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Sucrose and Ethylene Signaling Interact to Modulate the Circadian Clock[1[CC-BY]]

Michael J. Haydon,[a,b,c,2] Olga Mielczarek,[b,3] Alexander Frank,[b] Ángela Román,[c] and Alex A.R. Webb[b]

[a]School of BioSciences, The University of Melbourne, Parkville 3010, Australia
[b]Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, United Kingdom
[c]Department of Biology, University of York, York YO10 5DD, United Kingdom

ORCID IDs: 0000-0003-2486-9387 (M.J.H.); 0000-0001-9806-2186 (O.M.); 0000-0003-3457-999X (A.R.); 0000-0003-0261-4375 (A.A.R.W.).

Circadian clocks drive rhythmic physiology and metabolism to optimize plant growth and performance under daily environmental fluctuations caused by the rotation of the planet. Photosynthesis is a key metabolic process that must be appropriately timed to the light-dark cycle. The circadian clock contributes to the regulation of photosynthesis, and in turn the daily accumulation of sugars from photosynthesis also feeds back to regulate the circadian oscillator. We have previously shown that *GIGANTEA* (*GI*) is required to sustain Suc-dependent circadian rhythms in darkness. The mechanism by which Suc affects the circadian oscillator in a *GI*-dependent manner was unknown. Here, we identify that Suc sustains rhythms in the dark by stabilizing GI protein, dependent on the F-box protein *ZEITLUPE*, and implicate *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*), a negative regulator of ethylene signaling. Our identification of a role for CTR1 in the response to Suc prompted a reinvestigation of the effects of ethylene on the circadian oscillator. We demonstrate that ethylene shortens the circadian period, conditional on the effects of Suc and requiring *GI*. These findings reveal that Suc affects the stability of circadian oscillator proteins and can mask the effects of ethylene on the circadian system, identifying novel molecular pathways for input of sugar to the Arabidopsis (*Arabidopsis thaliana*) circadian network.

Circadian clocks have evolved to allow organisms to anticipate the predictable daily and seasonal variation in their environment. They regulate rhythmic physiology and metabolism and gate responses to occur at specific times of day. The current model for plant circadian clocks is a molecular oscillator comprised of multiple, interlocking regulatory feedback loops of transcriptional regulators with posttranslational control (Hsu and Harmer, 2014). The phase and pace of the oscillator is set by environmental cues such as light and temperature, and the oscillator components directly regulate key processes such as growth, flowering time, water balance, metabolism, and nutrient acquisition (Sanchez and Kay, 2016). There are also multiple examples of feedback from these rhythmic physiological and metabolic outputs to the circadian oscillator (Haydon et al., 2015).

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[2] Address correspondence to m.haydon@unimelb.edu.au.
[3] Current address: Babraham Institute, University of Cambridge, Cambridge CB22 3AT, UK.
The authors responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) are: Michael J. Haydon (m.haydon@unimelb.edu.au) and Alex A.R. Webb (arrw2@cam.ac.uk).
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The core circadian oscillator is principally defined by the sequential expression of a series of transcriptional repressors across the 24-h cycle. Two myb-like transcription factor genes, *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), are expressed at dawn (Alabadí et al., 2001), followed by sequential expression of *PSEUDO-RESPONSE REGULATOR9* (*PRR9*), *PRR7*, *PRR5* (Nakamichi et al., 2010), and the evening-active *TIMING OF CAB2 1* (Dixon et al., 2011; Gendron et al., 2012; Huang et al., 2012; Pokhilko et al., 2012). These PRR proteins act in a series of negative feedback loops to repress *CCA1* and *LHY*. A third myb-like transcription factor, REVIELLE8, acts in the afternoon to activate evening-expressed genes (Farinas and Mas, 2011; Rawat et al., 2011; Hsu et al., 2013), including *TOC1* and genes encoding components of the evening complex (EC) comprised of EARLY FLOWERING3 (ELF3), ELF4, and LUX ARRHYTHMO. The EC, in turn, represses morning-expressed genes during the night (Nusinow et al., 2011; Herrero et al., 2012). There is also posttranslational control of the core oscillator; TOC1 and PRR5 are degradation targets of ZEITLUPE (ZTL), an F-box protein and putative blue light photoreceptor (Más et al., 2003; Kiba et al., 2007). GIGANTEA (GI), a nucleocytoplasmic protein recently shown to have cochaperone activity to promote ZTL maturation (Cha et al., 2017), interacts with ZTL in the light and sequesters ZTL from its targets in the cytosol to contribute to amplitude of TOC1 rhythms (Kim et al., 2007).

GI also acts independently of ZTL in distinct pathways contributing to circadian rhythms, photoperiodic flowering, and light signaling (Fowler et al., 1999;

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Park et al., 1999; Huq et al., 2000; Mizoguchi et al., 2005; Martin-Tryon et al., 2007). GI physically interacts with proteins acting in these pathways, including the ZTL-related FLAVIN-BINDING, KELCH REPEAT, F-BOX 1, CONSTITUTIVE PHOTOMORPHOGENIC1, ELF3, and SPINDLY, an N-acetyl glucosamine transferase (Tseng et al., 2004; Sawa et al., 2007; Yu et al., 2008). The GI-ZTL interaction retains GI in the cytosol to inhibit nuclear functions of GI and sequester GI from other protein complexes (Kim et al., 2013). Thus, the spatiotemporal organization of these interactions is thought to permit GI to contribute these various pathways.

There is circadian regulation of photosynthesis in Arabidopsis (*Arabidopsis thaliana*; Harmer et al., 2000; Dodd et al., 2005; Noordally et al., 2013), and rhythmic photosynthesis feeds back to regulate the circadian oscillator (Haydon et al., 2013). Inhibition of photosynthesis lengthens circadian period through derepression of *PRR7* to adjust the phase of morning-expressed *CCA1* and contribute to entrainment of the clock (Haydon et al., 2013). Similarly, circadian period is lengthened in dim light conditions when photosynthetic activity is low. In these conditions, exogenous Suc shortens circadian period in the wild type, but not in *prr7-11* or *cca1-11* mutants (Haydon et al., 2013). This effect of exogenous Suc on circadian period is not observed in bright light, presumably because these sugar-responsive pathways are saturated by active photosynthesis.

Exogenous sugars can also sustain circadian rhythms in the absence of light (Dalchau et al., 2011; Haydon et al., 2013). This response to sugar requires GI (Dalchau et al., 2011), acting in a molecular pathway that appears to be distinct from that controlling *PRR7*-dependent phase adjustment, since the long circadian period in continuous dim light is shortened by Suc in *gi* mutants to a similar degree as in wild type (Haydon et al., 2013). Thus, there are multiple pathways for sugar input to the circadian oscillator, but these pathways have not yet been defined.

In multiple efforts to identify sugar-sensing pathways in Arabidopsis, a number of sugar-insensitive mutants have been isolated from genetic screens (Gibson, 2005). These mutants are typically resistant to growth inhibition and/or catabolite repression by high exogenous concentrations of Glc or Suc. Among these is *Glc insensitive 2* (*gin2*), a mutant in *HEXOKINASE1* (*HXK1*), which is proposed to function in sugar signaling in addition to its metabolic role in glycolysis (Moore et al., 2003; Cho et al., 2006). Other *gin* mutants have been identified that are affected in hormone biosynthesis or signaling genes, such as *ABSCISIC ACID DEFICIENT2* (*ABA2/GIN1*), *ABA3/GIN5*, and *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1/GIN4*), encoding a negative regulator of ethylene responses (Gibson et al., 2001; Xiong et al., 2001; González-Guzmán et al., 2002). Moreover, *Glc oversensitive* phenotypes have been described in *ethylene insensitive2* (*ein2*), *ein3*, *ein4*, and *ethylene receptor1* (*etr1*) mutants (Zhou et al., 1998; Gibson et al., 2001; Cheng et al., 2002; Yanagisawa et al., 2003). This suggests crosstalk between phytohormones and sugar-dependent signaling.

Here, we have investigated the role of GI in sustaining sugar-dependent circadian rhythms in the dark. We have discovered that mutants in *ZTL* or *CTR1* can sustain these rhythms in the absence of sugar in a GI-dependent manner. We show that GI protein is stabilized by Suc in the night by a mechanism that requires ZTL and provide evidence that ethylene can affect the circadian oscillator. Genetic analyses suggest that ZTL and CTR1 act in distinct pathways converging on GI to modulate a response of the clock to sugars.

RESULTS

Unique Role of GIGANTEA in Modulation of the Clock by Sugars

Circadian rhythms of transcriptional luciferase reporters of morning-expressed clock genes and outputs are absent or severely damped in continuous dark but can be sustained when Suc is added to growth media (Dalchau et al., 2011; Haydon et al., 2013). We previously used a mathematical modeling approach to explain this behavior and identified GI as an important circadian clock component mediating this response of the clock to Suc (Dalchau et al., 2011). The modeling predicted that reduced GI function should render the clock insensitive to Suc in continuous dark, which was confirmed by monitoring activity of the circadian output reporter *CHOROPHYLL A/B-BINDING PROTEIN2* (*CAB2*)*p:LUC* in *gi-11* null mutants (Dalchau et al., 2011). This effect is not specific to Suc, with *gi-11* mutants being similarly insensitive to Glc or Fru (Supplemental Fig. S1). In media containing a nonmetabolizable Glc analog, 3-*O*-methylglucose, or mannitol, reporter activity was similar to sugar-free control media in both Ws wild-type and *gi-11* seedlings (Supplemental Fig. S1).

To further investigate the *GI*-dependent effects of Suc on the core circadian oscillator, we introduced reporters for the clock genes *CCA1p:LUC* and *TOC1p:LUC* into *gi-2*, an independent allele in the Col-0 background, by crossing. Similar to the *CAB2* reporter, the morning-expressed clock reporter *CCA1p:LUC* was also insensitive to Suc in *gi-2* mutants (Fig. 1A). Since the low amplitude of CAB2p:LUC and CCA1p:LUC rhythms could potentially be explained by low availability of ATP in darkness for the luciferase enzyme, we also checked transcript levels in a light-dark cycle and the first true circadian cycle in continuous dark in Ws and *gi-11* seedlings and for 48 h in continuous dark in Col-0 and *gi-2* seedlings growing in media with or without Suc (Fig. 1B; Supplemental Fig. S2). Consistent with the luciferase reporter experiments, *CCA1* transcript amplitude was damped in wild type after 24 h in continuous dark but was completely abolished in *gi-11* and *gi-2* mutants (Fig. 1B; Supplemental Fig. S2). This suggests that the reporter phenotypes are not due to limiting ATP for the luciferase and that the reporter activity reflects the endogenous transcript levels.

Unlike *CCA1p:LUC* and *CAB2p:LUC*, reporters of evening-expressed clock genes are rhythmic in seedlings growing in continuous dark in media without added Suc

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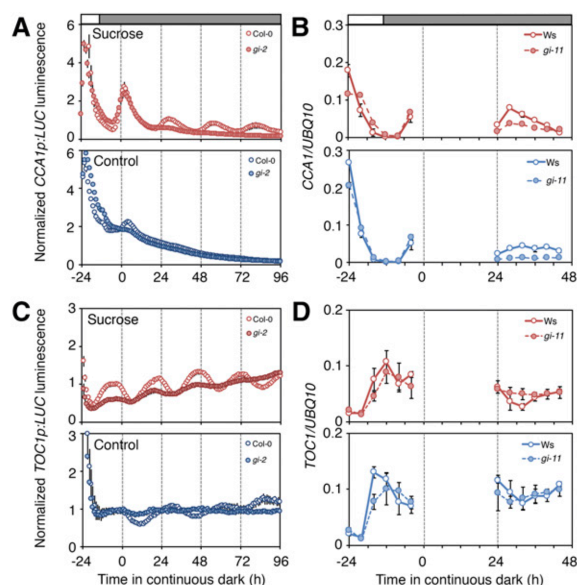

Figure 1. Luciferase reporter activity and transcript levels of *CCA1* and *TOC1* in *gi* mutants. A, Normalized CCA1p:LUC activity in Col-0 and *gi-2* and B, *CCA1* transcript levels in Ws and *gi-11* seedlings growing in media containing 90 mM Suc or control media without Suc. C, Normalized TOC1p:LUC activity in Col-0 and *gi-2* and D, *TOC1* transcript levels in Ws and *gi-11* seedlings growing in media containing 90 mM Suc or control media without Suc. Luciferase values are means ± SE, $n = 4$. Transcript levels are mean ± SD, $n = 3$. Light and dark periods are indicated by white and gray boxes, respectively.

(Haydon et al., 2013). Nevertheless, similar to the effects on CCA1p:LUC, TOC1p:LUC rhythms were not detected in *gi-2* mutants, and oscillation of *TOC1* transcript was absent in *gi-2* and *gi-11* in continuous dark with Suc (Fig. 1, C and D; Supplemental Fig. S2). In media without Suc, TOC1p:LUC rhythms were not detected in *gi-2*, consistent with *TOC1* transcript levels in *gi* mutants (Fig. 1, C and D; Supplemental Fig. S2). These experiments confirm the profound effect *gi* mutations have on sugar-dependent circadian rhythms in the dark in Arabidopsis.

Since *CCA1* and *PRR7* are required for the circadian oscillator to respond to sugars in the light (Haydon et al., 2013), we investigated whether other circadian clock genes in addition to *GI* are required for the response of the circadian oscillator to Suc in the dark. We measured rhythms of CAB2p:LUC or CCA1p:LUC in a collection of mutants in the core circadian oscillator in continuous dark with or without Suc (Fig. 2; Supplemental Fig. S3). To estimate the robustness of the oscillation with or without Suc, we measured the relative amplitude error (RAE) by fitting to a cosine wave using fast Fourier transform nonlinear least square analysis. A value of 0 represents a perfect fit, increasing to a maximum of 1. As expected, RAE values of reporter rhythms in *gi* mutants were close to 1 in media with or without Suc, consistent with loss of reporter rhythms in these mutants (Fig. 2A). By contrast, *cca1-11*, *toc1-1*, *toc1-2*, *prr9-10*, *prr7-11*, or *prr5-1* showed similar responsiveness to Suc as

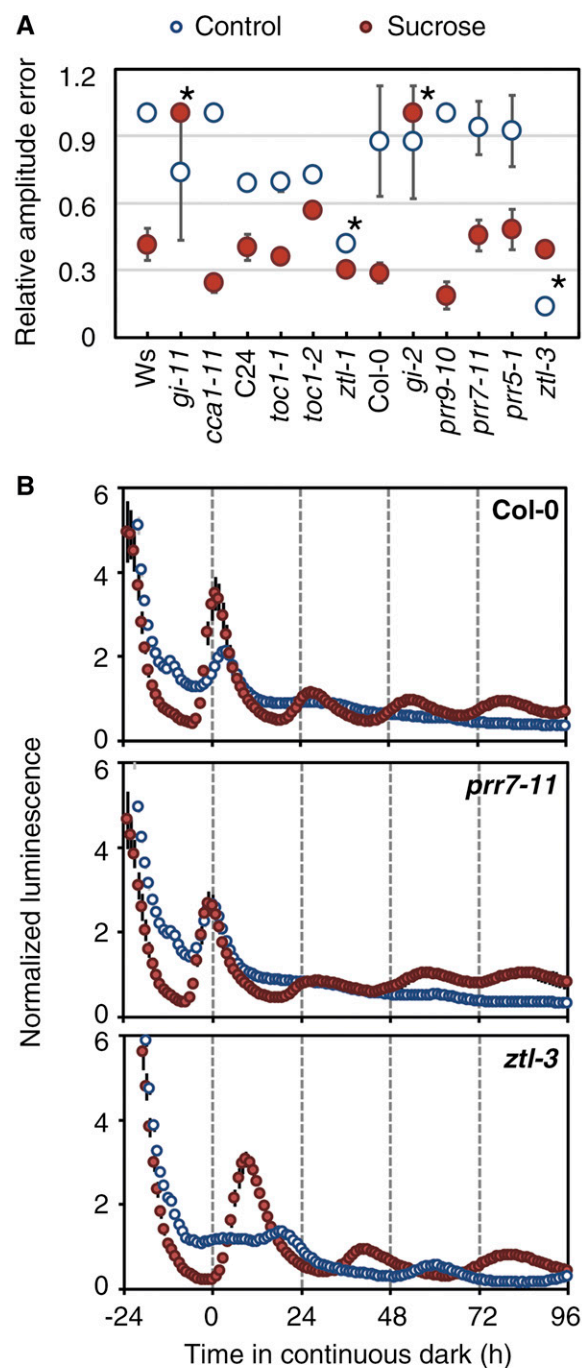

Figure 2. Robust circadian rhythms in *ztl* in continuous dark. A, Relative amplitude error of CCA1p:LUC rhythms in seedlings growing in continuous dark in media containing 90 mM Suc or media without Suc. Values are mean ± SD, $n = 4$. Asterisks indicate statistical difference from wild type by *t* test with Bonferroni correction for multiple comparisons ($P < 0.05$). B, Normalized CCA1p:LUC activity in Col-0, *prr7-11*, and *ztl-3* seedlings grown as in A (means ± SE, $n = 4$). Luciferase reporter activity plots for Ws, *gi-11*, *cca1-11*, C24, *toc1-1*, *toc1-2*, *ztl-1*, Col-0, *prr9-10*, and *prr5-1* are shown in Supplemental Figure S2.

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wild-type controls. RAE values in these mutants were not statistically significantly different from wild type in either condition; mean values were <0.6 in the presence of Suc and approaching 1.0 when Suc was absent from media (Fig. 2A). Thus, none of the clock mutants tested had a phenotype similar to *gi*, consistent with our previous mathematical modeling that suggested *GI* is the principal circadian clock gene associated with responsiveness of the oscillator to Suc in the dark (Dalchau et al., 2011). However, we observed a novel phenotype in *ztl* mutants in these experiments whereby robust, long-period reporter rhythms were detected in both *ztl-1* and *ztl-3* mutants in the absence of Suc (Fig. 2, A and B). ZTL is an evening active F-box protein that physically interacts with GI to regulate *TOC1* (Kim et al., 2007), suggesting a possible role in GI-dependent response to sugars. These robust rhythms of CAB2p:LUC and CCA1p:LUC in the absence of added Suc in these mutants also confirm that the damped circadian rhythms in continuous dark are not due to limited energy availability, and suggest that specific signaling events lead to repression of the circadian oscillator in these conditions.

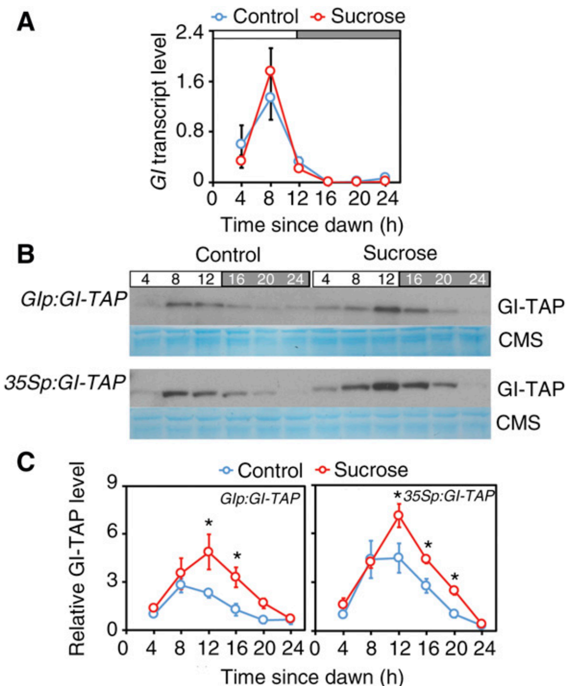

Figure 3. Suc stabilizes GI after dusk. A, *GI* transcript level, normalized to *PP2α* and *IPP2*, in Col-0 seedlings growing in a light-dark cycle in media containing 90 mM Suc or control media containing 90 mM mannitol (means ± SD, $n = 3$). No significant differences at any time point between treatments by *t* test. Light and dark periods are indicated by white and gray boxes, respectively. B, Immunoblots of GI-TAP in *GIp:GI-TAP* and *35Sp:GI-TAP* seedlings growing in a light-dark cycle in media containing 90 mM Suc or control media containing 90 mM mannitol. Coomassie-stained bands (CMS) are shown as a loading control. C, GI-TAP levels, relative to loading control, determined from immunoblots of triplicate experiments of seedlings grown as in B (means ± SE, $n = 3$). Asterisks indicate statistical differences between treatments determined by *t* test ($P < 0.05$).

Posttranscriptional Stabilization of GI by Suc Requires ZTL

Having established a unique role for GI in Suc-dependent effects on the circadian oscillator in continuous dark, we sought to better understand how GI modulates responsiveness of the clock to sugars in light-dark cycles. We first investigated regulation of *GI* transcript by Suc. We measured *GI* transcript levels by qRT-PCR over a 24-h light-dark cycle in wild-type seedlings growing on media supplemented with Suc or mannitol (Fig. 3A) and found no significant difference between treatments at any time point. This suggests that input of sugar to the clock through GI does not act through transcriptional regulation of *GI*.

To investigate whether there is posttranscriptional regulation of GI by sugars, we measured GI protein levels by western blot in *GIp:GI-TAP* and *35Sp:GI-TAP* transgenic lines (David et al., 2006). Similar to wild type, *GI* transcript levels in these transgenic lines were not affected by the presence of Suc in the growth media (Supplemental Fig. S4). By contrast, we detected a clear, reproducible increase in GI-TAP protein in seedlings grown on media supplemented with Suc compared to control conditions in both the *GIp:GI-TAP* and *35Sp:GI-TAP* lines (Fig. 3, B and C). The difference was most apparent around dusk (ZT12) and during the night. We also measured GI-TAP rhythms in continuous dark and detected robust rhythms of GI-TAP in the presence of Suc compared to heavily damped amplitude in media without Suc (Supplemental Fig. S5). This is despite a similar amplitude of *GI* transcript oscillation after 24 h in the dark either with or without Suc, albeit with the expected delayed peak expression in media without Suc (Supplemental Fig. S5). This is consistent with the proposed functional requirement of GI in sustaining circadian rhythms in these conditions. To further rule out an effect of *GI* transcript levels in sustaining circadian rhythms in continuous dark, we introduced *CCA1p:LUC* into *35Sp:GI-TAP* by crossing and found no difference in reporter rhythms from control seedlings with or without Suc (Supplemental Fig. S6). Thus, the observation of an effect of Suc on GI-TAP protein levels in both the *GIp:GI-TAP* and *35Sp:GI-TAP* lines, together with the absence of an effect of exogenous Suc on *GI* transcript level in these conditions or an effect of *GI* overexpression on the response of the oscillator to Suc, indicates that the effect of Suc to increase total GI protein level is acting posttranscriptionally.

Since ZTL physically interacts with GI to control protein stability (Kim et al., 2013) and *ztl* mutants could sustain circadian rhythms in the absence of Suc in the dark (Fig. 2; Supplemental Fig. S3), we crossed *35Sp:GI-TAP* into *ztl-1* to measure GI-TAP protein levels with and without Suc. Our choice of *35Sp:GI-TAP* allowed us to specifically examine posttranscriptional effects of Suc on GI-TAP expression in *ztl-1*. Rhythms of GI-TAP protein levels under the control of the constitutive promoter were absent in *ztl-1*, with dramatically reduced peak

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levels of GI-TAP. This is consistent with reduced amplitude oscillation of GI-TAP in *ztl-103* harboring *GIp:GI-TAP* reported in a previous study and a critical role for ZTL in rhythmic posttranscriptional regulation of GI (Kim et al., 2007). However, in contrast to the effect on GI-TAP in control lines, we did not detect increased GI-TAP levels in *ztl-1* when Suc was added to media (Fig. 4). This indicates that ZTL is required for the stabilization of GI protein by exogenous Suc.

Ethylene Signaling Contributes to Responsiveness of the Clock to Suc

To gain further insight into potential signaling pathways required for sugar-dependent circadian oscillations, we introduced *CCA1p:LUC* into a selection of sugar-insensitive mutants and measured reporter activity in continuous dark with and without Suc (Fig. 5; Supplemental Fig. S7). Mutants in *HXK1*, *ABA2*, and *ABA3* were similar to wild-type controls: arrhythmic in the absence of Suc (RAE approaching 1.0) and rhythmic with Suc (RAE < 0.6). By contrast, similar to the phenotype observed in *ztl* mutants, robust CCA1p:LUC rhythms were detected in *ctr1-12* in the absence of Suc (Fig. 5, A and B; period = 26.12 ± 0.57 h).

CTR1 is a negative regulator of ethylene signaling that has also been identified in screens for sugar-insensitive mutants (Kieber et al., 1993; Gibson et al., 2001). There are circadian rhythms of ethylene emission, but a role for ethylene signaling in circadian time-keeping has not been described (Thain et al., 2004). Mutants in *XAP5 CIRCADIAN TIMEKEEPER* (*XCT*) have a short circadian period (Martin-Tryon and Harmer, 2008) and *xct-2* phenocopies aspects of *ctr1-3* with respect to ethylene responses (Ellison et al., 2011). This connection between ethylene signaling and the circadian clock prompted us to measure circadian rhythms in *xct-2* mutants in continuous dark with and without Suc. *xct-2* harbors a *CCR2p:LUC* reporter, which behaves as morning-expressed reporters in continuous dark, requiring exogenous sugar supply to sustain circadian rhythms in wild-type seedlings (Haydon et al., 2013; Supplemental Fig. S7). Similar to *ctr1-12*, and in contrast to the wild-type control, robust reporter rhythms could be detected in *xct-2* in the absence of Suc (Fig. 5, A and C), suggesting CTR1 and XCT2 might contribute to a common pathway acting on the circadian clock.

To test whether ethylene directly contributes to maintenance of circadian rhythms in the dark, we measured *CCA1p:LUC* rhythms in continuous dark in the presence of the ethylene precursor, 1-aminocyclopropanecarboxylic acid (ACC). Similar to the effect of the *ctr1-12* mutant, addition of 10 μM ACC could sustain reporter rhythms in the absence of Suc (Fig. 5A; Supplemental Fig. S7). To investigate a broader role for ethylene in circadian time-keeping, we tested the effects of ACC on circadian rhythms in the light. In the presence of Suc, we found no effect of ACC on *TOC1p:LUC* activity rhythms (Fig. 6A), similar to previous reports (Thain et al., 2004; Hanano et al., 2006). However, in media without Suc, we observed a significantly shortened period of ~1.5 h in seedlings treated with 50 μM ACC in continuous light compared to untreated seedlings (Fig. 6, A and B). By contrast, no significant effect of ACC on period was detected in *gi-2* (Fig. 6, A and B), indicating that sensitivity of the oscillator to ACC requires GI.

Next, we introduced *CCA1p:LUC* into an *ein3* mutant. EIN3 is a transcription factor that acts downstream of CTR1 to promote ethylene responses (Chao et al., 1997). In continuous dark, both amplitude and period of rhythms in *ein3-1* were similar to wild-type controls with or without Suc (Fig. 5B; Supplemental Fig. S7; Col-0 26.48 ± 0.52 h, *ein3-1* 26.36 ± 0.66 h). In continuous light, circadian period of *CCA1p:LUC* in *ein3-1* was indistinguishable from wild type in the presence of Suc, consistent with a previous study (Fig. 6C; Thain et al.,

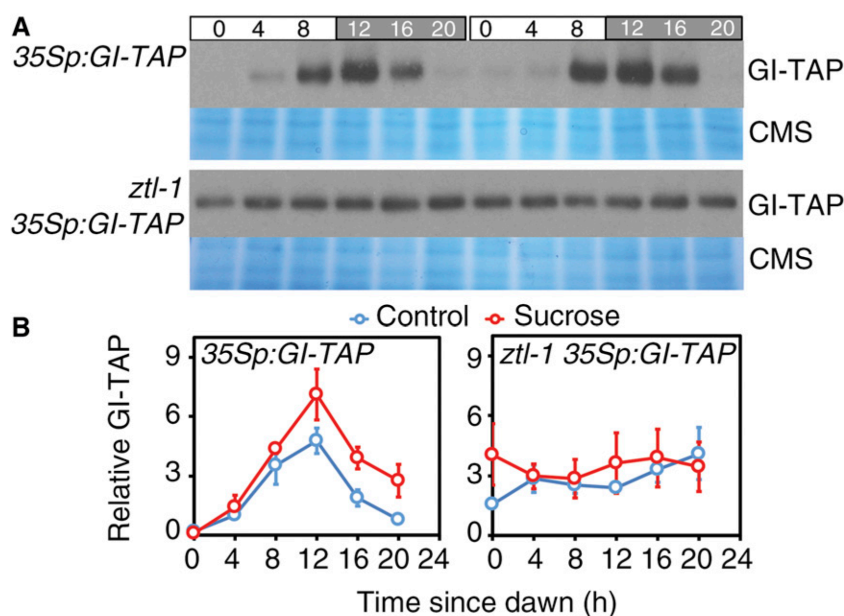

Figure 4. Stabilization of GI by Suc requires ZTL. A, Immunoblots of GI-TAP in *35Sp:GI-TAP* and *ztl-l 35Sp:GI-TAP* seedlings growing in a light-dark cycle in media containing 90 mM Suc or control media containing 90 mM mannitol. Coomassie-stained bands are shown as a loading control. B, GI-TAP levels, relative to loading control, determined from immunoblots of duplicate experiments of seedlings grown as in A (means ± SE, $n = 2$).

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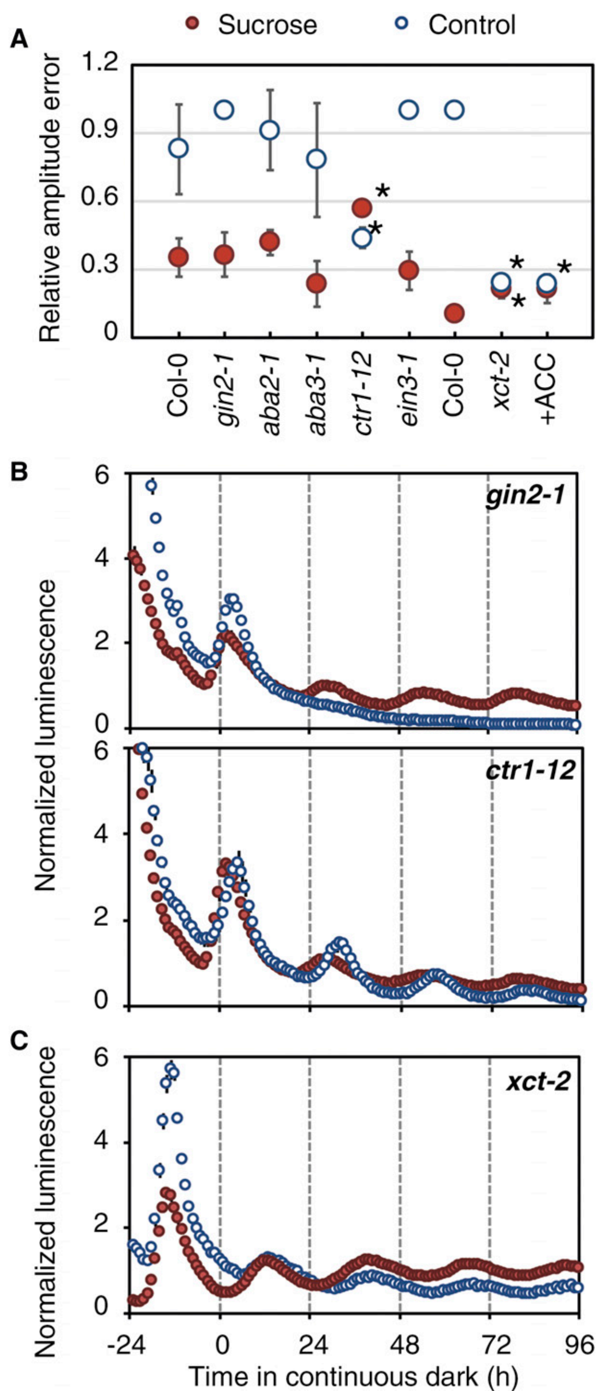

Figure 5. Robust circadian rhythms in *ctr1-1* in continuous dark. A, Relative amplitude error of CCA1p:LUC and CCR2p:LUC rhythms in seedlings growing in continuous dark in media containing 90 mM Suc or media without Suc. The reporter in *xct-2* is CCR2p:LUC, shown beside the Col-0 control. For +ACC, *CCA1p:LUC* seedlings were transferred to 10 μM ACC 2 d before release into continuous dark. Values are mean ± SD, $n = 4$. Asterisks indicate statistical difference from wild type by *t* test with Bonferroni correction for multiple comparisons ($P < 0.05$). B, Normalized CCA1p:LUC activity in *gin2-1* and *ctr1-12* seedlings grown as in A (means ± SE, $n = 4$). C, Normalized CCR2p:LUC activity in *xct-2* seedlings grown as in A (means ± SE, $n = 4$). Luciferase activity plots for Col-0, *aba2-1*, *aba3-1*, *ein3-1*, and ACC-treated Col-0 are shown in Supplemental Figure S5.

2004). However, without Suc in the media, the circadian period in *ein3-1* was significantly longer than wild type (Fig. 6C; Col-0 24.02 ± 0.99 h, *ein3-1* 25.44 ± 0.08 h; $P = 0.029$). These data suggest that exogenous Suc can mask the effects of ethylene signaling on the clock and that these effects require GI.

Sugar-Dependent Genetic Interactions between GI, ZTL, and CTR1

Since *ztl* and *ctr1* mutants were similarly able to sustain circadian rhythms without Suc in continuous dark, we sought to further investigate interactions between GI, ZTL, and CTR1 and establish whether ZTL and CTR1 contribute to sustaining sugar-dependent circadian rhythms by the same or distinct pathways. We generated pairwise combinations of double mutants containing *CCA1p:LUC* (Fig. 7). We first considered genetic interactions with respect to flowering time, since ZTL and GI both contribute to regulation of photoperiodic flowering (Park et al., 1999; Somers et al., 2000). In long-day conditions, *gi-2 ztl-3* was late flowering, indicating *gi-2* was epistatic to *ztl-3* (Fig. 7B). However, under short-day conditions, *ztl-3* was epistatic to *gi-2* (Fig. 7B), pointing toward conditional genetic interactions between these mutants. *ctr1-12* was also late flowering in both long and short days, as reported previously for *ctr1-1* (Achard et al., 2007). This effect was additive with late flowering of *gi-2* and to a lesser extent *ztl-3*, suggesting CTR1 contributes to flowering time through a GI-independent pathway.

We next measured CCA1p:LUC activity in continuous dark with and without Suc. Reporter rhythms could not be detected in either the *gi-2 ztl-3* or *gi-2 ctr1-12* double mutants (Fig. 7, C and D) in media with or without Suc, similar to *gi-2* single mutants. Thus, *gi-2* is epistatic to both *ztl-3* and *ctr1-12* in these conditions, indicating that the sustained circadian rhythms observed in continuous dark in *ztl-3* and *ctr1-12* without Suc both require GI. Furthermore, we could not detect reporter rhythms in *ztl-3 ctr1-12* double mutants either with or without Suc (Fig. 7, C and D), in stark contrast to each of the single mutants (Figs. 2 and 5; Supplemental Fig. S8). This unexpected additive phenotype in *ztl-3 ctr1-12* double mutants suggests that ZTL and CTR1 act in genetically distinct pathways with respect to sustaining rhythms in the dark without Suc, which converge on GI.

To further investigate the impact of these genetic interactions on the circadian oscillator, we measured CCA1p:LUC activity in continuous light with and without Suc (Fig. 7, E–G; Supplemental Fig. S8). Having previously established that these mutants do not contribute to the *PRR7*-dependent pathway for period adjustment in dim light, we performed these experiments in our standard light conditions in which circadian period is not shortened by Suc (Haydon et al., 2013). In media containing Suc, rhythms in *ztl-3 gi-2* double mutants were less robust than wild type (Fig. 7G) but were

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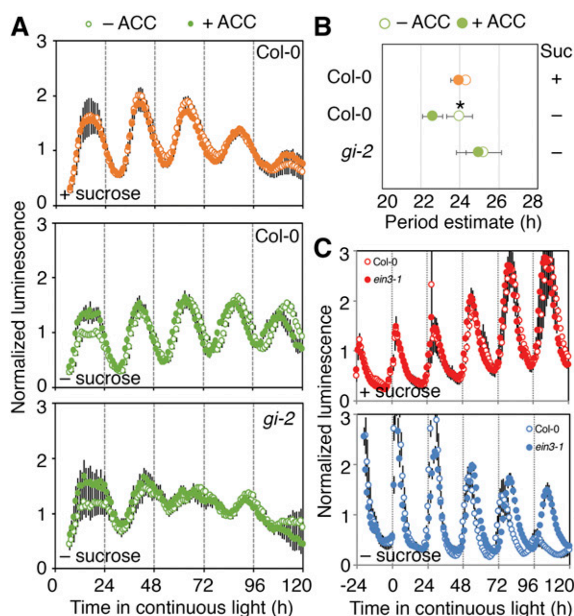

Figure 6. Suc masks effects of ethylene on the circadian oscillator. A, Normalized TOC1p:LUC luminescence in Col-0 and *gi-2* seedlings in media containing 90 mM Suc or media without Suc in continuous light with or without 50 μM ACC from 2 h (means ± SE, $n = 6$). B, Period estimates of TOC1p:LUC activity in seedlings grown as in A (means ± SD, $n = 6$). Asterisk indicates significant difference between +/−ACC by t test with Bonferroni corrections for multiple comparisons ($P < 0.05$). C, Normalized CCA1p:LUC activity in Col-0 and *ein3-1* seedlings growing in continuous light in media containing 90 mM Suc or control media without Suc (means ± SE, $n = 4$).

long period, similar to *ztl-3* and consistent with the long-period phenotype reported in *gi-200 ztl-105* and *gi-2 ztl-103* double mutants grown in Suc-containing media in previous studies (Martin-Tryon et al., 2007; Kim et al., 2007). However, in media without Suc, robust rhythms were detected in *ztl-3 gi-2* (Fig. 7G) and period was similar to *gi-2* and not significantly different from wild type (Fig. 7E). This indicates that the genetic interaction between GI and ZTL is conditional and implicates a specific involvement of sugar availability.

Reporter rhythms in *gi-2 ctr1-12* were long period in continuous light but not robust compared to the single mutants either with or without Suc (Fig. 7, E–G), indicating an additive contribution to circadian rhythms in these conditions. By contrast, *ztl-3 ctr-12* double mutants were long period, similar to *ztl-3* either with or without Suc (Fig. 7E). Thus, in contrast to the effects in continuous dark (Fig. 7C), we could not detect a genetic interaction between *ztl-3* and *ctr1-12* in continuous light. This is consistent with distinct contributions of ZTL and CTR1 to modulation of circadian rhythms.

DISCUSSION

We have previously shown that GI is required to sustain sugar-dependent circadian rhythms in continuous dark (Dalchau et al., 2011). Here, we have shown this is manifested through loss of rhythmicity of both morning- and evening-expressed clock transcripts in multiple *gi* mutants (Fig. 1; Supplemental Fig. S2). The effect of *gi* in these conditions is not specific to Suc but is also true for other metabolizable sugars (Supplemental Fig. S1). The insensitivity of the clock to sugars in *gi* in the dark is unique among single mutants of the core circadian oscillator genes tested (Fig. 2; Supplemental Fig. S3). However, we identified mutants in two genes, *ZTL* and *CTR1*, that are able to sustain robust, low amplitude circadian rhythms in continuous dark without added Suc (Figs. 2 and 5; Supplemental Figs. S3 and S7), which is essentially the opposite phenotype to *gi* mutants in these conditions. A similar phenotype has been reported in seedlings harboring a constitutively active allele of a red-light photoreceptor, phytochrome B (Jones et al., 2015). These observations indicate that loss of transcriptional circadian rhythms in continuous dark is not simply a consequence of limiting energy availability but is the result of specific regulatory or signaling events. Thus, describing the effect of sugars on circadian rhythms in continuous dark can be used to define these GI-dependent pathways affecting the circadian clock.

We previously reported that entrainment of the clock to photosynthetic rhythms of sugars acts through repression of *PRR7* during the day to modulate *CCA1* expression and adjust the phase of the oscillator (Haydon et al., 2013). It is notable that *cca1-11* and *prr7-11* mutants had no effect on responsiveness of the clock to Suc in continuous dark conditions (Fig. 2; Supplemental Fig. S3). This is perhaps not surprising, since PRR7p:LUC activity is low in these conditions and addition of Suc would act to repress *PRR7* further (Haydon et al., 2013). Nevertheless, the absence of a role for these core oscillator components suggests there are multiple, distinct pathways for input of sugars to the clock that act at different times of day or in different conditions.

ZTL is an F-box protein that interacts with GI and targets TOC1 for degradation by the 26S proteasome (Kim et al., 2007). The GI-ZTL interaction is stabilized by blue light, requires the ZTL light, oxygen, or voltage sensing (LOV) domain, and promotes localization of the complex in the cytosol (Kim et al., 2007, 2013). This interaction, in turn, inhibits nuclear and ZTL-independent functions of GI and sequesters ZTL away from its degradation target proteins, including TOC1 and PRR5 (Más et al., 2003; Kiba et al., 2007). We have shown that, similar to light, Suc promotes stabilization of GI (Fig. 3) and that this requires ZTL (Fig. 4). It remains to be determined whether this occurs by directly enhancing the GI-ZTL interaction and potentially inhibiting nuclear localization of GI. If so, it would be interesting to investigate whether this depends on the LOV domain, similar to the blue light-dependent interaction (Kim et al., 2013). Alternatively, the effect of Suc could depend on distinct GI and/or ZTL domains or require an intermediate or accessory protein(s).

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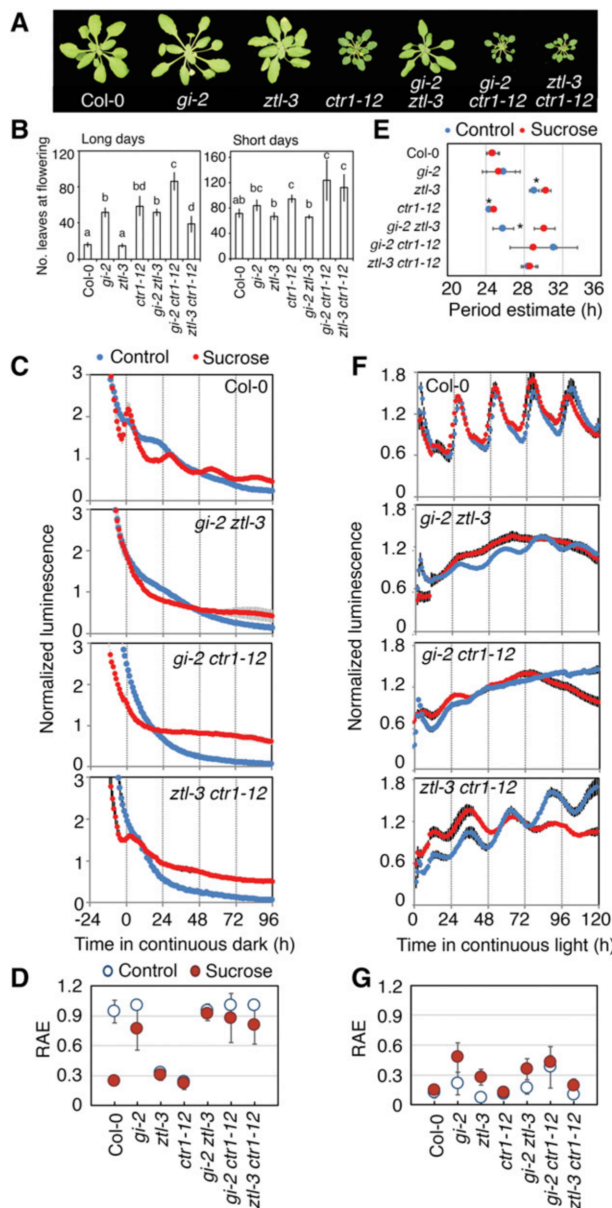

Figure 7. Conditional genetic interactions between *ztl-3* and *gi-2*. A, Four-week-old plants growing in long-day conditions. B, Total number of rosette leaves at flowering in plants growing in long-day or short-day conditions (means ± SD, $n = 6$). Different letters indicate significant difference by t test with Bonferroni corrections for multiple comparisons ($P < 0.05$). C, Normalized CCA1p:LUC activity in seedlings growing in continuous dark in media containing 90 mM Suc or control media without Suc (means ± SE, $n = 4$). D, RAE of CCA1p:LUC rhythms in seedlings growing as in C. Values are mean ± SD, $n = 4$. Activity traces of the single mutants are shown in Supplemental Figure S8. E, Period estimates of CCA1p:LUC activity in seedlings growing in continuous light in media containing 90 mM Suc or control media without Suc (means ± SD, $n = 4$). Asterisks indicate significant difference between conditions for each genotype by t test with Bonferroni correction for multiple comparisons ($P < 0.05$). F, Normalized CCA1p:LUC activity in seedlings grown as in E (means ± SE, $n = 4$). G, RAE of CCA1p:LUC rhythms in seedlings grown as in E. Values are mean ± SD, $n = 4$. Activity traces of the single mutants are shown in Supplemental Figure S8.

The opposite effects of *gi* and *ztl* mutants on amplitude of circadian rhythms in continuous dark could be explained by the posttranscriptional effects of the GI-ZTL interaction on TOC1 abundance. Although expression of morning-active clock genes is damped in continuous dark, rhythms of *TOC1* and *GI* transcript can be detected in media without Suc (Fig. 1; Supplemental Figs. S2 and S5; Haydon et al., 2013), suggesting there is a residual oscillator in these conditions comprised of evening-active components. In *gi* mutants, unbound ZTL will more readily degrade TOC1, leading to decreased amplitude of the residual oscillator. By contrast, in *ztl* mutants, degradation of TOC1 would be expected to be attenuated, which could permit sustained TOC1 rhythms driven by transcriptional rhythms of *TOC1* and *GI* in continuous dark in the absence of Suc.

The Suc-dependent effect on GI stability through ZTL also appears to manifest in conditional genetic interactions between *gi-2* and *ztl-3* (Fig. 7). A long-period phenotype in *gi-200 ztl-105* and *gi-2 ztl-103* double mutants in the presence of Suc has been reported previously (Martin-Tryon et al., 2007; Kim et al., 2007), consistent with our data (Fig. 7, E and F), and confirming that *ztl* is epistatic to *gi* in these conditions. However, in media without Suc, *gi-2 ztl-3* mutants had a robust shorter period, similar to *gi-2* single mutants, indicating that *gi-2* is epistatic to *ztl-3* in the absence of exogenous Suc. This is consistent with a model in which Suc promotes the GI-ZTL interaction; in the absence of Suc, GI will be unbound by ZTL and able to affect independent functions, whereas in the presence of Suc the effect of loss of GI function would be diminished compared to sequestered GI-ZTL in the wild type (Fig. 8). With respect to flowering time, *gi-2* is epistatic to *ztl-3* in long days, which is consistent with the circadian phenotypes in Suc-free media (Fig. 7). The reversed epistatic relationship in short days is unexpected but reflects the complexity of the pervasive GI-dependent regulatory network. The different effect in short days might be due to differing carbon metabolism or photoperiodic effects on GI-ZTL or relate to roles of GI and ZTL in a distinct regulatory pathway. *gi* mutants contain modestly elevated starch at dusk (Eimert et al., 1995), which could result in adjusted C status in short-day conditions. However, this starch excess phenotype would not be expected to affect C availability in continuous dark conditions, since starch is essentially exhausted by subjective dawn.

Similar to *ztl* mutants, we observed robust circadian rhythms in *ctr1-12* mutants in continuous dark in the absence of Suc (Fig. 5, A and B). CTR1 is a central, negative regulator of ethylene signaling (Kieber et al., 1993), and *ctr1-12* has been described as a sugar-insensitive mutant (Gibson et al., 2001). Similarly, we observed robust rhythms in the same conditions in *xct-2*, a short period clock mutant with ethylene-related phenotypes very similar to *ctr1* mutants (Ellison et al., 2011). Like CTR1, XCT has been proposed to be a negative regulator of ethylene

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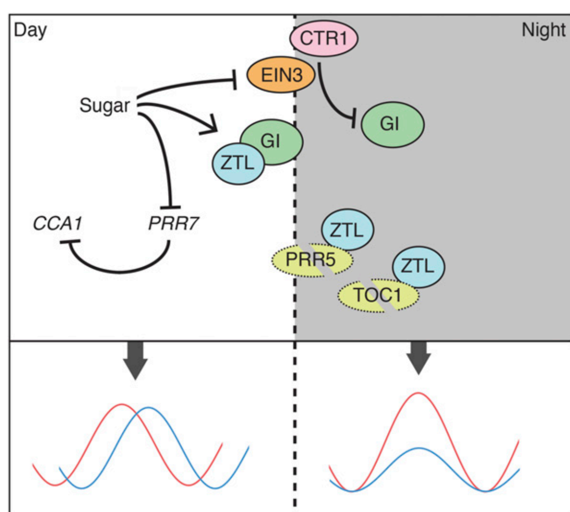

Figure 8. A proposed model of multiple pathways for modulation of the circadian oscillator by Suc. Sugars accumulate in plants cells during the day from photosynthesis. Sugars repress *PRR7* during the morning to adjust phase (Haydon et al., 2013). We propose here that sugars, in addition to light, contribute to the stabilization of GI-ZTL at the end of the day. As the cellular sugar concentration decreases, disassociation of the complex frees GI and ZTL to affect independent downstream functions, such as degradation of ZTL substrates, TOC1 and PRR5. This contributes to modulation of oscillator amplitude by sugars. Independent of the interaction with ZTL, the negative regulator of ethylene signaling, CTR1, acts upstream of GI. EIN3 protein is destabilized by sugar (Yanagisawa et al., 2003), suggesting a possible mechanism for Suc-dependent effects of ethylene signaling in the clock acting through GI.

responses (Ellison et al., 2011). Circadian rhythms of ethylene biosynthesis and emission in continuous dark and continuous light have been reported previously (Thain et al., 2004). However, no circadian period effects were observed in *etr1-1*, *ein4-1*, *eto2-1*, or *ctr1-1* mutants or in the presence of ethylene in continuous light in Suc-containing media in the previous study (Thain et al., 2004) and consistent with our data (Fig. 6). However, in Suc-free media, we observed a long-period phenotype in the ethylene-insensitive mutant *ein3-1* in continuous light (Fig. 6), similar to modestly lengthened circadian period in *gi-2* (Park et al., 1999). Consistent with this, ACC-treated seedlings without Suc had a short period compared to controls, and this effect was not observed in *gi-2* (Fig. 6). Furthermore, we detected CCA1p:LUC rhythms in continuous dark without Suc in the presence of ACC, similar to *ctr1-12* and *xct-2* (Fig. 5, B and C). Together, these data support a GI-dependent role for ethylene signaling in regulating the core circadian oscillator.

The precise relationship between input of ethylene and sugars to the oscillator remains unclear. Exogenous Suc increased total ethylene production in Arabidopsis seedlings (Ellison et al., 2011), but it is not known what effect Suc treatment has on circadian phase, period, or amplitude of ethylene production. Opposite to *ctr1*, *ein3* mutants are Glc oversensitive and EIN3 protein degradation is promoted by Glc (Yanagisawa et al., 2003; Fig. 8). It might be that the conditional circadian period phenotype of *ein3* mutants is due to enhanced EIN3 degradation in the presence of sugars, thus diminishing the functional effect of the mutation in these conditions. EIN3 is a master regulator of the transcriptional ethylene response with over 1,300 direct targets identified by ChIP-Seq experiments (Chang et al., 2013). Genes of the core circadian oscillator are absent among these identified targets. However, notable EIN3 targets are the circadian regulated Myb-like transcription factor *RVE2* (Zhang et al., 2007) and *TIME FOR COFFEE*, a nuclear protein of undefined function proposed to contribute to amplitude of circadian rhythms and metabolic signaling (Hall et al., 2003; Ding et al., 2007; Sanchez-Villarreal et al., 2013). These might represent pathways for ethylene signaling to the clock, or the effect of ethylene on the clock might act through indirect targets of EIN3.

The genetic analysis of *ctr1-12* double mutants suggests that CTR1 contributes to circadian clock function by a distinct pathway to ZTL. The loss of robust rhythms in continuous dark in *gi-2 ctr1-12* (Fig. 7, C and D), similar to *gi-2 ztl-3*, confirms that GI acts downstream of CTR1. However, since *ztl-3 ctr1-12* double mutants are also arrhythmic in these conditions (Fig. 7C), in stark contrast to both single mutants (Figs. 2 and 3), it seems likely that these contribute to rhythmicity by distinct pathways upstream of GI (Fig. 8). Furthermore, the long period phenotype of *gi-2 ctr1-12* in continuous light compared to the single mutants implies a genetic interaction that is not apparent in *ztl-3 ctr1-12* (Fig. 7, E and F).

In summary, we have shown that there is posttranscriptional regulation of GI by Suc that requires the GI-interacting F-box protein ZTL. The stabilization of this interaction by Suc, which is supported by the sugar-dependent genetic interactions between *GI* and *ZTL*, suggests a mechanism to sustain circadian rhythms in continuous dark. By examining mutants that affect sensitivity to sugars, we uncovered a role for ethylene in the regulation of the circadian oscillator, which had been previously obscured because earlier experiments had been performed in the presence of exogenous Suc (Thain et al., 2004). We have revealed that exogenous Suc masks the response of the oscillator to ethylene, which in the absence of exogenous Suc speeds up the oscillator to reduce circadian period. This sugar-sensitive response of the circadian oscillator to ethylene is dependent on GI, suggesting overlapping pathways by which Suc and ethylene regulate the oscillator. Our findings identify at least two distinct routes by which Suc affects the circadian oscillator (Fig. 8). In the evening, Suc regulates the amplitude of the oscillator posttranscriptionally through the stabilization of GI in a pathway that has overlapping elements with that by which ethylene can regulate the pace of the circadian oscillator. In the morning, Suc regulates

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the period of the circadian oscillator through a pathway independent of GI, through transcriptional changes in *PRR7* abundance (Haydon et al., 2013).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

CCA1p:LUC, *TOC1p:LUC*, and *CCR2p:LUC* are in Col-0 ecotype of Arabidopsis (*Arabidopsis thaliana*). *CAB2p:LUC* is in C24 and Ws ecotype. *gi-11*, *cca1-11* (Gould et al., 2006), *toc1-1*, *toc1-2* (Strayer et al., 2000), and *ztl-1* (Somers et al., 2000) mutants harbor *CAB2p:LUC* and *xct-2* (Martin-tryon and Harmer, 2008) harbors *CCR2p:LUC*. *CCA1p:LUC* and *TOC1p:LUC* were introduced into *gi-2* (Park et al., 1999) by crossing. *CCA1p:LUC* was introduced into *prr9-10*, *prr7-11*, *prr5-11* (Nakamichi et al., 2005), *ztl-3* (Jarillo et al., 2001), *gin2-1* (Moore et al., 2003), *abscisic acid deficient* (*aba2-1*/*gin1*; González-Guzmán et al., 2002), *aba3-1*/*gin5* (Xiong et al., 2001), *constitutive triple response1* (*ctr1-12*/*gin4*; Gibson et al., 2001), and *ein3-1* (Chao et al., 1997) by crossing. The *gin2-1* mutant in Landsberg *erecta* ecotype was backcrossed twice to *CCA1p:LUC* in Col-0. Homozygous F3 populations were selected for luciferase activity and visible mutant phenotypes, when applicable, and genotyped for the respective mutation. Double mutants were generated by pairwise crosses between *gi-2*, *ztl-3*, and *ctr1-12* mutants, each harboring *CCA1p:LUC*. F2 individuals were selected for visible single mutant phenotypes and then genotyped to identify double mutants. *GIp:GI-TAP* and *35Sp:GI-TAP* are in Col-0 (David et al., 2006). *35Sp:GI-TAP* was introduced into *ztl-1* by crossing. F2 populations were selected on kanamycin for *35Sp:GI-TAP* and genotyped for *ztl-1*. Surface-sterilized seeds were sown on half-strength Murashige and Skoog media, pH 5.7, and solidified with 0.8% (w/v) Bacto agar. After sowing, seeds were kept at 4°C in darkness for 2 d then grown in 12-h light/dark cycles under 50 μmol m^{-2} s^{-1} cool fluorescent white light at constant 19°C. Flowering time was measured in plants growing in soil under short days (8 h light) or long days (16 h light) at 21°C under 100 μmol m^{-2} s^{-1} cool fluorescent white light at 21°C. The number of rosette leaves at time of bolting was recorded.

Luciferase Activity Measurements

Clusters of 3 to 10 seedlings were grown in half-strength Murashige and Skoog media agar media and entrained in light-dark cycles (50 μmol m^{-2} s^{-1} light). Seedlings were dosed twice with 1 to 2 mM D-luciferin between 12 and 48 h before commencing luminescence measurements. Seedlings were released into continuous dark or continuous light after 7 to 11 d in light-dark cycles. Luminescence was detected for 600 s at each time point with an HRPCS4 (Photek) or a LB985 Nightshade (Berthold) camera. During photon counting, light was supplied from red (660 nm) and blue (470 nm) LEDs at 30 to 50 μmol m^{-2} s^{-1}. Where indicated, data were normalized to average counts across the experiment for each replicate. Period and RAE estimates where calculated on rhythms between 24 and 120 h in continuous conditions on non-normalized data using fast-Fourier transformed nonlinear least squares analysis, implemented in Biological Rhythms Analysis Software Suite (http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm). When no rhythm was detected by Biological Rhythms Analysis Software Suite, relative amplitude error was designated as 1.

Quantitative Real-Time PCR

Total RNA was extracted from frozen whole seedlings using RNeasy Plant Mini Kit (Qiagen) and RNase-free DNase on-column treatment (Qiagen). cDNA was synthesized from 1 μg RNA with RevertAid First Strand cDNA Synthesis Kit (Fermentas) using oligo(dT) primer. Technical replicates of gene-specific products were amplified in 10-μL reactions using Rotor-Gene SYBR Green PCR Kit (Qiagen) on a Rotor-Gene 6000 real-time PCR machine fitted with a Rotor-Disc 100 (Qiagen). Primers sequences are listed in Supplemental Table S1. Transcript levels were determined from C_t values, incorporating PCR efficiencies, relative to a reference transcript or the geometric mean of multiple reference transcripts as previously described (Haydon et al., 2012).

Immunoblotting

Protein extracts were prepared by grinding frozen plant tissue in extraction buffer (50 mM Tris-Cl, pH 7.5, 1 mM dithiothreitol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and cOmplete Protease Inhibitor cocktail [Roche]) followed by centrifugation at 16,000*g* for 20 min at 4°C. Protein concentration was determined in the supernatant by Bradford protein assay (Bio-Rad), and 5× loading dye (0.2 M Tris-Cl, pH 6.8, 10% SDS, 20% glycerol, and 0.05% [w/v] bromophenol blue) was added to 15 to 20 μg protein, denatured for 5 min at 95°C, and separated by 8% SDS-PAGE. Gels were cut at ~72 kD; the lower section was stained with Coomassie Brilliant Blue for quantification and the upper section transferred to polyvinylidene difluoride membrane (Amersham Hybond P, GE Healthcare). The membrane was blocked in 1× TBS (10 mM Tris-Cl, pH 8.0, and 150 mM NaCl) with 5% (w/v) nonfat dry milk, followed by sequential incubation with anti-Protein A antiserum (1:10,000; Sigma-Aldrich) and HRP-conjugated secondary antibody (1:10,000; Invitrogen). Protein bands were detected with Amersham ECL reagent (GE Healthcare) on x-ray film, developed with an X-O-Mat (Kodak).

To compare relative protein levels between treatments, equivalent time-point samples were loaded on the same gel (maximum 12 per gel). When separate gels were required, gels and membranes were processed simultaneously and developed on a single film. For GI-TAP quantification, gels were cut at ~72 kD; the upper gel section was used for transfer and the lower gel section was Coomassie stained. GI-TAP band intensity was quantified relative to unsaturated Coomassie-stained band intensity from the same lane with ImageJ software and normalized to the control treatment sample 4 h after dawn.

Accession Numbers

Sequence data from this article can be found in The Arabidopsis Information Resource database under the following accession numbers: *GI*, AT1G22770; *ZTL*, AT5G57360; *CTR1*, AT5G03730; *CCA1*, AT2G46830; *TOC1*, AT5G61380; *PRR5*, AT5G24470; *PRR7*, AT5G02810; *PRR9*, AT2G46790; *HXK1*, AT4G29130; *ABA2*, AT1G52340; *ABA3*, AT1G16540; *EIN3*, AT3G20770; and *XCT*, AT2G21150.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Circadian rhythms in *gi-11* mutants are insensitive to metabolizable sugars in continuous dark.

Supplemental Figure S2. *CCA1* and *TOC1* transcript levels in *gi-2* in continuous dark.

Supplemental Figure S3. Circadian rhythms in continuous dark in mutants of the core oscillator.

Supplemental Figure S4. *GI* transcript level in GI-TAP transgenic lines.

Supplemental Figure S5. Stabilization of GI protein by Suc in continuous dark.

Supplemental Figure S6. Circadian rhythms in continuous dark in *35Sp:GI-TAP*.

Supplemental Figure S7. Circadian rhythms in continuous dark in ABA and ethylene mutants.

Supplemental Figure S8. Circadian rhythms of single mutants in continuous light and continuous dark.

Supplemental Table S1. Primer sequences for qRT-PCR.

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