the myc-*AKIN10* pT175. In the wild type and non-
199 induced *pER8::myc-AKIN10* plants, only the endogenously expressed *AKIN10* pT175 was
200 detected (Figure 1B, lower bands). In β -estradiol treated *pER8::myc-AKIN10* plants, a myc-
201 *AKIN10* pT175 form was readily detected (Figure 1B, additional upper band). To further
202 confirm that expressed myc-*AKIN10* is biologically functional, the transcript level of
203 *AKIN10*-regulated genes were determined in *pER8::myc-AKIN10* plants. It is known that
204 *DARK INDUCIBLE 6 (DIN6)* and *SENESCENCE-ASSOCIATED PROTEIN 5 (SEN5)* are

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

210 **Overexpression of myc-AKIN10 lengthens clock period under light conditions**

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

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

237 We further investigated the effects of *AKIN10* on clock function under different light
238 conditions. For this, we determined circadian period under constant blue light (Bc), constant
239 red light (Rc), and in constant dark conditions. Consistent with constant R+B results in
240 Figure 2, *pER8::myc-AKIN10* plants displayed a significantly longer period than wild type in
241 response to external β -estradiol treatment under Bc and Rc conditions [P-value: 3.93E-8 (Bc),
242 1.8E-5 (Rc), ANOVA] (Figure 3A, 3B). In contrast, no period-lengthening effects were
243 observed by elevated *myc-AKIN10* in darkness. If anything, *pER8::myc-AKIN10* plants
244 displayed a slightly shorter period compared to the wild type when β -estradiol was applied,
245 but this was not statistically significant (P-value: 0.11, ANOVA) (Figure 3C). This could have
246 been because *myc-AKIN10* induction by β -estradiol treatment was restricted by darkness. To
247 explore this possibility, we examined myc-AKIN10 protein accumulation in response to β -
248 estradiol under Bc, Rc, and in dark conditions. myc-AKIN10 protein similarly accumulated
249 in darkness as under Bc and Rc conditions (Figure 4). The level of phosphorylated myc-
250 AKIN10 was also comparable regardless of light conditions (Figure 4), which implies
251 induced myc-AKIN10 has similar kinase activity under the differing conditions of these
252 experiments. Thus *AKIN10* activity and its effects in gene expression, as Baena-González *et*
253 *al.* (2007) showed for *DIN6* expression under darkness, could be equally independently of the
254 light conditions. Therefore, the lack of period lengthening phenotype of *pER8::myc-AKIN10*
255 plants in darkness does not appear to be caused by the failure of the β -estradiol-induced
256 *AKIN10* expression and/or light-specific post-translational modification of *AKIN10*.

257 Plants have been typically grown on 3% sucrose for luciferase reporter assays (Millar *et al.*,
258 1992). In previous studies, prolonged darkness, carbohydrate starvation, and induced
259 senescence, have been shown to promote SnRK1 activity (Baena-Gonzalez *et al.*, 2007,
260 Bhalerao *et al.*, 1999). However Jossier *et al.* (2009) described and increase in *AKIN10*
261 activity due to glucose addition. We thus examined the effects of the presence and/or type of
262 sugars on the *AKIN10*-mediated regulation of the circadian period in darkness. The rhythmic
263 period was determined from plants grown without exogenous sugar-, on 3% sucrose-, or 3%
264 glucose-containing media. Consistent with a previous report (Knight *et al.*, 2008), we
265 confirmed that sugar application shortens the circadian period (Figure 5). There were no
266 differences between sucrose and glucose on the regulation of period length, as previously

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

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

275 We next determined the transcript accumulation of several clock components in *myc-AKIN10*
276 overexpressing plants under diurnal conditions. *pER8::myc-AKIN10* plants were grown under
277 12L/12D conditions for 7 days, and transferred to β -estradiol-containing media for an
278 additional 2 days. *AKIN10* mRNA was not rhythmically expressed in control plants, nor in
279 plants treated with β -estradiol (Supplement Figure 1A, 1B). *AKIN10* was 42–153 fold
280 elevated by β -estradiol treatment for all time points measured (Supplement Figure 1B). *LHY*
281 (Figure 6A), *CCA1* (Figure 6B), *PRR7* (Figure 6C), *TOC1* (Figure 6E), *ELF4* (Figure 6F),
282 *PRR9*, *PRR5*, *ELF3*, and *LUX* (Supplement Figure 2) were similarly expressed in β -estradiol-
283 treated and non-treated plants. Therefore, under diurnal conditions, overexpressed *myc-*
284 *AKIN10* did not affect the gene-expression profiles of most clock genes. Exceptionally, we
285 found that *GI* expression peaked at ZT12 (ZT: *Zeitgeber* time, ZT12 indicates 12 h after
286 lights on) in β -estradiol-treated plants, whereas it peaked at ZT8 in non-treated plants (Figure
287 6D). Under diurnal conditions, *myc-AKIN10* induction appeared to specifically delay the
288 peak expression phase of *GI*.

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

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

309 ***AKIN10* genetically interacts with *TIC* in periodicity determination**

310 Altered clock activity in *myc-AKIN10* overexpressing plants is the opposite phenotype of
311 plants having a mutation in the clock regulator gene *TIC*. *tic* is known to express *GI* around
312 4-h earlier than the wild type, has extensive developmental and metabolic phenotypes
313 (Sánchez-Villarreal *et al.*, 2013), and displayed a shorter rhythmic period (Ding *et al.*, 2007,
314 Hall *et al.*, 2003). These observations led us to test if there is a genetic relationship between
315 *AKIN10* and *TIC* in period determination. We first examined *AKIN10* transcript accumulation
316 in the *tic* mutant. *AKIN10* mRNA similarly accumulated in *tic* as in the wild type, both under
317 diurnal and free-running conditions (Supplement Figure 3A). Therefore, *TIC* did not affect
318 *AKIN10* expression at the transcript level. We next generated *tic-2 pER8::myc-AKIN10* plants
319 by crossing *pER8::myc-AKIN10* into *tic-2*, then determined clock gene expression both in
320 *AKIN10* induced and non-induced plants. We confirmed that *tic-2 pER8::myc-AKIN10* plants
321 express *AKIN10* at similar patterns as *pER8::myc-AKIN10* in response to β -estradiol both
322 under diurnal and free-running conditions (Supplement Figure 3B). These results indicate that
323 the capacity of the *pER8* promoter to generate overexpressed *myc-AKIN10* is comparable in
324 *tic-2* and the wild type. Consistent with previous reports in *tic* (Ding *et al.*, 2007), *GI*
325 transcript accumulation reached to its maximum at ZT4 in *tic-2 pER8::myc-AKIN10* under β -
326 estradiol non-treated conditions (Figure 8A). Notably, we found that elevation of *AKIN10*
327 expression in the *tic* mutant no longer delayed the peak phase of *GI*. Rather, it displayed a
328 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

343 **Discussion**

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

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

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

376 *AKIN10* was shown to specifically lengthen circadian period only under light conditions
377 (Figure 2, Figure 3, and Figure 7). Although *myc-AKIN10* overexpressing plants displayed a
378 long period under light conditions, the peak and trough transcript levels of clock genes were
379 similar to those of control plants, and the rhythm was precisely maintained (Figure 2, Figure
380 6, Figure 7 and Supplemental Figure 2) albeit with a slight increase in amplitude in evening
381 expressed genes *LUX*, *TOC1*, *ELF4*, and *ELF3*. Based on our results, *AKIN10* seems to act in
382 the circadian-input pathway rather than functioning in the core oscillator. In darkness,
383 elevated *myc-AKIN10* did not lengthen the clock period regardless of the presence and type
384 of sugars supplied to the media (Figure 3E–3F, Figure 5). Thus *AKIN10* effect on clock
385 period seems is not solely dependent on sucrose, but rather the kinase effect on the clock
386 additionally requires light. Under our assay conditions, *myc-AKIN10* protein levels and its
387 phosphorylation status were not significantly changed in darkness, compared to light
388 conditions (Figure 4). It is possible that other SnRK1 complex subunits are also involved in
389 the regulation of the clock function, and their expression, availability, and/or activity is
390 modulated depending on the light conditions. Indeed, it has been shown that the expression of
391 three SnRK1 β subunits is differentially regulated according to environmental conditions,
392 organs, and developmental stages (Polge *et al.*, 2008). Furthermore tissue expression
393 specificity by *AKIN10* and *AKIN11* (SnRK1.1 and SnRK1.2, respectively) as well as
394 responses to carbohydrates and developmental effects has been shown (Williams, 2014). The
395 detailed molecular and biochemical relationships should be further investigated to reveal the
396 underlying mechanism of the light-dependent effects of *AKIN10* on the regulation of the
397 clock.

398 In our luciferase-reporter assays, the control plants displayed around 27 h free-running period
399 (Figure 2, Figure 3). This could be due to low intensity of light [$\sim 2 \mu\text{E}/\text{m}^2/\text{s}$ (red) and ~ 2
400 $\mu\text{E}/\text{m}^2/\text{s}$ (blue)] used under free-running conditions, whereas these plants were entrained
401 under higher intensity of white light ($\sim 75 \mu\text{E}/\text{m}^2/\text{s}$). Indeed, it is well established that the
402 circadian period becomes longer as light intensity decreases [reviewed in (Bujdosó & Davis,
403 2013)]. Thus period estimates from Figure 2 and Figure 3 obtained under low intensity blue
404 and red light cannot be directly compared to periods derived from quantitative RT-PCR, as in

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

409 We found a genetic interaction between *AKIN10* and *TIC*. Similar to *AKIN10*, *TIC* was
410 shown to be required to lengthen the clock period and delay the peak expression phase of *GI*
411 under diurnal conditions. Moreover, overexpression of *AKIN10* in the *tic* background did not
412 restore the *tic* mutant phenotype. *tic-2 pER8::myc-AKIN10* plants periodicity were rather
413 comparable to the *tic-2* mutant (Figure 8 and Supplemental Figure 4). These data consistently
414 indicate that *tic* is genetically epistatic to *AKIN10* overexpression. Previously, we have shown
415 that *TIC* is involved in stress responses (Shin *et al.*, 2013, Shin *et al.*, 2012, Sánchez-
416 Villarreal *et al.*, 2013), and it has been also observed that *TIC* contributes to starch
417 metabolism as its mutation results in a starch-excess phenotype (Sánchez-Villarreal *et al.*,
418 2013). It is interesting to note that *TIC* and *GI* share circadian and metabolic intersections, as
419 they are both involved in starch metabolism and oxidative stress (Fornara *et al.*, 2015,
420 Sánchez-Villarreal *et al.*, 2013). These studies together reinforce the genetic relationship
421 between *AKIN10* and *TIC* with connections to *GI*. It will be interesting to test if *TIC* alters
422 *AKIN10* kinase activity in the regulation of the circadian clock. Another equally plausible
423 scenario is a regulatory mechanism where *TIC* promotes the function of *AKIN10*, thereby
424 *AKIN10* physiological activity on the clock is attenuated in the *tic* mutant. These need not be
425 mutually exclusive possibilities.

426 In animal systems, defects in AMPK complexes are known to trigger various disorders, such
427 as metabolic syndrome, insulin resistance, obesity, cardiovascular diseases, and cancer
428 (Hardie, 2015). The plant circadian-clock system is also critical to increase fitness, and
429 promote growth and development in a metabolic-dependent manner (Dodd *et al.* 2005,
430 Fukushima *et al.*, 2009, Lai *et al.*, 2012). Our study highlights a possible role of SnRK1 on
431 circadian-clock function, and therefore, could affect plants performance. Furthermore the
432 recent discovery of magnesium fluxes, both in the unicellular alga *Ostreococcus* and human
433 cell lines, affect the cells energy balance through ATP (Feeney *et al.* 2016). This again
434 highlights the role of energy balance in coordinating clock function. The genetic interactions
435 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

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440 **Accession Numbers**

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

446

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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, 5 μ M 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 5 μ M 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 AKIN10 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

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

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

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

499 Circadian rhythmicity of *GI::LUC* in Col and *pER8::myc-AKIN10* plants in constant darkness.
 500 Col and *pER8::myc-AKIN10* plants harboring *GI::LUC* construct were entrained under
 501 12L/12D conditions for 8 days, and transferred into constant darkness. β -estradiol was added
 502 to plants 36 h before releasing into free-running conditions. Error bars indicate standard error.

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

506 Quantitative RT-PCR of *LHY* (A), *CCA1* (B), *PRR7* (C), *GI* (D), *TOC1* (E), and *ELF4* (F)
 507 relative to *PP2A* under diurnal conditions. *pER8::myc-AKIN10* plants were grown under
 508 12L/12D for 9 days in total, and treated or not with 5 μ M β -estradiol for the last 2 days as

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

512

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

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

522

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

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

533

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

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

542

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

548

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

556

557 **Supplement Figure 4. A functional *TIC* gene is necessary for *AKIN10* overexpression to**
558 **have an effect on the circadian clock.** (A and B) Normalized luminescence of *CCA::LUC*
559 traces under free running conditions for Col-0, *pER8::myc-AKIN10*, *tic-2* and *tic-*
560 *2/pER8::myc-AKIN10* without or with 5 μ M β -estradiol induction. Plants were grown under
561 12L/12D for 7 days and then transferred to media with or without ~~not~~ 5 μ M β -estradiol. 24
562 hours after plants were placed under constant B/R light. (C) Period length for Col-0,
563 *pER8::myc-AKIN10*, *tic-2* and *tic-2/pER8::myc-AKIN10* with or without application of 5 μ M
564 β -estradiol for the induction of the *AKIN10* expression.

565

566 **Supplement Table 1. Primers**

567

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Figure 1

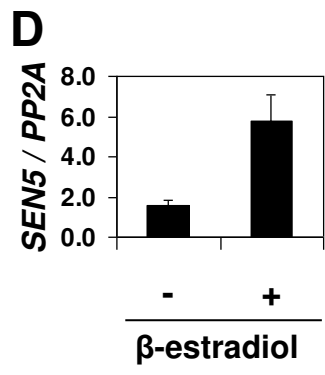
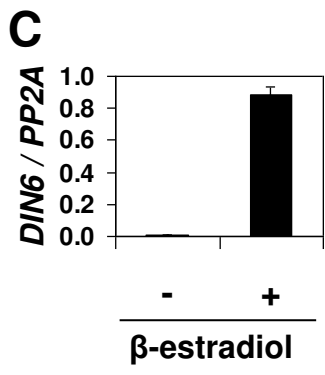
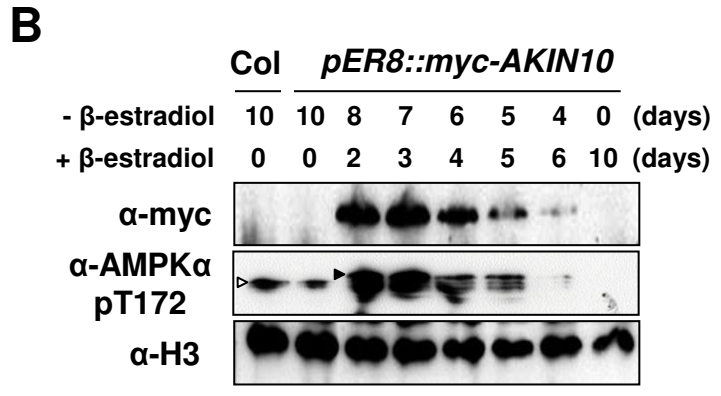
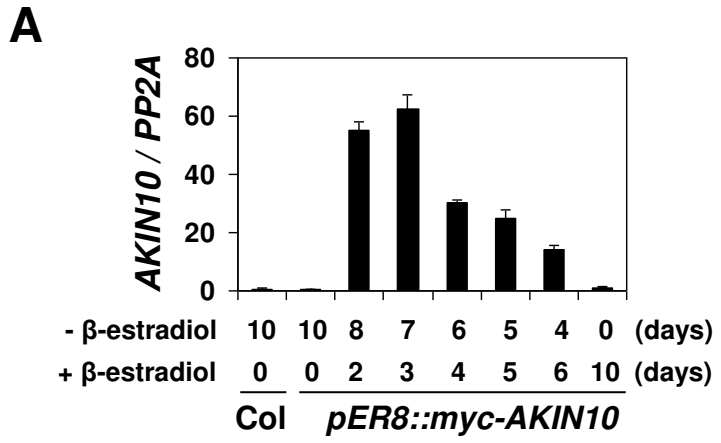


Figure 2

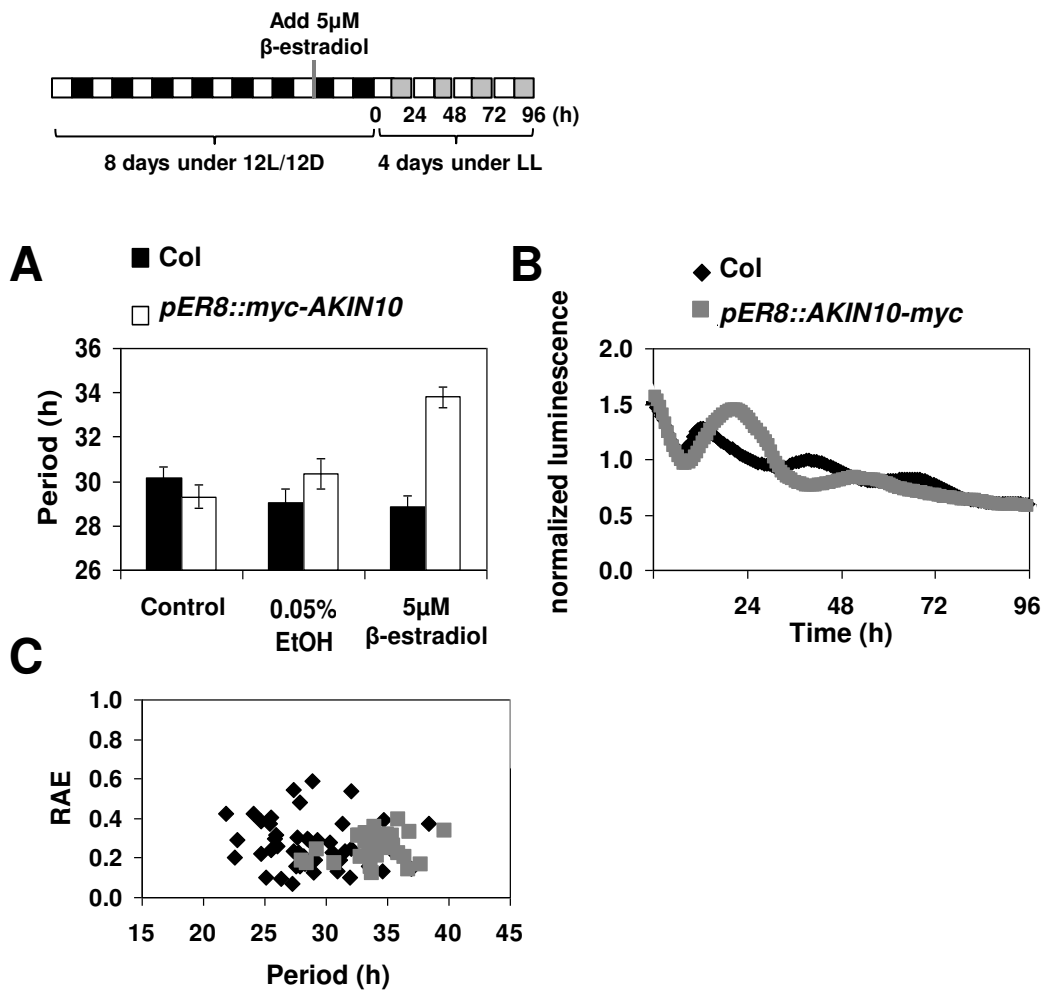


Figure 3

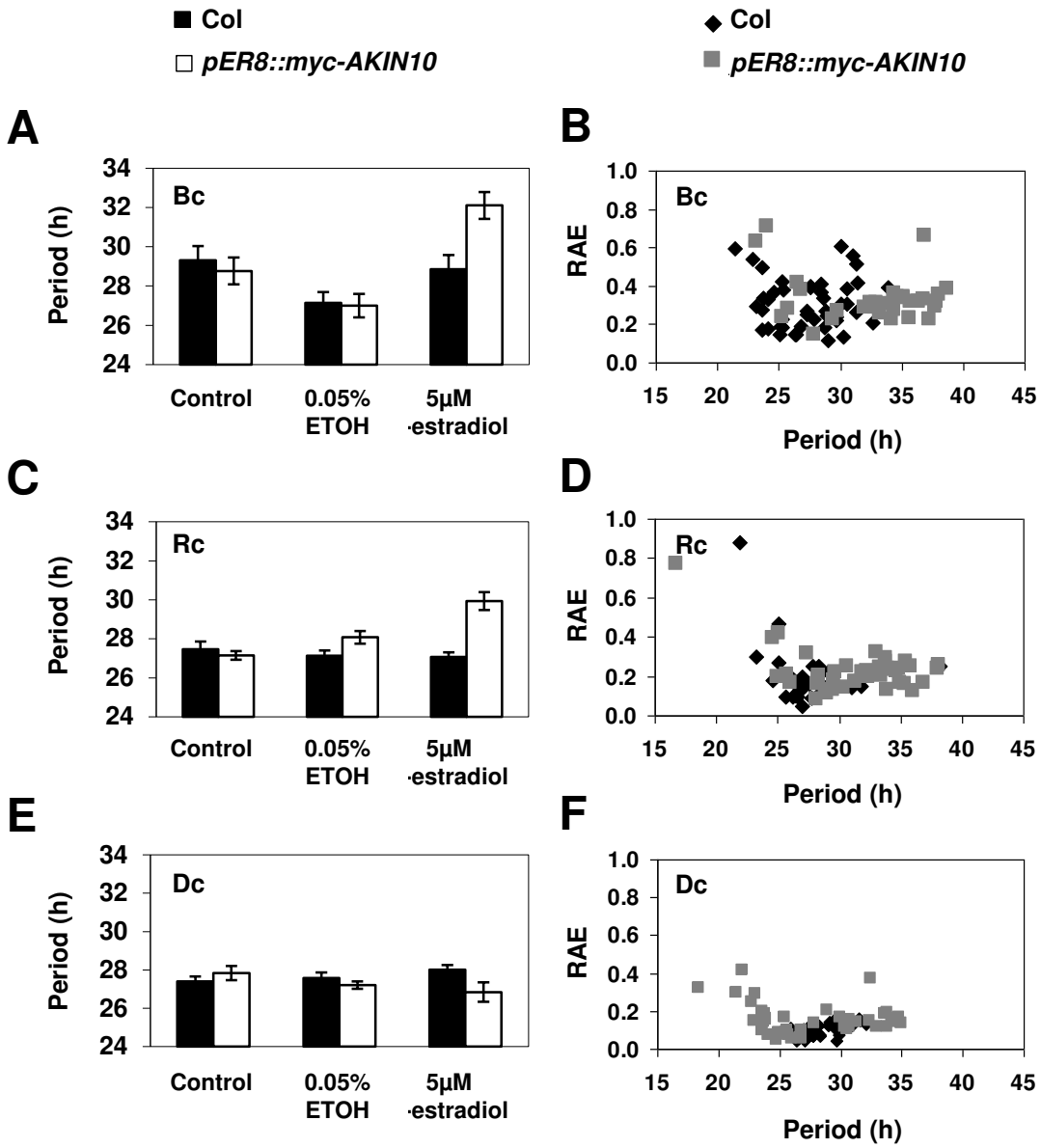


Figure 4

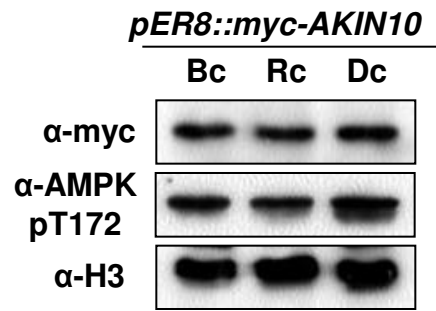
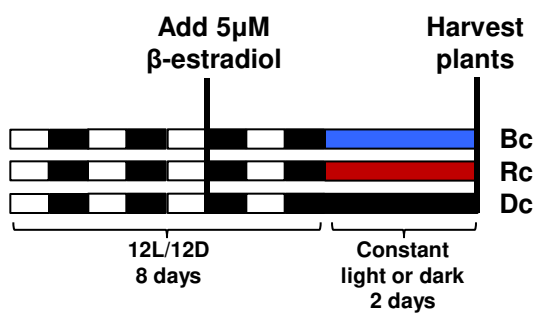


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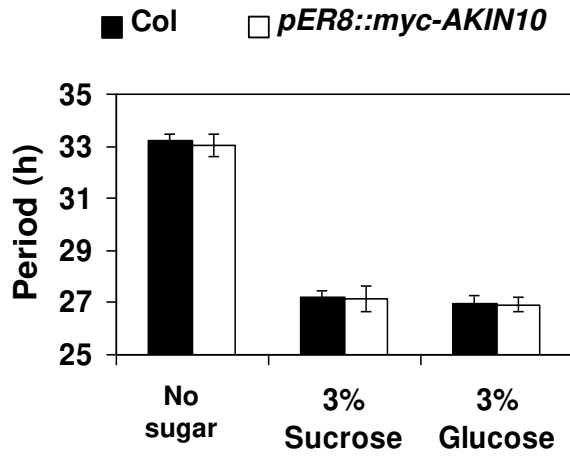


Figure 6

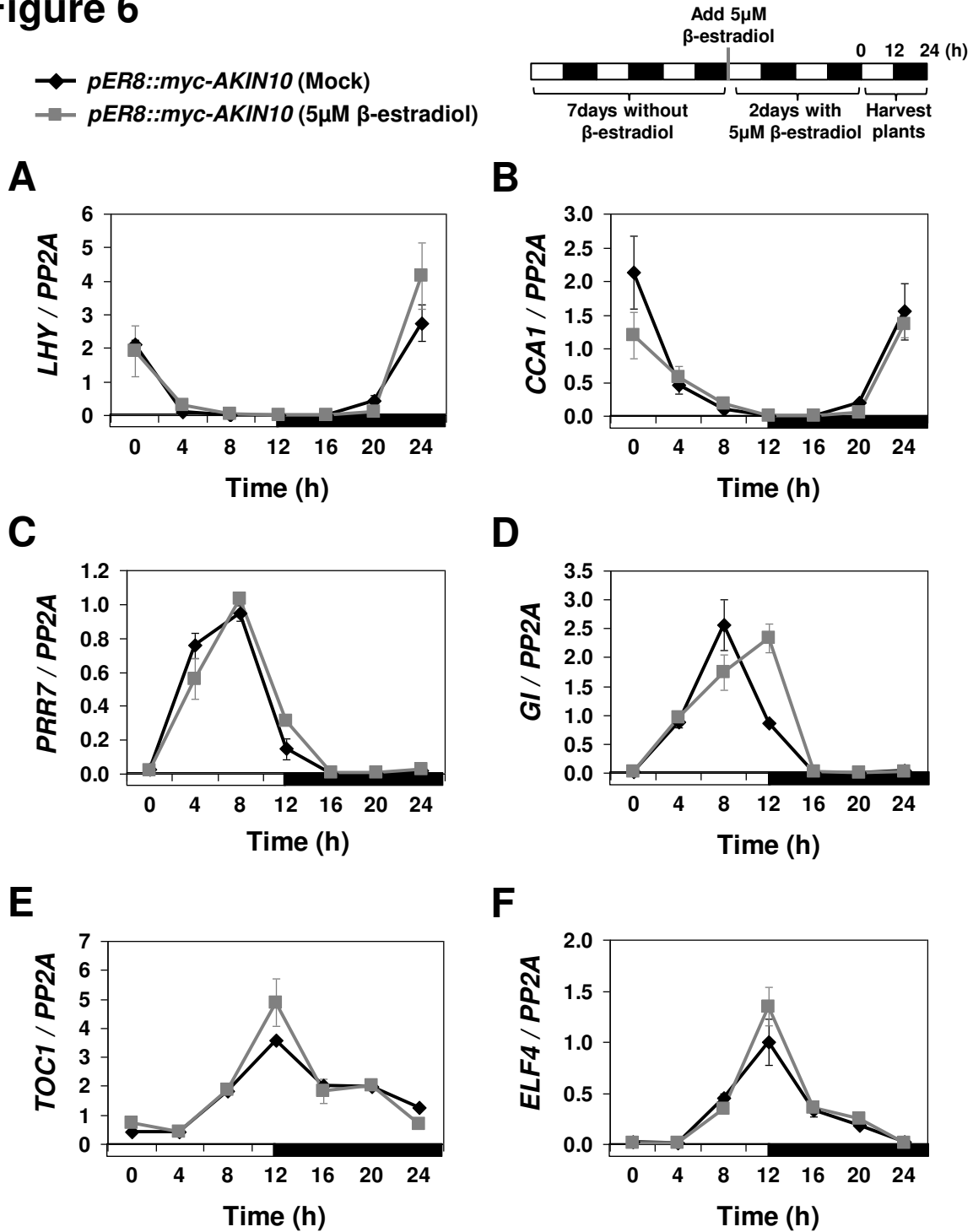


Figure 7

◆ *pER8::myc-AKIN10* (Mock)
■ *pER8::myc-AKIN10* (5μM β-estradiol)

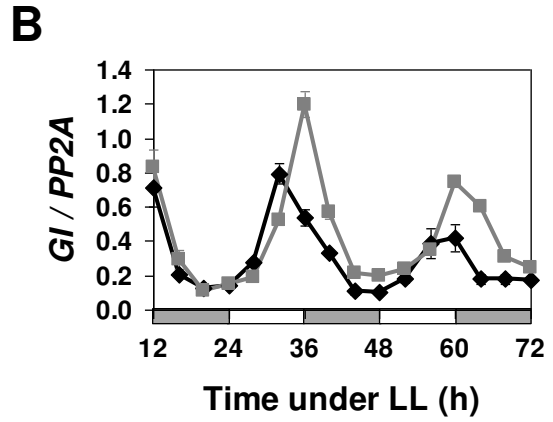
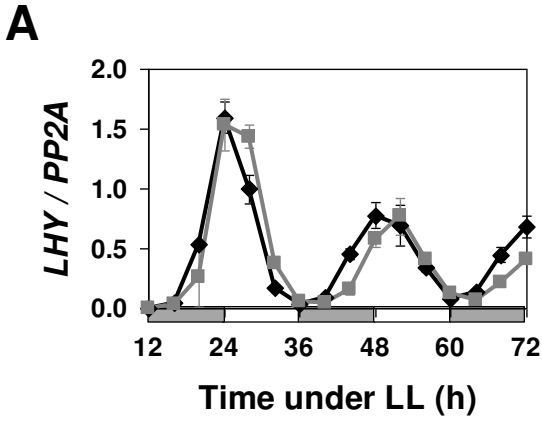
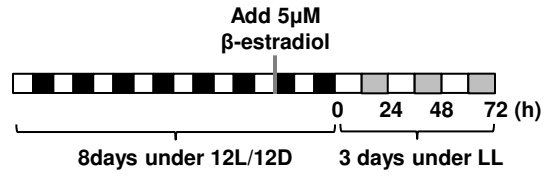
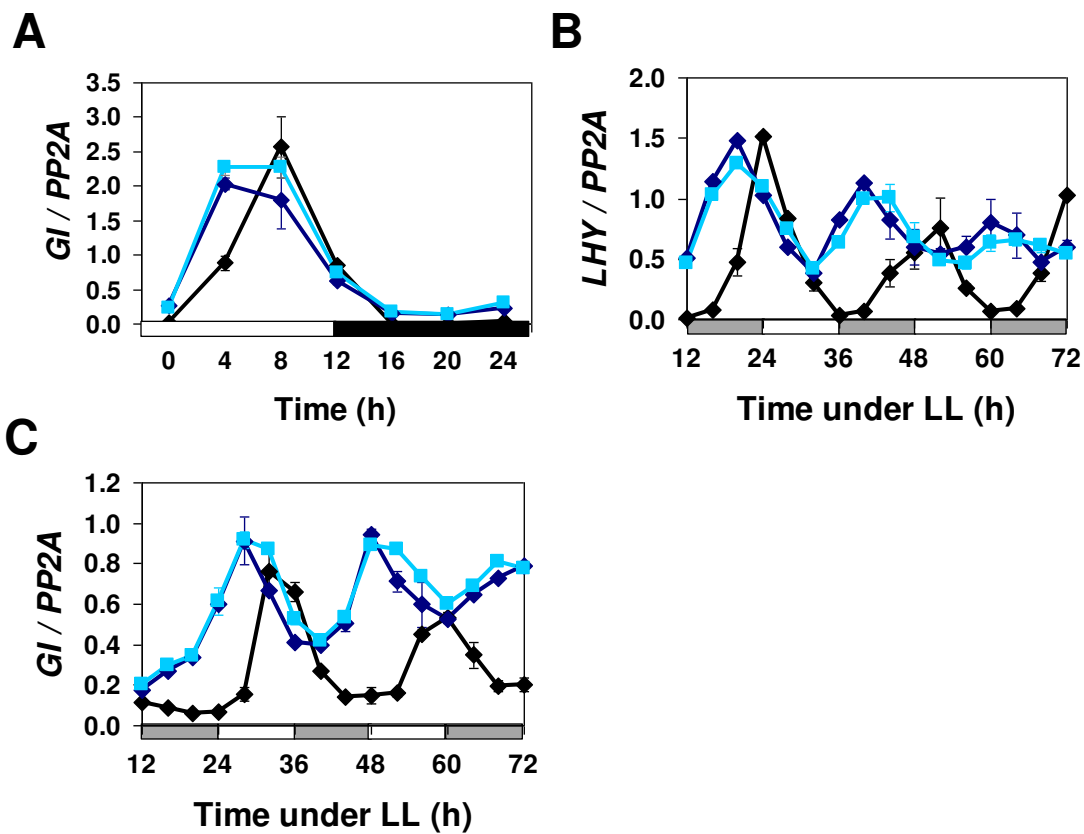


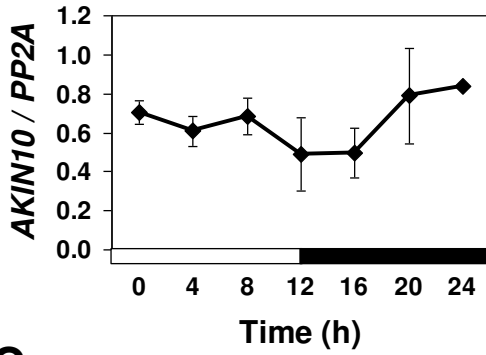
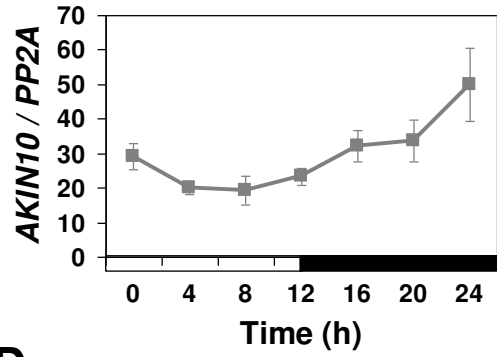
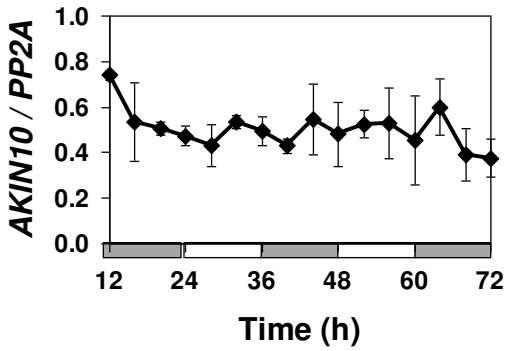
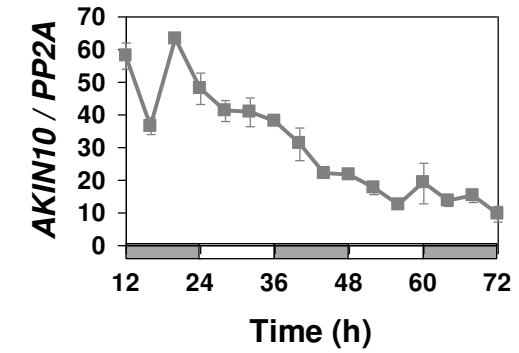
Figure 8

- ◆ *pER8::myc-AKIN10* (Mock)
- ◆ *tic-2 pER8::myc-AKIN10* (Mock)
- ◆ *tic-2 pER8::myc-AKIN10* (5 μ M β -estradiol)



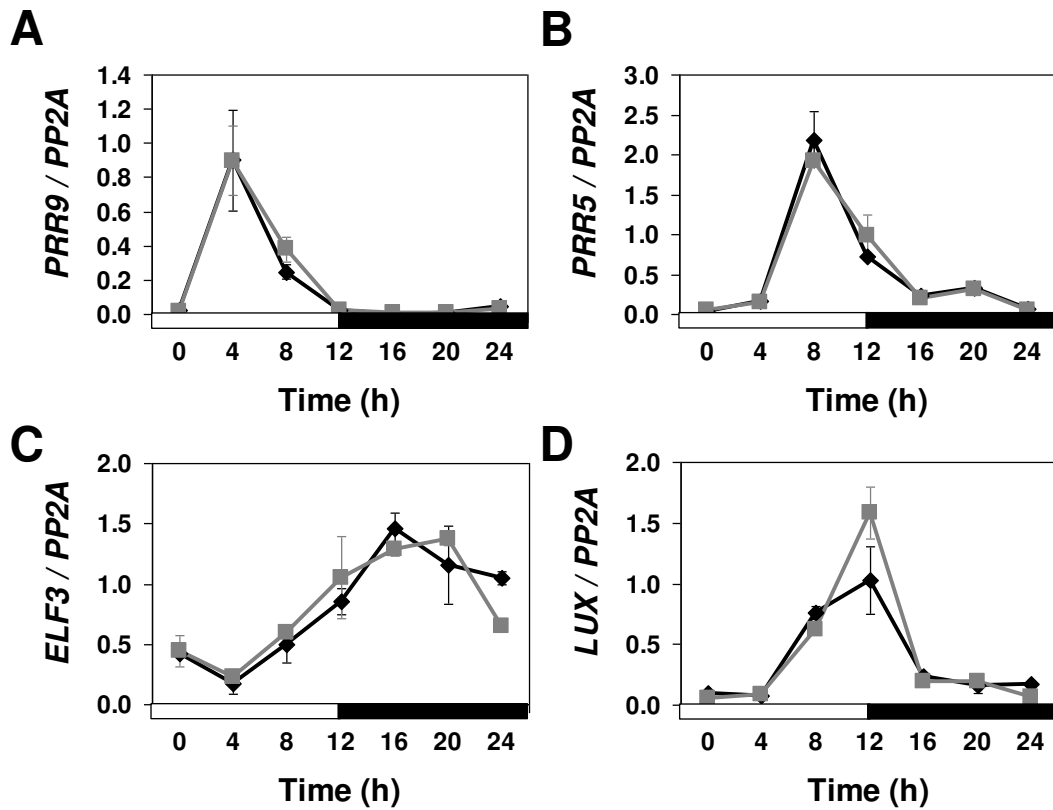
Supplement Figure 1

◆ *pER8::myc-AKIN10* (Mock)
■ *pER8::myc-AKIN10* (5 μ M β -estradiol)

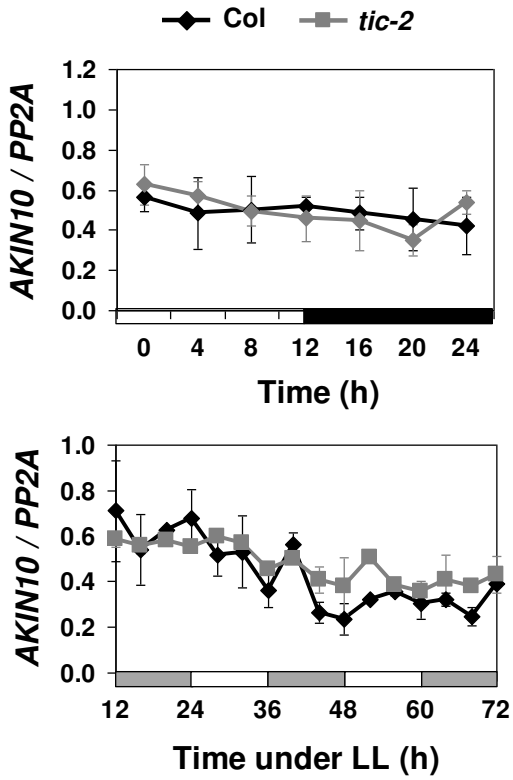
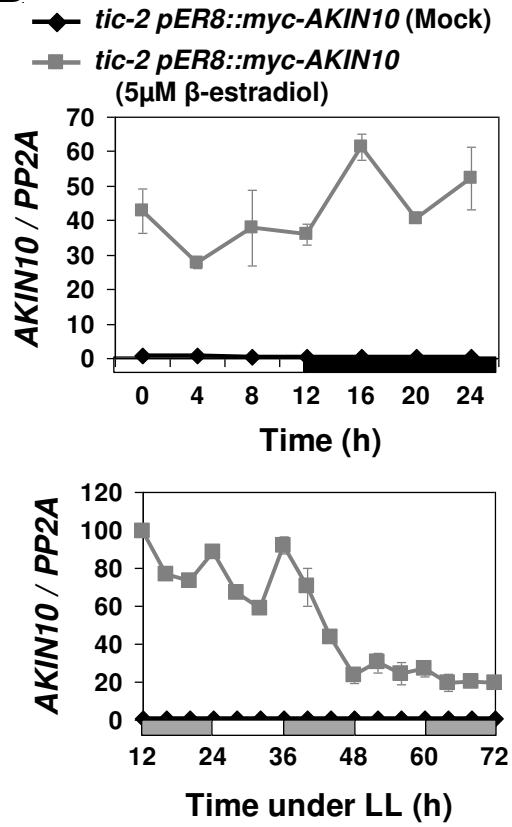
A**B****C****D**

Supplement Figure 2

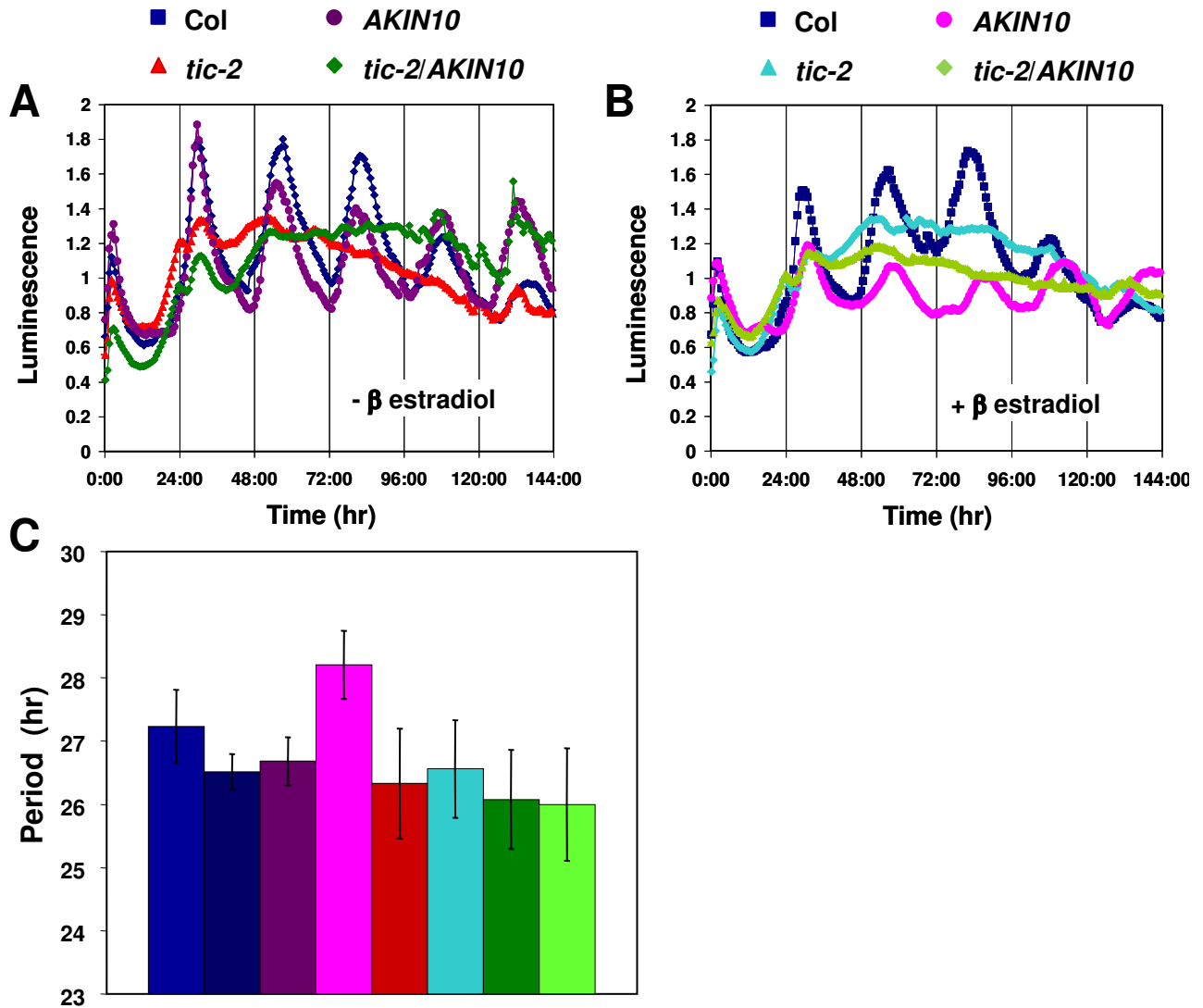
◆ *pER8::myc-AKIN10* (Mock) ■ *pER8::myc-AKIN10* (5 μ M β -estradiol)



Supplement Figure 3

A**B**

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	TCTGTGTCTGACGAGGGTTCGAATT
CCA1 RP2	ACTTTGCGGCAATACCTCTCTGG
LHY LP2	CAACAGCAACAACAATGCAACTAC
LHY RP2	AGAGAGCCTGAAACGCTATACGA
PRR7 LP	TGAAAGTTGGAAAAGGACCA
PRR7 RP	GTTCCACGTGCATTAGCTCT
PRR9 LP	GCACAGAGAAACCAAAGGAA
PRR9 RP	CTTTCCTCGAGGACGTTGT
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