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

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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.

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 (Bujdosó & 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 ARRhythMO (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, Kolmøs et al., 2011, Kolmøs 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.* 2013).

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 *SENSENCE-ASSOCIATED PROTEIN 5* (*SENP5*) 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 – 192 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), 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, Bhlerao 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-
estriadiol 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
myc-AKIN10 (Figure 8A). These results suggest that TIC is necessary for the action of AKIN10 on clock periodicity.

Such a genetic interaction between AKIN10 and TIC was further observed under free-running conditions. As already reported (Ding et al., 2007), we confirmed that tie-2 mutants display under LL a short period for both the morning and evening clock genes, LHY and GI, respectively (Figure 8B, 8C). myc-AKIN10 overexpression no longer lengthened circadian period in the tie-2 background (Figure 8B, 8C). In addition, we evaluated clock periodicity with a luciferase reporter in tie-2 pER8::myc-AKIN10 CCA1::LUC plants under free running conditions after induction with β-estradiol. Different from the longer period in pER8::myc-AKIN10 after the induction of AKIN10, the period length in tie-2 pER8::myc-AKIN10 seedlings was not increased even when AKIN10 was over expressed after induction (Supplemental figure 4A-C). These data collectively indicate that tie is genetically epistatic to AKIN10 overexpression for regulating the circadian periodicity.
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/m²/s (red) and ~2 μE/m²/s (blue)) used under free-running conditions, whereas these plants were entrained under higher intensity of white light (~75 μE/m²/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
will be worth to define if AKIN10 is an evolutionarily conserved zeitgeber within eukaryotic clocks, which serves conserved energy signaling using a same type of kinases of diverse organisms.
Accession Numbers

Sequence data from this article can be found in TAIR databases under the following accession numbers: \textit{AKINJ0} (AT3G01090), \textit{SEN5} (AT3G15450), \textit{DIN6} (AT3G47340), \textit{LHY} (AT1G01060), \textit{CCA1} (AT2G46830), \textit{PRR7} (AT5G02810), \textit{GI} (AT1G22770), \textit{TOC1} (AT5G61380), \textit{ELF4} (AT2G40080), \textit{PRR5} (AT5G24470), \textit{ELF3} (AT2G25930), \textit{LUX} (AT3G46640), \textit{PP2A} (AT1G13320).
Acknowledgements

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Figure legends

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

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

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

Col and *pER8::myc-AKIN10* plants harboring *GI::LUC* construct were entrained under 12L/12D conditions for 8 days, and transferred into constant light conditions. β-estradiol was added to plants 36 h before releasing into free-running conditions. (A) Effect on period length by AKNI10 gene expression induction. Error bars indicate standard error. (B) Normalized bioluminescence of *GI::LUC* under constant R+B conditions after β-estradiol induction. (C) Period versus relative amplitude error (RAE) of individual wild type and *pER8::myc-AKIN10* plants treated with β-estradiol.
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
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 placed 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 placed 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 8 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 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.
Supplement Table 1. Primers
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Figure 2

A. Bar graph showing the effect of different treatments on the period of Col and pER8::myc-AKIN10 lines. The treatments include control, 0.05% EtOH, and 5μM β-estradiol. The bars indicate the mean ± standard error of the normalized luminescence.

B. Line graph showing the normalized luminescence over time for Col and pER8::AKIN10-myc lines.

C. Scatter plot showing the relationship between period (h) and RAE for Col and pER8::AKIN10-myc lines.

Legend:
- Col
- pER8::myc-AKIN10
- pER8::AKIN10-myc
Figure 3

A

B

C

D

E

F

![Graphs showing period (h) and RAE for different conditions](image)

- **A**: Col and pER8::myc-AKIN10
  - Bc
  - Control
  - 0.05% ETOH
  - 5µM estradiol

- **B**: Col and pER8::myc-AKIN10
  - Bc
  - Period (h)
  - RAE

- **C**: Col and pER8::myc-AKIN10
  - Rc
  - Control
  - 0.05% ETOH
  - 5µM estradiol

- **D**: Col and pER8::myc-AKIN10
  - Rc
  - Period (h)
  - RAE

- **E**: Col and pER8::myc-AKIN10
  - Dc
  - Control
  - 0.05% ETOH
  - 5µM estradiol

- **F**: Col and pER8::myc-AKIN10
  - Dc
  - Period (h)
  - RAE
**Figure 4**

**Diagram Description:**
- **Add 5 μM β-estradiol**
- **Harvest plants**

**Table:**

<table>
<thead>
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<th>Dc</th>
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**Legend:**
- Bc: Black Carbon
- Rc: Red Carbon
- Dc: Dark Carbon

**Timeline:**
- 12L/12D: 8 days
- Constant light or dark 2 days
Figure 5

No sugar

Sucrose

Glucose

Period (h)

Col

pER8::myc-AKIN10
Add 5µM β-estradiol

7 days without β-estradiol
2 days with 5µM β-estradiol
Harvest plants

Figure 6

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

A

LHY / PP2A

B

CCA1 / PP2A

C

PRR7 / PP2A

D

GI / PP2A

E

TOC1 / PP2A

F

ELF4 / PP2A

0 4 8 12 16 20 24

Time (h)
Figure 7

A) 

- \textcolor{red}{pER8::myc-AKIN10 (Mock)}
- \textcolor{gray}{pER8::myc-AKIN10 (5\mu M \beta\text{-estradiol})}

\begin{align*}
LHY / PP2A \\
\text{Time under LL (h)}
\end{align*}

B) 

\begin{align*}
GI / PP2A \\
\text{Time under LL (h)}
\end{align*}
Figure 8

- pER8::myc-AKIN10 (Mock)
- tic-2 pER8::myc-AKIN10 (Mock)
- tic-2 pER8::myc-AKIN10 (5µM β-estradiol)

A

B

C
Supplement Figure 3

A

B

tic-2 pER8::myc-AKIN10 (Mock)
tic-2 pER8::myc-AKIN10
(5µM β-estradiol)

AKIN10 / PP2A

Time (h)

Time under LL (h)

AKIN10 / PP2A

Time (h)

Time under LL (h)
### GATEWAY cloning primer

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### qRT-PCR primer

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