Running title: GI acts in blue light, clock, and flowering regulation

Author to whom all correspondence and proofs should be sent:
Stacey L. Harmer, Section of Plant Biology, University of California Davis, One Shields Ave, Davis, CA 95616
(530) 752-8101; fax (530) 752-5410
slharmer@ucdavis.edu

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**GIGANTEA** acts in blue light signaling and has biochemically separable roles in circadian clock and flowering time regulation

Ellen L. Martin-Tryon¹, Joel A. Kreps², Stacey L. Harmer¹

¹Section of Plant Biology, College of Biological Sciences, University of California, Davis, USA
²Diversa Corporation, 4955 Directors Place, San Diego, California, USA
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Corresponding author:
Stacey L. Harmer,
slharmer@ucdavis.edu
fax (530) 752-5410
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Abstract:
Circadian clocks are widespread in nature. In higher plants, they confer a selective advantage, providing information regarding not only time of day but also time of year. Forward genetic screens in Arabidopsis thaliana have led to the identification of many clock components, but the functions of most of these genes remain obscure. In order to identify both new constituents of the circadian clock and new alleles of known clock-associated genes, we performed a mutant screen. Using a clock-regulated luciferase reporter, we isolated new alleles of ZEITLUPE (ZTL), LATE ELONGATED HYPOCHOTYL (LHY) and GIGANTEA (GI). GI has previously been reported to function in red light signaling, central clock function, and flowering time regulation. Characterization of this and other GI alleles has helped us to further define GI function in the circadian system. We found that GI acts in photomorphogenic and circadian blue light signaling pathways and is differentially required for clock function in constant red versus blue light. Gene expression and epistasis analyses show that TOC1 expression is not solely dependent upon GI and that GI expression is only indirectly affected by TOC1, suggesting that GI acts both in series with and in parallel to TOC1 within the central circadian oscillator. Finally, we found that the GI-dependent promotion of CONSTANS (CO) expression and flowering is intact in a gi mutant with altered circadian regulation. Thus GI function in the regulation of a clock output can be biochemically separated from its role within the circadian clock.
Introduction:
Circadian clocks, found widely in nature, act to coordinate biological processes with rhythmic changes in the environment. Light input plays an important role in adjusting the clock’s phase as well as maintaining its pace, allowing for perception of both daily and seasonal information (Aschoff, 1979). The central biological oscillator interprets this environmental information and transmits the signal to a diverse set of outputs. A substantial portion of the Arabidopsis thaliana transcriptome is clock-regulated, with estimates ranging from 2% to 36% of expressed genes (Harmer et al., 2000; Schaffer et al., 2001; Michael and McClung, 2003a; Edwards et al., 2006; MF Covington and SL Harmer, personal communication).

In the current model of the Arabidopsis oscillator, TIMING OF CHLOROPHYLL A/B BINDING PROTEIN/PSEUDO RESPONSE REGULATOR1 (TOC1/PRR1) is believed to act as part of a central negative feedback loop with CIRCADIAN CLOCK ASSOCIATED (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) (Alabadí et al., 2001). TOC1 positively regulates expression of CCA1 and LHY by an unknown mechanism, and CCA1 and LHY in turn negatively regulate TOC1 expression. CCA1 and LHY are homologous myb-like transcription factors thought to directly repress TOC1 expression by interacting with the evening element motif in the TOC1 promoter (Alabadí et al., 2001; Harmer and Kay, 2005). This gives rise to an anti-phasic pattern of expression, with CCA1 and LHY messages peaking near dawn (Schaffer et al., 1998; Wang and Tobin, 1998) and TOC1 near dusk (Strayer et al., 2000). Mutation or altered expression of any of these three genes leads to severe circadian phenotypes, supporting their central roles in the clock (Millar et al., 1995; Schaffer et al., 1998; Wang and Tobin, 1998; Green and Tobin, 1999; Alabadí et al., 2002; Mizoguchi et al., 2002; Más et al., 2003a).

This simple model, however, does not accommodate all published data (Salomé and McClung, 2004); further, computer modeling suggests this loop is insufficient to drive the rhythms observed in Arabidopsis (Locke et al., 2005). It seems likely that the plant clock consists of multiple interlocking feedback loops, as has been suggested for animal
circadian oscillators (Emery and Reppert, 2004). Proposed components of other Arabidopsis circadian loops include the TOC1 homologues PRR3, 5, 7 and 9 (Mizuno, 2004; Farré et al. 2005); EARLY FLOWERING4 (Kikis et al., 2005); LUX ARRHYTHMO/PHYTOCLOCK1 (Hazen et al., 2005; Onai and Ishiura, 2005); and GIGANTEA (GI) (Locke et al., 2005; Mizoguchi et al., 2005, Gould et al., 2006). Computer modeling suggests that GI in particular may play an important role in the central clock, acting in series with or parallel to TOC1 (Locke et al., 2005; Mizoguchi et al., 2005, Gould et al., 2006).

GI was originally discovered as a late-flowering mutant (Rédei, 1962; Koornneef et al., 1991; Araki and Komeda, 1993), and positively regulates expression of the flowering time genes CONSTANS (CO) and FLOWERING LOCUS T (FT) (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Suarez-Lopez et al., 2001), suggesting a function for GI in clock output. One allele, gi-100, was isolated through a screen for photomorphogenic mutants, suggesting GI may also function in light input pathways involving phytochrome (Huq et al., 2000). Recent experiments suggest that GI has separable roles in the circadian clock and flowering time regulation (Mizoguchi et al., 2005, Gould et al., 2006).

Although circadian rhythms persist in the absence of environmental cues, in a natural environment the clock must be reset by signals such as changes in light or temperature to allow for seasonal time measurement as well as daily coordination. Light input to the clock is mediated by phytochrome (PHY) and cryptochrome (CRY) photoreceptors (Somers et al., 1998a; Devlin and Kay, 2000). Light input may also be carried out by ZEITLUPE (ZTL), a member of a small family of proteins with a distinctive assortment of domains (Nelson et al., 2000; Somers et al., 2000; Schultz et al., 2001). These proteins each have an F-box domain, an N-terminal LOV domain, and a series of six C-terminal kelch repeats. This domain structure suggests that ZTL might be a light-regulated protein involved in the proteasome-dependent degradation of protein substrates (Somers et al., 2000). Indeed, ZTL has been shown to cause the dark dependent degradation of TOC1 through the proteasome (Más et al., 2003b; Han et al., 2004).
Most of the above components were isolated through forward-genetic screens, as is true of most clock components discovered in other model systems (Young and Kay, 2001). In order to isolate new clock components and gain insight into known regulatory components, we screened plants mutagenized with ethyl methane sulfonate (EMS) for mutants exhibiting clock dysfunction in constant darkness. We found new alleles of $GI$, $LHY$, and $ZTL$. Characterization of the new short period $GI$ allele, $gi-200$, revealed that $GI$ acts in both red and blue light input to the clock and that its roles in these pathways are distinct. Expression analysis in $gi-200$ and a $gi$ T-DNA allele, $gi-201$, revealed that these mutations had minor effects on $TOC1$ expression, suggesting that $GI$ is not solely responsible for promotion of $TOC1$ expression. No significant changes in $GI$ expression levels were observed in $toc1-2$ mutants; moreover, epistasis analysis between $gi$ and $toc1$ and $ztl$ mutants suggests that $GI$ and TOC1 proteins may act in parallel pathways. Finally, unlike most $gi$ mutants, we found that $gi-200$ maintains its ability to relay timing signals from the clock to the day-length dependent flowering pathway through $CO$ and $FT$. Combined with its short period phenotype, this causes normal flowering in long days but early flowering in non-inductive short days. Thus $GI$ plays multiple, and in at least some cases, biochemically separable roles in clock input, the central oscillator, and clock output pathways.

Results:

Screening and initial characterization of mutants

A successful screen for clock mutants with altered expression of a circadian-regulated promoter luciferase reporter ($CAB2::LUC$) had previously been performed in constant light conditions (LL) (Millar et al., 1995). To complement this earlier screen, we assayed mutagenized seedlings in constant dark conditions (DD). We monitored luciferase activity rhythms in Columbia-0 (Col) plants that expressed luciferase under the control of the $COLD$–$CIRCADIAN RHYTHM$–$RNA BINDING 2/GLYCINE-RICH RNA-BINDING PROTEIN 7$ ($CCR2/GRP7$) promoter ($CCR2::LUC$) (Strayer et al., 2000). In contrast to $CAB2::LUC$, rhythmic $CCR2::LUC$ activity persisted in plants even after several weeks in constant darkness (Strayer et al., 2000, and Figure S1). Therefore, we mutagenized Col...
**CCR2::LUC** seedlings with EMS, screened 10,000 M2 plants for alterations in period length (Figure S2), and identified mutant lines for further study. Prior to further phenotypic analysis, confirmed mutants were backcrossed four to five times to the parental Col **CCR2::LUC** strain to remove extraneous EMS-induced mutations. Mutant M3 plants were also outcrossed to Landsberg *erecta* (*Ler*) to establish populations for SSLP mapping (Lukowitz *et al*., 2000, Jander *et al*., 2002).

**Isolation of genes responsible for short- and long-period phenotypes**

The mapping population of a short period line demonstrated strong linkage to the top of chromosome I. Because of the proximity to the known clock locus **LHY** and the similar short period phenotype observed for *lhy-20* (Michael *et al*., 2003b), we sequenced the **LHY** locus in the mutant and identified a nonsense mutation, C2136T, predicted to cause a stop codon 359 amino acids after the translation start site (Figure S3). If translated, this would result in a truncated protein containing the myb-like DNA-binding domain but missing the C-terminal half of the wild-type gene product. The possibility that this mutation was responsible for the short-period phenotype was confirmed by lack of complementation by *lhy-20* (Table SI) and we therefore designated the nonsense allele *lhy-100*. Like *lhy-20*, period length is shortened in *lhy-100* in constant red or blue light as well as in the dark (Table SII), and *lhy-100* exhibits a normal period response to increased fluence (data not shown).

Five long-period mutants demonstrated strong linkage to the bottom of chromosome V, near the **ZTL** locus. **ZTL** mutants have been previously reported to have a long-period phenotype both in LL and DD (Somers *et al*., 2000; Somers *et al*., 2004), consistent with the phenotype of these alleles (Table SII). Surprisingly, three of the five mutants (*ztl-100, ztl-101,* and *ztl-104*), all isolated from different pools of M2 seeds, had alterations at the same nucleotide within the ZTL locus, C1977T. This mutation is predicted to cause a proline to serine mutation at amino acid 383, a residue conserved within the ZTL kelch repeats. In *ztl-102* we found that the corresponding conserved proline within a different kelch repeat was predicted to be altered, causing a P331L mutation. The fifth allele, *ztl-103*, had a G to A transition at nucleotide 1680, causing a premature stop codon 280
amino acids past the translation initiation site at the start of the kelch repeats. In addition, we have characterized *ztl-105* (SALK-069091), which contains a T-DNA insertion located near the beginning of the kelch repeats (Figure S3). *ztl-105* failed to complement each of the EMS alleles (Table SI). ZTL protein can be detected at near normal levels in *ztl-101* and *ztl-102* but is undetectable in *ztl-103* and *ztl-105* (D. Somers, personal communication). As previously described for almost all *ztl* mutants (Somers *et al.*, 2000; Somers *et al.*, 2004; Kevei *et al.*, 2006), the alleles we characterized exhibited a steeper fluence response curve in red light than wild type (data not shown), indicating that all our alleles have defects in red light signaling to the clock.

An additional short period mutant (Table I and Figure 1) mapped to a 63kb region on chromosome I encompassing the *GI* locus. Since *GI* has previously been implicated in clock function (Fowler *et al.*, 1999; Park *et al.*, 1999; Mizoguchi *et al.*, 2005; Gould *et al.*, 2006), the *GI* locus was sequenced as the most likely candidate gene within the region. A mutation (G3704A) was found that is predicted to induce a serine to alanine (S932A) amino acid change (Figure S3). This serine residue is conserved across monocots and dicots (Edwards *et al.*, 2005), suggesting it may play an important role in GI function. Since the short-period phenotype of this mutant was not complemented by the T-DNA allele *gi-201* (SALK-092757) (Table SI) we designated this mutant *gi-200* (Figure S3). The T-DNA insertion in *gi-201* is predicted to be within the second *GI* exon; if translated, the *gi-201* protein product would contain only 66 of the normal 1174 amino acids in the GI protein (Figure S3). No *GI* mRNA downstream of the insertion site could be detected in *gi-201* plants by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Figure 5D and data not shown), suggesting this is likely to be a null allele. Gene expression phenotypes similar to those seen for *CCR2::LUC* activity were also observed when we examined *CCR2* mRNA levels by qRT-PCR in these *gi* mutants (Figure 1D).

**GI acts in blue light signaling to the clock**

*gi-200* plants exhibited a short-period *CCR2::LUC* expression phenotype under all light conditions tested (Table 1 and Figure 1), similar to the gene expression phenotypes
previously reported for gi-1 and gi-3 (Park et al., 1999; Mizoguchi et al., 2005), two alleles with premature stop codons near the 3’ end of GI (Fowler et al., 1999, Figure S3). Luciferase activity was greatly reduced in the T-DNA mutant gi-201 (Figure 1A); however, nearly normal levels of CCR2 mRNA could be detected (Figure 1D), indicating that CCR2 expression is not damped low in these plants and that luciferase activity is reduced through an unknown mechanism. For more accurate measurements, we monitored the luciferase activity of clusters of gi-201 plants and found that CCR2::LUC rhythms were lower amplitude, damped more rapidly, and had a slightly altered period relative to wild-type (Figure 1 and Table I). These phenotypes are similar to gene expression phenotypes in the likely null alleles gi-2 and gi-11 (Park et al., 1999; Gould et al., 2006) further supporting gi-201 as a null allele.

Since it had previously been reported that gi-1 mutants displayed a low amplitude phenotype in constant white light (Fowler et al., 1999; Park et al., 1999; Sothern et al., 2002; Tseng et al., 2004; Mizoguchi et al., 2005) but not in constant darkness (Park et al., 1999), whereas gi-3 rhythms are low amplitude in both conditions (Mizoguchi et al., 2005), we examined the light-dependence of the gi-200 phenotype. Similar to previous reports for gi-1 (Park et al., 1999), the severity of the period and amplitude phenotypes of gi-200 plants was reduced when they were maintained in DD (Table I and data not shown), however expression of CCR2::LUC still cycled with a short period.

To investigate the previous reports of variability in period phenotypes in gi-2 mutants (Park et al., 1999), we examined rhythmic CCR2::LUC expression in a variety of light conditions. In constant red light, the rhythmic amplitude of CCR2::LUC activity in gi-200 plants was significantly lower than that of wild type (p=0.0013) (Figure 2A), however, in constant blue light there was no difference in amplitude (p=0.72) (Figure 2B). Interestingly, when held in red plus blue light, allowing for light perception through multiple pathways, gi-200 rhythms had an increased amplitude when compared to Col (p=0.0013) (Figure 2C). Thus the gi-200 clock phenotype is wavelength-dependent, suggesting GI acts in both red and blue light input pathways to the clock and that these pathways may be differentially compromised by the gi-200 mutation. In contrast, the gi-
T-DNA mutants displayed very low-amplitude rhythms that damped rapidly in constant red, constant blue, or the combination of constant red and blue light (Figure 1). Interestingly, gi-201 displayed a slightly long period phenotype in constant red light, but had a short period in constant blue light or the combination of red and blue light (Table I), further supporting functionally distinct roles for GI in blue and red light signaling.

Although the circadian clock does not require light input to function, higher fluences of light cause the Arabidopsis clock to run with a shorter period, as is true for many other organisms (Aschoff, 1979). Because gi-1 was reported to demonstrate a lack of response to increasing red light (Park et al., 1999), we determined whether light input to the circadian clock was altered in the gi-200 mutant by examining the free-running period under various intensities of light. As previously reported (Somers et al., 1998a; Devlin and Kay, 2000), higher fluences of monochromatic red or blue light caused the clock to run with a shorter period in wild-type plants (Figure 3). In contrast, there was no statistical difference (by one-way ANOVA or Student’s t-test) in the period length of gi-200 plants when grown in constant red, blue, or red plus blue light across a wide fluence range (Figure 3). Additionally, a significant interaction between genotype and fluence could be detected (p<0.05 in red and p<0.01 in both blue and red plus blue; ANOVA for general linear fixed-effects model), indicating the response to both red, blue, and the combination of red plus blue is altered in gi-200 relative to wild type. Our study extends the role of GI beyond red light signaling to indicate a function for GI in blue light input to the clock.

It has previously been reported that gi-1, gi-2 and gi-100 exhibit elongated hypocotyls in red light at all fluences tested and that gi-3 plants are tall when grown in blue light at elevated temperature (Huq et al., 2000; Paltiel et al., 2006). To further investigate the role of GI in photomorphogenesis, we examined hypocotyl elongation in many GI alleles under constant light of a variety of wavelengths and fluences. All alleles tested, in both the Col and Ler backgrounds, had significantly elongated hypocotyls (p<0.02 by Student’s t-test) when grown in constant white light or constant monochromatic red or blue light (Figure 4), but were not significantly different from wild type when grown in
constant darkness (data not shown). The degree of significance varied across a wide range of fluences, but the same trend of long hypocotyls was observed in all wavelengths of constant light examined (Figure S4). This tall hypocotyl phenotype of these six GI alleles in blue light further supports the role for GI in blue light signaling.

**Complex interaction between GI and TOC1**

Both GI and TOC1 have been suggested to function positively in regulation of CCA1 and LHY transcription (Fowler et al., 1999; Park et al., 1999; Alabadí et al., 2001; Mizoguchi et al., 2005). We therefore examined expression of CCA1 and LHY in gi mutants. Both expression levels and rhythmic amplitude of cycling were reduced for CCA1 and LHY in gi-201 and gi-200 plants, although the gi-200 phenotypes were less severe (Figures 5A and 5B). The short period phenotype seen for CCR2::LUC and CCR2 mRNA rhythms (Figure 1) is also apparent for CCA1 and LHY expression (Figures 5A and 5B) in gi-200.

Two models have recently placed GI within the central clock oscillator, one suggesting GI promotes TOC1 expression and thus acts in series with TOC1 (Locke et al., 2005) and the other suggesting that GI and TOC1 act in parallel pathways (Mizoguchi et al., 2005). To help clarify this issue, we examined TOC1 expression in gi mutants and GI expression in toc1 mutants. TOC1 expression levels were similar in gi-200 and wild-type plants but had a shorter period in gi-200 (Figure 5C), consistent with the observed pattern of CCA1 and LHY expression (Figures 5A and 5B). Interestingly, TOC1 message was also easily detected in the putative null allele gi-201, with mRNA levels damping towards the median level of wild-type expression rather than to trough levels as would be expected if GI were the only factor promoting TOC1 expression (Figure 5C). Therefore factors other than GI must positively regulate TOC1 gene regulation. However, these observations are consistent with the possibility that GI normally provides a portion of TOC1-activating activity (Locke et al., 2005, Figures S5A and S5B).

We also examined GI expression in the strong loss-of-function allele toc1-2. Since it has previously been reported that overexpression of TOC1 causes GI message levels to damp to low levels (Makino et al., 2002), it was of interest to observe that GI message levels
were very similar to wild type in *toc1*-2 mutants, although they revealed the expected short-period phenotype (Figures 5D). A recent model has suggested that TOC1 negatively regulates *GI* expression, leading to the prediction that *GI* levels would damp high in a *toc1* loss-of-function mutant (Locke *et al.*, 2005, Figures S5D). In contrast, our data suggest that TOC1 does not directly regulate *GI* expression levels.

To further examine the relationship between *TOC1* and *GI*, we generated double *gi-200 toc1*-2 mutants and compared their phenotypes to those of the single mutants. We observed that in both monochromatic red and blue light the short period phenotypes of *gi-200* and *toc1*-2 were nearly additive in the double mutant (Figures 6A and 6B and Table I). Similarly, the tall hypocotyl phenotype seen in both single mutants in constant red light was more exaggerated in the double mutant (data not shown). Furthermore, *gi-201 toc1*-2 mutants are largely sterile, a phenotype not present in either single mutant (data not shown). Thus a number of phenotypes are more extreme in the double mutant than in either single mutant, suggesting the two proteins have parallel functions rather than acting solely in series with each other.

TOC1 is targeted for degradation by the F-box protein ZTL; in *ztl* mutants, there is a sustained accumulation of TOC1 protein and a consequent increase in free-running period length (Más *et al.*, 2003b). Consistent with the regulated degradation of TOC1 being an important function of ZTL, the short-period phenotype of a *toc1* loss-of-function allele is epistatic to the long-period *ztl-1* phenotype in plants mutant for both genes (Más *et al.*, 2003b). We generated *gi-200 ztl-105* double mutants to further explore the relationship between *GI*, *TOC1*, and *ZTL*. *ztl-105* is a T-DNA insertion allele (SALK-069091) which produces no detectable ZTL message by northern blot or RT-PCR (data not shown) nor any detectable ZTL protein (D. Somers, personal communication). The free-running period estimates for *gi-200 ztl-105* double mutants held in constant red or blue light were intermediate between the estimates for either single mutant, but were more similar to *ztl-105* (Figures 6C and 6D and Table I). Additionally, we found that the hypocotyl lengths of the double mutants in constant red light were slightly shorter than Col, but taller than *ztl-105* (data not shown), again demonstrating an additive rather than
epistatic effect of the two mutations. Thus unlike TOC1, a GI allele is not epistatic to ZTL, further suggesting that TOC1 and GI may act in parallel.

**GI functions in the central clock and in regulation of a clock output are biochemically separable**

Previously-characterized lesions in GI cause a near complete loss of photoperiodism, resulting in late flowering in inductive photoperiods (Rédei, 1962; Koornneef et al., 1991; Araki and Komeda, 1993; Park et al., 1999; Huq et al., 2000). Consistent with these results, the T-DNA allele gi-201 flowered late in long days (LD) but with the normal number of leaves in short days (SD) (Figure 7A). But to our surprise, we found that under inductive photoperiods gi-200 flowered normally, and actually flowered earlier than wild type under SD (Figure 7A). Therefore, although gi-200 mutants have a similar clock phenotype to the previously characterized gi-1 and gi-3 alleles, gi-200 plants completely lack the late-flowering phenotype that gave GI its name.

The early flowering phenotype in gi-200 plants is reminiscent of that seen in the short-period mutant toc1-1, which is caused by the inappropriately early phase of CO expression and the consequent upregulation of FT expression in SD (Yanovsky and Kay, 2002). Hypothesizing that the short-period phenotypes of gi-200 and toc1-2 might have a similar effect on the phase of CO expression, we used qRT-PCR to examine CO message in these plants. gi-200 and toc1-2 displayed near wild-type levels of CO transcript (Figure 7B), whereas CO levels were decreased in gi-201, similar to previous reports for the late-flowering mutant gi-3 (Suarez-Lopez et al., 2001). Notably, CO expression was detectable late in the day in gi-200 and toc1-2 mutants but not in Col or gi-201 plants (Figure 7B, see time points 7 and 31). FT mRNA was very low in Col and gi-201 but was similarly high in gi-200 and toc1-2 plants, consistent with their early flowering phenotypes in SD (Figures 7C and 7D). Thus a coincidence between light and CO expression in both toc1-2 and gi-200 in SD is correlated with high FT expression and early flowering. The flowering phenotype in gi-200 mutants is therefore due to a change in the phase in CO expression and thus results from the short-period clock phenotype of these plants, rather than being caused directly by a change in CO expression levels. This
indicates gi-200 retains its normal biochemical function in CO regulation but lacks the ability to properly regulate circadian timing, demonstrating that the roles of GI in clock function and CO regulation are separable.

**Discussion:**

**GI functions in red and blue light signaling**

GI has previously been reported to be involved in red light signaling, both in photomorphogenesis and input to the circadian clock (Park et al., 1999; Huq et al., 2000). We now report phenotypes showing defects in blue light signaling in gi mutants. All the gi mutants we examined showed a decrease in photomorphogenesis in constant blue light (Figures 4 and S4), consistent with a previous report that gi-3 shows defects in blue-light induced photomorphogenesis under unusual temperature conditions (Paltiel et al., 2006), and suggesting that multiple domains of GI are required to inhibit hypocotyl elongation in the light. In addition, the change in free-running period of the circadian clock in response to increased blue light intensity is compromised in gi-200 (Figure 3), indicating that GI functions in blue light input to the circadian clock. Since cryptochromes are the blue light receptors involved in photomorphogenesis and input to the clock (Ahmad and Cashmore, 1993; Lin et al., 1998; Devlin and Kay, 2000), it is likely that GI is involved in cryptochrome signaling. However, the flattening of the fluence response curve to blue light seen in gi-200 (Figure 3) was not observed in cry1 cry2 double mutants (Devlin and Kay, 2000), indicating that the gi light input defects are not solely due to a decrease in blue light signaling through cryptochrome.

GI has been proposed to act within the central clock, either as a positive regulator of TOC1 expression or in parallel with TOC1 (Locke et al., 2005; Mizoguchi et al., 2005, Gould et al., 2006). Like gi mutants, tocl loss-of-function alleles also have long hypocotyls in red light (Más et al., 2003a). However, the semi-dominant short period tocl-1 mutant has no hypocotyl phenotype in red light despite its circadian phenotype (Somers et al., 1998b). These data suggest TOC1 plays biochemically distinct roles in phytochrome signaling and central clock function. Since gi mutants have clock (but not hypocotyl) phenotypes even in DD (Park et al., 1999; Mizoguchi et al., 2005; Table I,
and data not shown), GI’s functions in red and blue light signaling are likely distinct from its role in the central circadian oscillator. Notably, all gi mutants examined had similar hypomorphic hypocotyl phenotypes (Figures 4 and S4) but disparate and often antimorphic circadian phenotypes (Table I; Park et al., 1999; Mizoguchi et al., 2005). In particular, we found that the long-period clock phenotype of the likely null gi-201 was recessive while the short-period phenotype of the EMS allele gi-200 was semi-dominant (Table SI), consistent with the short-period phenotype of plants overexpressing GI (Mizoguchi et al., 2005). Taken together, these data support the hypothesis that the biochemical roles of GI in light signaling and the circadian clock are separable (Mizoguchi et al., 2005). The observation that gi but not toc1 mutants have hypocotyl phenotypes in blue light (Más et al., 2003a; Figures 4 and S4) also suggests that in at least blue light signaling GI and TOC1 act independently of each other.

**GI acts within the central clock**

Rhythmic luciferase activity quickly damped in gi-201 plants transferred to constant light, a more extreme phenotype than observed in the gi-200 mutant (Figure 1) and consistent with the suggestion that GI may act in the central clock along with TOC1, CCA1 and LHY (Locke et al., 2005; Mizoguchi et al., 2005). TOC1 and GI are both evening-phased genes whose expression is negatively regulated by CCA1 and LHY (Fowler et al., 1999; Alabadí et al., 2001). Further analogies with TOC1 are suggested by the wavelength-dependent phenotype of gi-200 plants: like toc1 loss-of-function mutants (Más et al., 2003a), gi-200 demonstrates a more severe clock phenotype in constant red than in constant blue light (Figures 1, 2, 6A, and 6B). Finally, both TOC1 and GI are required for normal peak levels of CCA1 and LHY expression (Figures 5A and 5B, Fowler et al., 1999; Park et al., 1999; Alabadí et al., 2001; Mizoguchi et al., 2002).

This and other data was incorporated into a recent model of the plant circadian oscillator. In this model, a component termed ‘Y’ positively regulates TOC1 expression, and the expression of ‘Y’ is in turn negatively regulated by TOC1, LHY, and CCA1. It has been proposed that GI represents all or part of component ‘Y’ (Locke et al., 2005). Our data indicate that GI is not the only protein that positively regulates TOC1 expression and thus
cannot constitute all of the ‘Y’ activity, since TOC1 mRNA levels do not show a marked reduction in the strong gi-201 mutant (Figure 5C). However, the apparent damping of TOC1 levels towards the median in this mutant is consistent with GI providing a portion of ‘Y’ activity (Figures S5A and S5B). On the other hand, a different prediction of the two-loop model is not supported by our data. The model predicts that a strong loss-of-function tocl allele would cause expression of ‘Y’ to be high and arrhythmic (Locke et al., 2005, Figure S5D), but we found GI transcript levels were very similar in tocl-2 and wild-type plants (Figures 5D and S5C). This suggests that either the decreased GI expression seen in TOC1 overexpressing plants (Makino et al., 2002) is a non-specific effect or that another factor acts redundantly with TOC1 to inhibit GI expression.

The circadian phenotype of gi-200 tocl-2 double mutants is more severe than that observed in either single mutant and is in fact nearly additive (Figures 6A and 6B and Table I). Since neither allele is a null, this result must be interpreted with caution. However, combined with the observation that overexpression of TOC1 causes a long period while overexpression of GI causes a short period phenotype (Más et al., 2003a; Mizoguchi et al., 2005), the extreme short-period phenotype in the double mutant suggests that GI has functions in the circadian clock independent of regulation of TOC1 expression. This possibility is reinforced by the observation that CCA1 and LHY message levels are reduced in both gi-200 and gi-201 (Figures 5A and 5B) despite the different period phenotypes of these mutants (Table I and Figure 1). Furthermore, 35S::GI plants demonstrate near wild type levels of CCA1 and LHY despite their short period phenotype (Mizoguchi et al., 2005). Since loss of LHY or CCA1 function causes a shortened period (Green and Tobin, 1999; Michael et al., 2003b), these data suggest that GI plays a role within the central clock that is independent of the TOC1/CCA1/LHY feedback loop. We therefore propose that GI acts both in series and in parallel to TOC1 within the central oscillator.

Further distinctions between TOC1 and GI were revealed by epistasis analysis with ZTL. The double gi-200 ztl-105 mutant has a long period phenotype (Figure 6, Table I), unlike the tocl-like short period phenotype seen in tocl ztl double mutants (Más et al., 2003b).
It therefore seems unlikely that accumulation of GI protein contributes significantly to the *ztl* long-period phenotype. This conclusion is underscored by the observation that plants that constitutively overexpress *GI* have a short period, in contrast to the long period seen in *ztl* mutants (Mizoguchi *et al.*, 2005; Somers *et al.*, 2000). These data suggest that GI is not a substrate for ZTL-mediated degradation and that the dark-induced proteolysis observed for GI (David *et al.*, 2006) is likely directed by another mechanism.

**GI has separable functions within the circadian clock and in the regulation of a clock output**

GI has been implicated in flowering time regulation, red light signaling, and central clock function (Rédei, 1962; Koornneef *et al.*, 1991; Araki and Komeda, 1993; Huq *et al.*, 2000; Locke *et al.*, 2005; Mizoguchi *et al.*, 2005; Gould *et al.*, 2006). Since both plant responses to red light and photoperiodic control of flowering are regulated by the clock, it has been difficult to determine whether all *gi* phenotypes are secondary to disruption of clock function. This issue has been clarified by our characterization of *gi-200* mutants. These plants have a low-amplitude, short-period phenotype similar to *gi-1* and *gi-3* (Park *et al.*, 1999; Tseng *et al.*, 2004; Mizoguchi *et al.*, 2005); however, *gi-1* and *gi-3* flower late in LD while *gi-200* flowers normally in inductive photoperiods (Figure 7A). The late flowering of *gi-3* plants is due to reduced levels of *CO* expression (Suarez-Lopez *et al.*, 2001), whereas *CO* levels are similar to wild-type in *gi-200* plants (Figure 7B). *gi-200*, however, demonstrates a phase shift in *CO* expression due to the increased pace of the clock (Figure 7B), indirectly causing early flowering in SD. Thus *gi-200* has a defect in clock function but is nonetheless able to promote *CO* expression in LD. This shows that the genetically separable functions of GI in the circadian clock and flowering time regulation (Mizoguchi *et al.*, 2005) in fact reflect distinct biochemical requirements for GI activity in these two pathways.

Similar to *gi-200*, a previous study reported that *gi-611* mutants had a short free-running period and flowered early in SD, although the basis for the flowering phenotype was not explored (Gould *et al.*, 2006). *gi-611* is altered at leucine 281 with a change to phenylalanine. Another allele, *gi-596*, exhibited a long period with normal flowering in
long days (Gould et al., 2006). Like gi-200, gi-596 is a missense allele with serine 191 changed to phenylalanine. gi-200 has a similar alteration, with serine 932 changed to alanine, but has a short-period phenotype. Therefore the normal function of GI within the circadian clock requires sequences at both the N- and C-termini of the protein. Serine 191 is conserved in all known GI homologues, both in monocots and dicots. Serine 932 is conserved in all dicot sequences and those of most monocots, but in two Lemma homologues the corresponding residue is a glycine. These serine residues might be important for the structure or activity of GI or might be the sites of post-translational modification (Tseng et al., 2004).

Comparison of the phenotypes of gi mutants may help shed light on its mode of action in the clock. Period is lengthened when GI is not present, as in gi-2 or gi-201 (Park et al., 1999; Table I). However, two mutants with premature stop codons near the C-terminus (gi-1 and gi-3) both have short-period phenotypes (Park et al., 1999; Mizoguchi et al., 2005), similar to plants overexpressing GI (Mizoguchi et al., 2005). This suggests that the N-terminus of GI acts to stimulate the pace of the clock and that the C-terminus may function to block N-terminal activity. Alteration of a C-terminal residue in gi-200 leads to a semi-dominant short-period phenotype, suggesting that the Ser932A mutation impairs the putative negative regulatory role of the C-terminus.

**Conclusions:**
The isolation and characterization of new alleles of known clock-associated genes has provided insights into the functioning of GI in the circadian system. GI has a wavelength-dependent role in circadian clock function, acting in both red and blue light signaling to the clock. Characterization of a missense allele revealed that its action in the clock is biochemically distinct from its regulation of the flowering time pathway. In fact, it may be that most plant clock proteins also act in non-clock dependent processes (Más et al., 2003b; Kevei et al., 2006). Further biochemical characterization of GI function will shed light on the mechanisms underlying its diverse roles in plant signaling.

**Materials and Methods:**
Mutagenesis and screening

Plants of the Columbia-0 ecotype were transformed with the CCR2::LUC reporter construct (Strayer et al., 2000) which confers gentamycin resistance. Seeds homozygous for CCR2::LUC were mutagenized with EMS by soaking two grams of seeds in 0.25% EMS at 21°C for 19 hours. Approximately 10,000 M1 plants were grown in pots of about 100 plants, each pot producing one pool of M2 seeds. M2 seeds were sterilized and plated on MS containing 3% sucrose and stratified for three to four days at 4°C before release to 12:12 light:dark cycles. Five days post-germination, seedlings were transferred to 96-well Packard plates and entrained for 3 more days. Seedlings were then assayed in constant darkness in a Packard multi-well scintillation counter for 6 days. See Harmer and Kreps (2001) for more details.

Data collected from the assay was analyzed for rhythmicity following the luciferase activity analysis methods found in Plautz et al. (1997). Due to the high frequency of arrhythmic plants in DD, only plants with period estimates greater than 2 standard deviations from the variance weighted mean of the population were isolated and allowed to self-fertilize for the M3 generation. Screening of approximately 10,000 M2 plants yielded nearly 200 M3 families, of which 14 exhibited heritable alterations in period length in constant darkness. Only 10 mutant lines demonstrated sufficiently robust phenotypes to allow for further characterization. Mutant lines were outcrossed to Landsberg erecta for mapping and backcrossed to the parental Col CCR2::LUC line at least 4 times to remove extraneous EMS mutations before further characterization. Mutations were confirmed with CAPS markers according to supplemental protocols.

SALK T-DNA alleles

T-DNA insertion mutants ztl-105 (SALK-069091) and gi-201 (SALK-092757) were obtained from the Arabidopsis Biological Resources Center (Alonso et al., 2003). See supplemental protocols on the web for the primers sequences used for genotyping.

Period phenotype assay
Seeds were plated on MS (MP Biomedicals) 0.7% agar (Sigma A1296) plates containing 3% sucrose (EMD chemicals) and 75mg/L gentamycin (EMD chemicals) and stratified at 4°C for 4 days before 12:12 light/dark entrainment at 22°C. After 6 days in light/dark cycles plants were sprayed with 3mM D-luciferin (Biosynth AG) and monitored with a cooled CCD camera (either an ORCA II ER (Hamamatsu) or a DU434-BV (Andor Technology)). Plants were grown at a constant 22°C. Light was provided by red and/or blue LED SnapLites (Quantum Devices) or cool white fluorescent bulbs. Neutral density filters (RoscoLux #98 and #398) were used to obtain the different fluence levels for the period length fluence response curves. Images were analyzed with MetaMorph (Molecular Devices) software and the pattern of luciferase activity was fit to a cosine wave through Fourier Fast Transform-Non-Linear Least Squares (FFT-NLLS) (Plautz et al., 1997) allowing for estimates of period length, amplitude, and phase.

Hypocotyl analysis
Hypocotyl length was assayed by sowing seeds on MS-agar plates containing 3% sucrose before stratification at 4°C for 4 days. After a four hour white light pulse (100 µmol m\(^{-2}\)s\(^{-1}\)), seeds were released to constant white light (cool white fluorescent bulbs, Sylvania and Phillips), or constant red or blue light (LED SnapLites, Quantum Devices) at 22°C under neutral density filters (RoscoLux #98 and #398) for 6 days before collection. On day 6 seedlings were transferred to transparencies and scanned. Individual measurements were obtained from the scans using the Seed Vigor Index System (Hoffmaster et al., 2003) with program modifications implemented by Kikuo Fujimura and Lijie Xu (manuscript in preparation).

Flowering time analysis
Seeds were stratified in water for 4 days before sowing directly to soil. Flats were then placed in either long days of 16 hours light and 8 hours dark (~100 µmol m\(^{-2}\)s\(^{-1}\)) or short days of 8 hours light and 16 hours dark (~200 µmol m\(^{-2}\)s\(^{-1}\)) provided by cool white fluorescent bulbs (Sylvania and Phillips). After germination flats were weeded allowing only one plant per pot and monitored daily for bolting. When a one centimeter bolt was present, the number of rosette leaves was noted.
**qRT-PCR assay for expression of CCA1, LHY, TOC1, GI, CCR2, and PP2a**

Approximately 40 plants of each genotype for each time point were germinated on Whatman filter paper atop MS-agar, 3% sucrose plates and entrained in 12:12 light/dark cycles under cool white fluorescent bulbs (55 µmol m⁻²s⁻¹) for 8 days before release to constant light (55 µmol m⁻²s⁻¹) and sample collection at three hour intervals. Total RNA was prepared with TRIzol reagent (Invitrogen) and 3 µg each were used for cDNA synthesis from oligo-dT₁₈ with SuperScript II Reverse Transcriptase (Invitrogen) following manufacturers protocol. Real time quantitative RT-PCR was performed using an iCycler (Bio-Rad) in 40 mM Tris HCL pH 8.4, 100 mM KCl, 6 mM MgCl₂, 8% Glycerol, 20 nM fluorescein, 0.4X SYBR Green I (Molecular Probes), 1XBSA (New England Biolabs), 1.6 mM dNTPs, 2.5 µM each primer, and 10% diluted cDNA using Taq polymerase. Samples were run in duplicate and starting quantity was estimated from critical thresholds compared to the standard curve of amplification. Data presented are normalized to PP2a expression level. All primer sets contain one primer which bridges an intron to reduce genomic amplification, melt curve analysis was performed following amplification to confirm specificity of products over primer dimers, and a no RT control was used to ensure products detected were from cDNA rather than genomic. See supplemental protocols for primer sequences.

**FT and CO expression analysis**

cDNA samples were generated as for qRT-PCR analysis except that plants were entrained in short days (8:16) under cool white fluorescent bulbs (200 µmol m⁻²s⁻¹) for 8 days before collection. cDNA samples were diluted 1:5 for FT expression and 1:20 for CO expression. FT expression was monitored by PCR using FT and UBQ10 specific primers (see supplemental protocols for primer sequences). We found 30 cycles for FT and 20 cycles for UBQ-10 to be within the log-linear phase of amplification on a template dilution series. RT-PCR products were visualized on agarose gels with ethidium bromide staining and quantified using ImageQuant (GE Healthcare) software. CO expression was monitored by qRT-PCR as described above (see supplemental protocols for primer sequences).
Supplemental Material:
Supplemental Protocols: See this section for a description of the CAPS markers used to genotype the EMS alleles described in this study, primer sequences for used for genotyping lines obtained from ABRC, the sequences of primers used in qRT-PCR experiments, and the sequences of primers used for semi-quantitative RT-PCR, and supplemental references.

Figure S1: CCR2::LUC plants maintain rhythmic luciferase activity after more than two weeks in constant darkness.

Figure S2: Period distribution of EMS mutagenized Col CCR2::LUC population.

Figure S3: Location of ZTL, LHY, and GI mutations utilized in this study.

Figure S4: Fluence response of hypocotyl phenotypes in gi mutants grown in constant red, blue, or white light.

Figure S5. Comparison of predicted and observed effects of toc1 and gi mutations on gene expression.

Table SI: Complementation testing of gi, ztl, and lhy mutants.

Table SII: CCR2::LUC expression phenotypes in ztl and lhy mutants.

Supplemental Protocols
EMS allele CAPS markers
EMS alleles generated through this screen can be detected with CAPS or dCAPS markers. ztl-100, ztl-101, and ztl-104 were amplified with CAPS primers F-5’-AGC AAG GTT TGC TGA ACG AT-3’ and R-5’-CAG GCA GCT GGA ATC TCT CT-3’ followed by digest with AccI (New England Biolabs); wild type produces a 238bp band and the mutant produces two bands of 186bp and 52bp. ztl-102 can be detected with dCAPS primers F-5’-TAC CTT TGT TTT AGA TCT GAA TTC TGA TTA CG-3’ and R-5’-CAA GTA GGC GGC TTA GCA TC-3’ followed by digest with HpyCH4IV (New England Biolabs); wild type produces a band of 195bp and the mutant produces bands of 165bp and 30bp. ztl-103 can be detected with primers F-5’-TGG TGC TAA AAG ACT CGG TTG-3’ and R-5’-CAA CCA GAT TGG AGC CAT TT-3’ followed by digest with MboII (New England Biolabs); wild type reveals a 305bp band and mutant gives two
bands of 227bp and 78bp. *lhy-100* can be detected with primers F-5’-TGG AGT TGG AAC TGC AAC AG-3’ and R-5’-CTT ATG CGA GTG TCG GGA AT-3’ followed by digest with HphI (New England Biolabs); wild type produces a 155bp and a 36bp band and mutant produces three bands of 82bp, 73bp, and 36bp. *gi-200* can be detected with dCAPS primers F-5’-GAT TGC TGC TCC TGA AAT CCA GCC CAC CGC AGA AG-3’ and R-5’-ACT GTA AGC GAG CTG GGA AA-3’ followed by digest with MboII (New England Biolabs); wild type produces a 103bp band and mutant produces two bands of 60bp and 43bp.

**SALK T-DNA alleles**

Insertions were confirmed using the T-DNA left border primer LBb1 5’-TGG TTC ACG TAG TGG GCC ATC G-3’and gene specific primers flanking the insertion site. Primer sequences for SALK-069091 were F-5’- GCT TTG ATC TTA CAG GAG GAG C-3’ and R-5’- CAT GAG CTG TGC CAT GAT CT-3’, and for SALK-092757 were F-5’- TTT TTC CCG AAT CAT TTG ATG-3’ and R-5’-AAA TGC GAA GGA TTT CTC CAC-3’.

**qRT-PCR assay for expression of CCA1, LHY, TOC1, GI, CCR2, CO, PP2a**

Real time quantitative PCR was performed using an iCycler (Bio-Rad) and detected with SYBR Green (Molecular Probes). Primers used for *CCA1*, *LHY*, *TOC1*, *GI*, and *CO*, were as described in Mockler et al. (2004), *PP2a* primers were described in Czechowski et al. (2005) and *CCR2* primers were F-5’-CTT GAT CTT CCA GTC TCA CGA T-3’ and R-5’-CGA CGT TAT TGA TTC CAA GAT CA-3’.

**FT expression analysis**

Semi-quantitative RT-PCR was preformed using *FT* specific primers F-5’-ACT ATA TAG GCA TCA TCA CCG TTC GTT ACT CG-3’ and R-5’-ACA ACT GGA ACA ACC TTT GGC AAT G-3’ (Blázquez and Weigel, 1999) for 30 cycles. A dilution series indicated cDNA concentrations were within the log-linear phase of amplification.

*UBQ10* specific primers F-5’-TCA ATT CTC TCT ACC GTG ATC AAG-3’ and R-5’-
TTA CAT GAA ACG AAA CAT TGA ACT TC-3’ (J. Callis, personal communication) amplified for 20 cycles were within the log-linear phase of amplification.

**Supplemental Refences**

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Table I – Phenotypes in different light conditions at 22°C. Variance weighted mean period ± variance weighted standard error (n) was calculated for the period of the free-running rhythms of CCR2::LUC expression. The indicated genotypes were entrained as described in Figure 1 and then transferred to the indicated light conditions. Period estimates were generated using the method described by Plautz et al. (1997) and statistical comparisons were made using a two-tailed Student’s t-test.

| genotype   | Dark     | Red Light (55 µmol m⁻² s⁻¹) | Blue Light (12 µmol m⁻² s⁻¹) | Red+Blue Light (55 + 12 µmol m⁻² s⁻¹) |
|------------|----------|------------------------------|------------------------------|---------------------------------------|
| Col        | 27.48 ±0.52 (17) | 24.69 ±0.03 (35) | 24.88 ±0.11 (36) | 25.12 ±0.07 (15) |
| gi-200     | 25.40 ±0.21 (53) | 21.08 ±0.06 (16) | 21.84 ±0.11 (36) | 22.47 ±0.08 (15) |
| gi-201     | N/D      | 26.14 ±0.42 (18) | 22.25 ±0.81 (7)  | 24.44 ±0.62 (15) |
| toc1-2     | N/D      | 22.54 ±0.20 (27) | 21.00 ±0.10 (11) | N/D                     |
| gi-200 toc1-2 | N/D    | 18.78 ad±0.17 (38) | 18.87 ad±0.06 (49) | N/D                     |
| ztl-105    | 30.83 ±0.74 (2/36) | 29.69 ad±0.31 (6) | 28.92 ±0.11 (36) | N/D                     |
| gi-200 ztl-105 | N/D    | 27.57 ad±0.11 (39) | 27.10 ad±0.24 (15) | N/D                     |

a period estimate significantly different from Col (p<0.001)
b period estimate significantly different from Col (p<0.01)
c period estimate marginally different from Col (p=0.06)
d period estimates are significantly different from both single mutant parental strains (p<0.01)
e period estimates were generated for only the fraction of plants indicated due to low luciferase activity
f period estimates represent pools of approximately 10 plants each
N/D not determined.
Table SI – Complementation testing of period mutants. The indicated F1 seedlings were entrained as described in Figure 1 before being assayed in constant red light of 55 µmol m⁻²s⁻¹ at 22°C. Data represents variance weighted mean period ± variance weighted standard error (n) of the period of CCR2::LUC cycling.

| genotype X Col | gi-201 | ztl-105 | lhy-20 |
|---------------|--------|---------|--------|
| Col           | 24.69±0.03 (35) | 24.34±0.12 (36) | 27.24±0.13 (15) | 23.97±0.06 (31) |
| gi-200        | 23.17±0.15 (37) | 20.84±0.08 (41) |        |        |
| ztl-100       | 26.40±0.08 (25) |        | 27.99±0.12 (26) |        |
| ztl-101       | 26.49±0.06 (21) |        | 28.36±0.05 (27) |        |
| ztl-102       | 26.24±0.10 (26) |        | 28.07±0.05 (53) |        |
| ztl-103       | 26.74±0.10 (18) |        | 27.54±0.11 (24) |        |
| ztl-104       | 26.43±0.08 (11) |        | 28.37±0.09 (18) |        |
| lhy-100       | 24.06±0.06 (92) |        |        | 22.20±0.18 (22) |

* p<0.01 as compared to Col control, † p<0.01 as compared to the F1 of the mutant x Col backcross
Table SII – Mutant phenotypes in different light conditions at 22°C. Plants were entrained as described in Figure 1 and transferred to the indicated light conditions for period determination. Variance weighted mean period ± variance weighted standard error (n) are reported for the period of CCR2::LUC cycling. All mutant phenotypes were significantly different from Col (p<0.001).

| genotype | Dark        | Red Light           | Blue Light           |
|----------|-------------|---------------------|----------------------|
|          | (55 µmol m^2·s^-1) |                     | (12 µmol m^2·s^-1)  |
| Col      | 27.48±0.52 (17) | 24.69±0.03 (35)     | 24.88±0.11 (36)     |
| ztl-100  | 31.32±0.10 (50) | 28.38±0.05 (84)     | 28.21±0.09 (36)     |
| ztl-101  | 32.72±0.24 (48) | 28.41±0.05 (39)     | 28.05±0.10 (54)     |
| ztl-102  | 30.73±0.25 (63) | 27.52±0.06 (38)     | 27.31±0.10 (54)     |
| ztl-103  | 33.38±0.66 (21) | 28.70±0.11 (38)     | 29.97±0.21 (51)     |
| ztl-104  | 31.32±0.10 (50) | 28.57±0.09 (50)     | 28.42±0.06 (68)     |
| ztl-105  | 30.83±0.74 (2/36) | 29.69±0.31 (6)       | 28.92±0.11 (36)     |
| lhy-100  | 25.03±0.30 (29) | 21.81±0.03 (50)     | 22.68±0.08 (36)     |
| lhy-20   | N/D          | 22.18±0.03 (72)     | N/D                 |

^a period estimates were generated for only the fraction of plants indicated due to low luciferase activity
^b period estimates represent pools of approximately 10 plants each
N/D-not determined.
Figure captions:

Figure 1. *CCR2* expression is altered in *gi* mutants. Plants were entrained for eight days in 12 hours light/12 hours dark cycles at 22°C before release to constant light conditions at 22°C. A, B, C. Rhythmic *CCR2::LUC* expression was monitored in (A) constant red light of 55 µmol m⁻² s⁻¹ (n=11-12), (B) constant blue light of 12.5 µmol m⁻² s⁻¹ (n=11-12), and (C) the combination of constant red and blue light of the above fluence rates, delivering a total fluence rate of 67.5 µmol m⁻² s⁻¹ (n=15). Data depicted are indicative of at least two independent assays. *gi-201* pool is data collected from groups of approximately 10 seedlings. D. Endogenous *CCR2* expression was examined in constant white light of 55 µmol m⁻² s⁻¹ at 22°C using qRT-PCR and normalizing *CCR2* levels to *PP2a* expression (n=3).

Figure 2. Amplitude of *CCR2::LUC* expression varies in *gi-200* in different light conditions. Plants were entrained as described in Figure 1 and then released to constant red, blue, or red plus blue light. Luciferase activity was monitored in (A) constant red light of 55 µmol m⁻² s⁻¹ (n=11-12), (B) constant blue light of 12.5 µmol m⁻² s⁻¹ (n=11-12), and (C) the combination of constant red and blue light of the above fluence rates, delivering a total fluence rate of 67.5 µmol m⁻² s⁻¹ (n=15). cRL = constant red light, cBL = constant blue light, and cR+BL = constant red plus blue light. Amplitude was determined using FFT-NLLS as described in Plautz et al., (1997). Data are indicative of at least two independent assays. Statistical comparisons were made using a two-tailed Student’s t-test.

Figure 3. Fluence response of rhythmic *CCR2::LUC* expression is altered in *gi-200*. Plants were entrained as described in Figure 1 and then luciferase activity was monitored in plants transferred to continuous (A) red (n=11-12), (B) blue (n=11-12), or (C) red+blue (n=15) light of various fluences. cRL = constant red light, cBL = constant blue light, and cR+BL = constant red plus blue light. Data are representative of at least two independent assays.
Figure 4. The hypocotyl elongation response to many light conditions is altered in gi mutants. Plants were grown in constant white light of 4.3 µmol m\(^{-2}\)s\(^{-1}\), constant red light of 45 µmol m\(^{-2}\)s\(^{-1}\), or constant blue light of 4.4 µmol m\(^{-2}\)s\(^{-1}\) for 6 days before measurement. * indicates a significant difference from WT (p<0.02) calculated by Student’s t-test. Data are representative of at least two independent assays.

Figure 5. Expression of central clock components in constant white light. Plants were grown for 8 days in light/dark (12:12) cycles before release to constant white light of 55 µmol m\(^{-2}\)s\(^{-1}\). Tissue was collected for RNA extraction every 3 hours. Levels of (A) CCA1, (B) LHY, (C) TOC1, and (D) GI mRNA were monitored by qRT-PCR and normalized to PP2a. These data represent the average of two or three replicate experiments.

Figure 6. CCR2::LUC expression in (A,B) gi-200 toc1-2 and (C,D) gi-200 ztl-105 double mutants. Plants were entrained as described in Figure 1 and then monitored in (A,C) constant red light of 55 µmol m\(^{-2}\)s\(^{-1}\) (n=33-48) or (B,D) constant blue light of 12.5 µmol m\(^{-2}\)s\(^{-1}\) (n=12-48). Luciferase activity (but not CCR2 mRNA levels) were reduced in ztl-105 plants; therefore, clumps of approximately 10 seedlings rather than individual seedlings were analyzed in the ztl-105 and gi-200 ztl-105 genotypes. Data are representative of at least two independent assays; period estimates are shown in Table I. Data between light conditions are not directly comparable as monitoring was performed for 20 minutes per image (A, C) using a DU434-BV (Andor Technology) or for 15 minutes per image (B, D) using an ORCA II ER (Hamamatsu).

Figure 7. gi-200 retains the ability to stimulate flowering through CO and FT. (A) Leaf number at bolting in long days and short days; * indicates the leaf number is significantly different from the appropriate wild-type control (p<0.05 (n=15-18)). (B) qRT-PCR for CO normalized to PP2a (n=2). (C) Semi-quantitative RT-PCR gel images and (D) quantification of FT expression normalized to UBQ-10. For panels (B – D), plants were grown in short days. These experiments were performed twice with similar results.
Figure S1. CCR2::LUC plants maintain rhythmic luciferase activity after more than two weeks in constant darkness. CCR2::LUC and CAB2::LUC plants were entrained for eight days in 12:12 light/dark cycles before release to constant darkness. Luciferase activity was monitored with a Packard TopCount luminometer.

Figure S2. Representative period distributions for CCR2::LUC activity in parental and EMS-mutagenized M2 populations. Period estimates were generated as described in Table I.

Figure S3. Locations of mutations. 5’-UTR are represented by downward diagonal lines, exons are dark, introns are white, and 3’-UTR are indicated by upward diagonal lines. (A) ZTL EMS mutations are indicated: ztl-103 is G1680A, ztl-102 is C1822T, ztl-100, ztl-101, and ztl-104 are C1977T, and ztl-105 is SALK T-DNA line 069091 with a predicted insertion before nucleotide 1717. (B) lhy-20 is a T-DNA line with the insertion predicted to occur before nucleotide 623, and the EMS-generated allele lhy-100 has a C2136T alteration. (C) The EMS-generated GI allele gi-200 alters G3704A; gi-201 is a T-DNA line (SALK-092757) with a predicted insertion before nucleotide 281; gi-1 contains a deletion between bases 4327 and 4331 causing a predicted premature stop codon; gi-2 contains a deletion between nucleotides 670 and 677 causing a frame shift and truncation of the protein; gi-3 contains a C3929T transition leading to a premature stop codon; and gi-4 is altered at G4750T, the splice junction of intron 12, with unknown effects on the protein.

Figure S4. Fluence response of hypocotyl phenotypes in gi mutants grown in constant red, blue, or white light. (A,B) constant red light (cRL), (C,D) constant blue light (cBL), (E,F) constant white light (cWL). Plants were grown in the indicated conditions for six days before measurement.

Figure S5. Comparison of double mutant expression data with simulation data derived from the interlocking loop model using the program Circadian Modelling 2.0 (described in Locke et al., 2005; software available at http://millar.bio.ed.ac.uk/). Experimental
samples were collected and analyzed as described in Figure 5. For modeling, the default parameters were used except as noted. (A) Observed pattern of TOC1 expression in Col, gi-200, gi-201, and toc1-2. (B) Simulation of TOC1 expression in wild type and a gi mutant. The gi-201 mutant was simulated as a 50% loss of function in ‘Y’ activity (rate constant of mRNA translation = 0.124 hr⁻¹), and the toc1-2 mutant simulated as a 90% loss-of-function allele (rate constant of mRNA translation = 0.432 hr⁻¹) as toc1-2 has been shown to have at least 6% of normally spliced TOC1 message (Strayer et al., 2000). (C) Observed pattern of GI expression. (D) Simulated pattern of ‘Y’ expression with the same rate constants as in B.
A

B

C

Period estimate (hours)

amplitude

cRL

amplitude

cBL

amplitude

cR+BL

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Fluence rate ($\mu$mol m$^{-2}$s$^{-1}$)

| Fluence rate (µmol m$^{-2}$s$^{-1}$) | Period estimate (hours) |
|--------------------------------------|-------------------------|
| 20                                   | 21                      |
| 21                                   | 22                      |
| 22                                   | 23                      |
| 23                                   | 24                      |
| 24                                   | 25                      |
| 25                                   | 26                      |
| 26                                   | 27                      |
| 27                                   | 28                      |

A: Col
B: cRL
C: cBL

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A

Time in constant white light (hours)

Relative expression (CCA1/PP2a)

Col
- gi-200
- gi-201
- toc1-2

B

Relative expression (LHY/PP2a)

Col
- gi-200
- gi-201
- toc1-2

C

Relative expression (TOC1/PP2a)

Col
- gi-200
- gi-201
- toc1-2

D

Relative expression (GI/PP2a)

Col
- gi-200
- gi-201
- toc1-2

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