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GIGANTEA and *EARLY FLOWERING 4* in *Arabidopsis* Exhibit Differential Phase-Specific Genetic Influences over a Diurnal Cycle

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ABSTRACT The endogenous circadian clock regulates many physiological processes related to plant survival and adaptability. GIGANTEA (GI), a clock-associated protein, contributes to the maintenance of circadian period length and amplitude, and also regulates flowering time and hypocotyl growth in response to day length. Similarly, *EARLY FLOWERING 4* (*ELF4*), another clock regulator, also contributes to these processes. However, little is known about either the genetic or molecular interactions between *GI* and *ELF4* in *Arabidopsis*. In this study, we investigated the genetic interactions between *GI* and *ELF4* in the regulation of circadian clock-controlled outputs. Our mutant analysis shows that *GI* is epistatic to *ELF4* in flowering time determination, while *ELF4* is epistatic to *GI* in hypocotyl growth regulation. Moreover, *GI* and *ELF4* have a synergistic or additive effect on endogenous clock regulation. Gene expression profiling of *gi*, *elf4*, and *gi elf4* mutants further established that *GI* and *ELF4* have differentially dominant influences on circadian physiological outputs at dusk and dawn, respectively. This phasing of *GI* and *ELF4* influences provides a potential means to achieve diversity in the regulation of circadian physiological outputs, including flowering time and hypocotyl growth.

Key words: microarray; *LHY*; endogenous clock; *GI*; *ELF4*.

INTRODUCTION

The Earth's rotation is responsible for the day–night cycle. Living organisms recognize the external changes produced by this cycle and generate their own endogenous daily rhythms known as a circadian rhythm. This endogenous rhythm having almost 24-h period can be entrained by day–night cycles and be sustained without external stimuli. Living organisms use circadian rhythms to adapt themselves to the environment (Dodd et al., 2005). The endogenous clock is composed of a series of interlocking molecular feedback loops that are conserved in most organisms (Harmer, 2009; Young and Kay, 2001).

Plant clock recognizes photoperiods and uses daily rhythms in many developmental processes from seedling growth to flowering, as demonstrated in *Arabidopsis thaliana* (de Montaigu et al., 2010). A recent model for the *Arabidopsis* clock described a series of multiple interlocking feedback loops referred to as the morning, core, and evening loops. These loops are interlocked in a complex manner: (1) the core loop including *TIMING OF CAB EXPRESSION 1* (*TOC1*), *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), and *LATE ELONGATED HYPOCOTYL* (*LHY*) (Alabadi et al., 2001; Locke et al., 2005a, 2005b); (2) the morning loop, inducing *PSEUDO RESPONSE REGULATOR 9* (*PRR9*) and *PSEUDO RESPONSE REGULATOR 7* (*PRR7*), which are linked to *CCA1/LHY* (Locke et al., 2006; Zeilinger et al., 2006); and (3) the evening loop, including GI and ZEITLUPE (ZTL), which are connected to *TOC1* in the core loop (Pokhilko et al., 2010). In addition,

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EARLY FLOWERING 3 (*ELF3*) acts as a component of the circadian clock input pathway (McWatters et al., 2000) and *EARLY FLOWERING 4* (*ELF4*) has been suggested as another component in the core loop (Doyle et al., 2002; McWatters et al., 2007).

Genes such as *TOC1*, *CCA1*, and *LHY* were isolated via direct circadian screens. However, *GI*, *ELF3*, and *ELF4* were identified using non-circadian forward genetic screens, such as for altered flowering time (Hall and McWatters, 2005). *GI* was initially isolated as a photoperiodic flowering regulator and *gi* mutants show an aberrant flowering phenotype caused by altered circadian rhythms (Fowler et al., 1999; Park et al., 1999). Although *GI* encodes a protein with domains of unknown functions, recent reports have suggested that GI stabilizes the photoreceptor protein, ZTL, via the formation of a blue light-dependent complex (Kim et al., 2007). GI also interacts with ELF3, which recruits CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1), leading to GI protein degradation during the night (Yu et al., 2008). Additionally, GI can be found at *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) promoters where interactions between GI and FLAVIN-BINDING, KELCH REPEAT F-BOX 1 (FKF1) modulate *CO* mRNA expression through degradation of the *CO* repressor, CYCLING DOF FACTOR 1 (CDF1) (Sawa et al., 2007). GI interacts with SHORT VEGETATIVE PHASE (SVP), TEMPRANILLO (TEM) 1, and TEM2 *in vivo* and controls expression at the *FT* promoter (Sawa and Kay, 2011). Furthermore, *GI* also functions in hypocotyl growth at the seedling stage via an unknown molecular mechanism (Huq et al., 2000; Nozue et al., 2007).

ELF4-deficient mutants show an early flowering phenotype with increased *CO* expression, while *ELF4* overexpressors exhibit delayed flowering (Doyle et al., 2002; McWatters et al., 2007). *ELF4* is also involved in *PHYTOCHROME B* (*PHYB*)-dependent seedling growth (Khanna et al., 2003) and a recent study revealed that an ELF3/ELF4/LUX complex binds to *PHYTOCHROME INTERACTING FACTOR* (*PIF*) *4* and *PIF5* promoters to control their expression (Nusinow et al., 2011). *ELF4* is involved in many of the same physiological processes as *GI* but the *ELF4*/*GI* interaction has been rarely investigated. Studies in pea (*Pisum sativum*) suggest that *DIE NEUTRALIS* (*DNE*) and *LATE BLOOMER 1* (*LATE1*), orthologs of *ELF4* and *GI*, respectively, interact genetically to regulate flowering time (Liew et al., 2009). *late1* is epistatic to *dne* in regulating flowering time, but flowering by both *DNE* and *LATE* 1 seemed not to regulate *CO*-like genes in pea (Hecht et al., 2007; Liew et al., 2009).

In this study, we investigated the genetic interactions between *GI* and *ELF4* in the regulation of the circadian clock, flowering time, and hypocotyl growth in *Arabidopsis*. The results show diverse genetic interactions between *GI* and *ELF4* in regulation of these and other processes. Furthermore, differential dominant influences between *GI* and *ELF4* were confirmed by genome-wide gene expression analysis of *gi*, *elf4*, and *gi elf4* mutants at dawn and dusk. The results suggest that differences in the functional dominance of *GI* and *ELF4* during a day may provide a means by which a diverse set of circadian physiological outputs can be coordinated.

RESULTS AND DISCUSSION

gi Is Epistatic to *elf4* in Photoperiodic Flowering Time Regulation

In plants, flowering represents the transition from vegetative to reproductive development. The determination of flowering time is regulated by multiple pathways including the aging, autonomous, vernalization, gibberellin, ambient temperature, and photoperiodic pathways (Mouradov et al., 2002; Fornara et al., 2010). Plants with defects in maintaining circadian rhythms often show an altered flowering time in response to photoperiod (Schaffer et al., 1998; Park et al., 1999; Strayer et al., 2000). In *Arabidopsis*, *GI* and *ELF4* mutants have late and early flowering time phenotypes, respectively. The flowering time phenotypes in both mutants were ascribed to the altered circadian rhythms (Park et al., 1999; Doyle et al., 2002). The orthologs of *GI* and *ELF4* in Pea, *LATE1*, and *DNE*, respectively, control flowering time through a linear hierarchy in which *dne* acts upstream of *late1* (Liew et al., 2009).

We attempted to confirm whether this interaction is conserved in *Arabidopsis* using *gi-2* and *elf4-209* alleles, respectively (Fowler et al., 1999; Kolmos et al., 2009). The *elf4-209* mutant (*elf4*) contains a premature stop codon and produces a 26 amino acid polypeptide (Kolmos et al., 2009). However, the *elf4-209* mutant was generated by Targeting Induced Local Lesions IN Genomes (TILLING) so, to exclude the possible effects of the other mutations, we backcrossed *elf4-209* with Col-0 (WT) for three generations and then introduced this allele into the *gi-2* background to generate *gi elf4* double mutants.

The *elf4* and *gi* single mutants showed early- and late-flowering phenotypes, respectively (Figure 1A), as reported previously (Fowler et al., 1999; Doyle et al., 2002). The flowering time in *gi elf4* double mutants was similar to that of *gi* single mutant under LD (Figure 1A). We further quantitatively evaluated the flowering time of all mutant combinations by counting total leaf number (rosette and cauline) at the appearance of the first flower (Figure 1B). The early flowering of *elf4* in SD was statistically significant, but not as dramatic as previously described in Ws (Doyle et al., 2002; Khanna et al., 2003). The late-flowering phenotype in *gi* mutants was much more obvious in LD than that in SD as previously described (Fowler et al., 1999; Park et al., 1999; Mizoguchi et al., 2005). The flowering times in *gi elf4* double mutants under both LD and SD were not statistically different from those of *gi* single mutants. Therefore, the results indicate that *gi* is epistatic to *elf4* in photoperiodic flowering time regulation, thus forming a hierarchy similar to that described in pea (Hecht et al., 2007; Liew et al., 2009).

CO is an integrator in the photoperiodic pathway that accelerates flowering in response to long days, and its expression is under the control of the endogenous clock (Suarez-Lopez et al., 2001). During daylight, the CO protein directly induces *FT* as a florigen signal, and *FT* then promotes flowering

(Valverde et al., 2004). To confirm and extend the epistatic effects of *gi* over *elf4* in the regulation of flowering time at the molecular level, we examined *CO* and *FT* mRNA levels under LD and SD conditions in the single and double mutants. In WT plants, the *CO* expression level was lower under SD than under LD, and the shoulder shape of *CO* expression near dusk in LD disappeared under SD (Figure 1C and 1E). The *FT* expression level in WT was also reduced under SD, compared to that under LD (Figure 1D and 1F). *CO* and *FT* expression levels in *gi* mutants were almost completely eliminated under both LD and SD conditions. The *CO* and *FT* expression patterns in *gi elf4* double mutants mirrored those in *gi* single mutants. The data showed a mild increase in expressions of *CO* and *FT* in *elf4* mutant, relative to WT, in contrast to the data previously reported (Doyle et al., 2002). Nonetheless, these results indicate that *gi* is epistatic to *elf4* in the regulation of *CO* and *FT* expression.

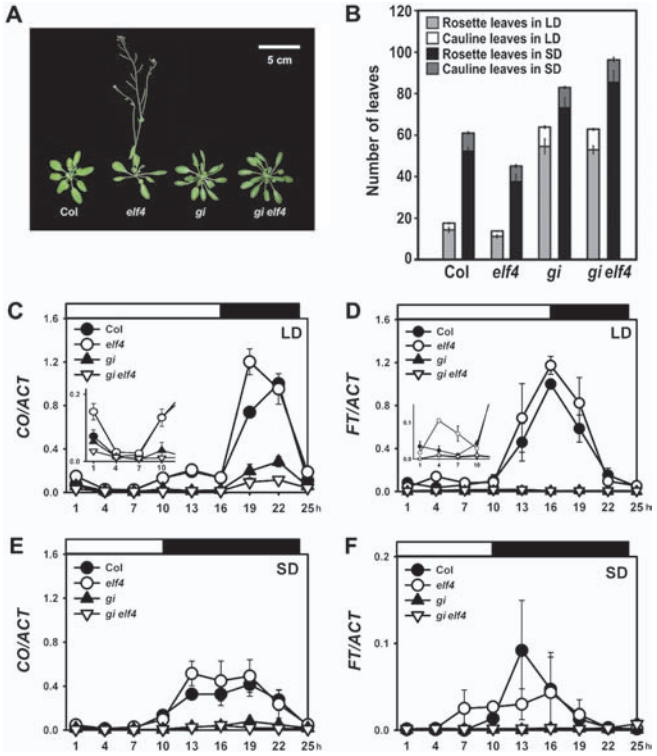

Figure 1. Photoperiodic Flowering Time in WT and Mutants.

(A) Whole plant images. Plants were grown under LD for 4 weeks: Col (WT), *elf4*, *gi*, and *gi elf4* mutants. The scale bar represents 5 cm.

(B) Total numbers of leaves. Total leaves including rosette and cauline leaves were counted at the first flower bloom. White and black bars represent LD and SD, respectively. Data represent the means ± 95% confidence interval (CI) from at least 16 plants. Asterisks indicate statistically significant differences (Col versus *elf4*; $P < 0.01$).

(C, E) *CO* mRNA expression levels under LD (C) and SD (E).

(D, F) *FT* mRNA expression levels under LD (D) and SD (F). Total RNA was isolated from 7-day-old seedlings, and *CO* and *FT* mRNA were measured by quantitative PCR and normalized against *Actin 2* (*ACT*). White and black bars represent day and night, respectively. Data represent the means ± standard error (SE) from biological triplicates.

elf4 Is Epistatic to *gi-2* in Hypocotyl Growth Regulation

Hypocotyl growth is coordinately controlled by external stimuli and the endogenous clock (Dowson-Day and Millar, 1999). The circadian clock regulates the rhythmic growth of seedlings and gates the activity at night. *PIF4* and *PIF5* are key factors in controlling these phenomena (Nozue et al., 2007). *GI* and *ELF4* also play roles in seedling growth (Huq et al., 2000; Khanna et al., 2003; Nozue et al., 2007). Thus, we examined the genetic interactions of *GI* and *ELF4* in the regulation of hypocotyl growth. When 7-day-old seedlings were grown under LD, *elf4* mutants showed significantly longer hypocotyls than WT plants and *gi* mutants showed slightly longer hypocotyls than the WT (Figure 2A). The hypocotyl length in *gi elf4* double mutants was similar to that of *elf4* single mutants. When the seedlings were grown under SD, hypocotyl lengths in WT were longer than those under LD (Figure 2B). Similarly to the LD condition, *elf4* seedlings under SD had significantly increased hypocotyl lengths, compared to WT, and hypocotyl lengths in *gi elf4* double mutants were similar to those in the *elf4* single mutants. The results imply that *elf4* is epistatic to *gi* in regulating seedling growth.

Hypocotyl growth increases dramatically late at night. Growth at night is controlled by the endogenous clock, which

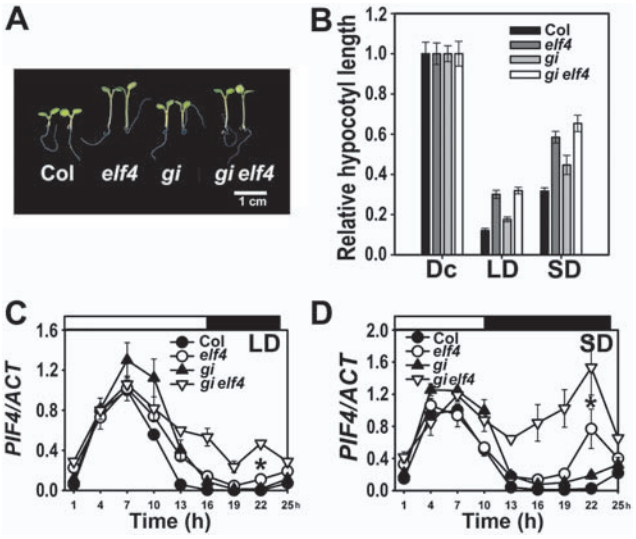

Figure 2. Seedling Growth in WT and Mutants.

(A) Seedling images of Col (WT), *elf4*, *gi*, and *gi elf4* seedlings. Plants grown under LD for 7 d were imaged. The scale bar represents 1 cm.

(B) Relative hypocotyl lengths under LD and SD. Hypocotyl lengths were measured and normalized to the mean hypocotyl length under continuous dark (DD) conditions. Data represent the means ± 95% CI from at least 20 seedlings.

(C, D) *PIF4* mRNA expression levels under LD (C) and SD (D). Total RNA was isolated from 7-day-old seedlings, and *PIF4* mRNA expression levels were measured by quantitative PCR and normalized to that of *ACT*. White and black bars represent day and night, respectively. Data represent the means ± SE from experiments performed in triplicate. Asterisks indicate statistically significant differences (Col versus *elf4*; $P = 0.02$ in LD and $P = 0.12$ in SD).

induces *PIF4* expression (Nozue et al., 2007). The *gi* and *elf4* mutants exhibited defects in photoperiod dependent hypocotyl growth. To examine the relationship between *elf4* and *gi* in the regulation of hypocotyl growth at the molecular level, we examined *PIF4* expression levels under diurnal conditions (Figure 2C and 2D). *gi* and *elf4* mutants showed differential effects on *PIF4* expression level during day and night. We first focused on their effects during the night when hypocotyl growth is most apparent. In the *elf4* mutant, increased *PIF4* expression levels at night were significantly increased under SD ($P = 0.02$ between WT and *elf4* at ZT22; Figure 2D), but not significantly so under LD ($P = 0.12$ between WT and *elf4* at ZT22; Figure 2C), indicating that *ELF4* suppresses *PIF4* expression mostly at night. In contrast, the *PIF4* expression levels in *gi* mutants during the night were similar to those in WT under LD (Figure 2C), and only marginally higher under SD (Figure 2D). Interestingly, the *PIF4* expression levels in *gi elf4* double mutants at night were much higher than those in either *gi* or *elf4* single mutants under both photoperiods (Figure 2C and 2D). The elevated expression levels of *PIF4* in *elf4* appear to be sufficient to accelerate hypocotyl growth, such that the much higher levels of *PIF4* in the *gi elf4* background do not contribute significantly to increasing hypocotyl elongation (Figure 2A and 2B). Therefore, while our results show that *elf4* is epistatically dominant to *gi* in the regulation of hypocotyl growth, the synergistic effect on *PIF4* expression suggests that there is another mechanism governing hypocotyl expansion, independently of *PIF4* levels.

GI and *ELF4* Function Together in Circadian Regulation

GI is known to function in both the core circadian clock and input pathways (Fowler et al., 1999; Park et al., 1999; Kim et al., 2007), and *ELF4* also has a role in the core pathway (Doyle et al., 2002; Kikis et al., 2005; McWatters et al., 2007). To better understand interactions between these two genes in circadian clock regulation, we measured the period and robustness of the clock by a leaf movement assay and a luminescence assay using *COLD, CIRCADIAN RHYTHM*, and *RNA BINDING 2* (*CCR2*) promoter-luciferase reporters under the free-running conditions.

Plant leaves close at night and open during the daytime repetitively and, after light/dark entrainment, these movements persist under continuous light (LL) or dark (DD) conditions. Compared to WT, *gi* mutants showed a circadian period shortened by about 1 h. Moreover, they showed an irregular circadian rhythm with reduced robustness as indicated by the relative amplitude error (RAE) values larger than those of WT (Figure 3A and 3B, and Table 1) (Plautz et al., 1997). Similarly, *elf4* mutants also exhibited a dramatic loss in robustness, with high RAE values as previously reported (Doyle et al., 2002; McWatters et al., 2007). In the *gi elf4* double mutants, the circadian amplitude is further diminished, indicating a synergistic or additive effect of *GI* and *ELF4* on the regulation of circadian rhythmicity (Figure 3B and Table 1). Approximately 50% of the *gi elf4* double mutants appear arrhythmic, and thus the period and RAE could not be assessed. The remaining 50% of plants showed irregular rhythms with significantly reduced robustness (Figure 3A and Table 1). These results indicate that *GI* and *ELF4* together contribute strongly to the regulation of circadian rhythmicity.

To evaluate the collective regulation of circadian rhythm at the molecular level by *GI* and *ELF4*, we further measured the endogenous clock activity by tracking the promoter activity of the clock-controlled gene, *CCR2*, in WT, *gi*, *elf4*, and *gi elf4* plants. In constant light, the *CCR2* promoter showed maximum activity at dusk in WT plants (Figure 3C). However, both *gi* and *elf4* mutants showed significantly irregular rhythms with reduced robustness (Figure 3D), consistent with the reduced robustness shown by leaf movement (Figure 3A). Furthermore, *gi elf4* double mutants had a much longer period and showed a further severe loss of robustness than either the *gi* or *elf4* single mutants (Figure 3C and 3D, and Table 1). In constant dark (DD), the robustness of rhythmic plants was not statistically different among the three types of mutants (Figure 3E and 3F, and Table 1). However, the numbers of rhythmic plants were reduced in both *gi* and *elf4* mutants, compared to those of WT plants, and further reduced in *gi elf4* double mutants (Figure 3F and Table 1). Also, both *gi*, *elf4*, and *gi elf4* mutants exhibited longer period lengths, compared to WT (Figure 3E and 3F, and Table 1), which were not significantly different among the three types of mutants. The results (the RAE values under LL and the reduced numbers of rhythmic plants under DD) indicate that *GI* and *ELF4* have the synergistic or additive effect on the circadian rhythmicity of *CCR2* promoter activity in both LL and DD conditions.

Finally, we measured the mRNA expression levels of a core circadian clock gene, *LHY*, under light/dark cycles to further examine the molecular effects of *GI* and *ELF4* on circadian rhythmicity (Figure 3G and 3H). *LHY* is a core component of the central oscillators of the *Arabidopsis* circadian clock in the morning feedback loop (Alabadi et al., 2001; Locke et al., 2006; Zeilinger et al., 2006). *LHY* expression in *gi* and *elf4* mutants was lower than that of WT as previously reported (Park et al., 1999; Khanna et al., 2003; Mizoguchi et al., 2005; McWatters et al., 2007), and it decreased further to near undetectable levels in *gi elf4* double mutants (Figure 3G and 3H). Taken together, all the data above collectively indicate that *GI* and *ELF4* function either additively or synergistically in *Arabidopsis* circadian clock regulation.

Gene Expression Profiling Reveals Differential Temporal Predominance of GI and ELF4 on the Regulation of Flowering Time, Hypocotyl Growth, and Circadian Rhythmicity

Recent studies revealed that GI induces *CO* at dusk (Sawa et al., 2007) and ELF3/ELF4/LUX complex directly suppresses *PIF4*/*PIF5* expression at night (Nusinow et al., 2011). These results imply that flowering time regulation by GI is modulated during the daytime and hypocotyl growth is regulated by ELF4 complex during the night. Based on these results, we hypothesized that the genetic interactions between *GI* and *ELF4* could be circadian phase-dependent and that the influence of the two

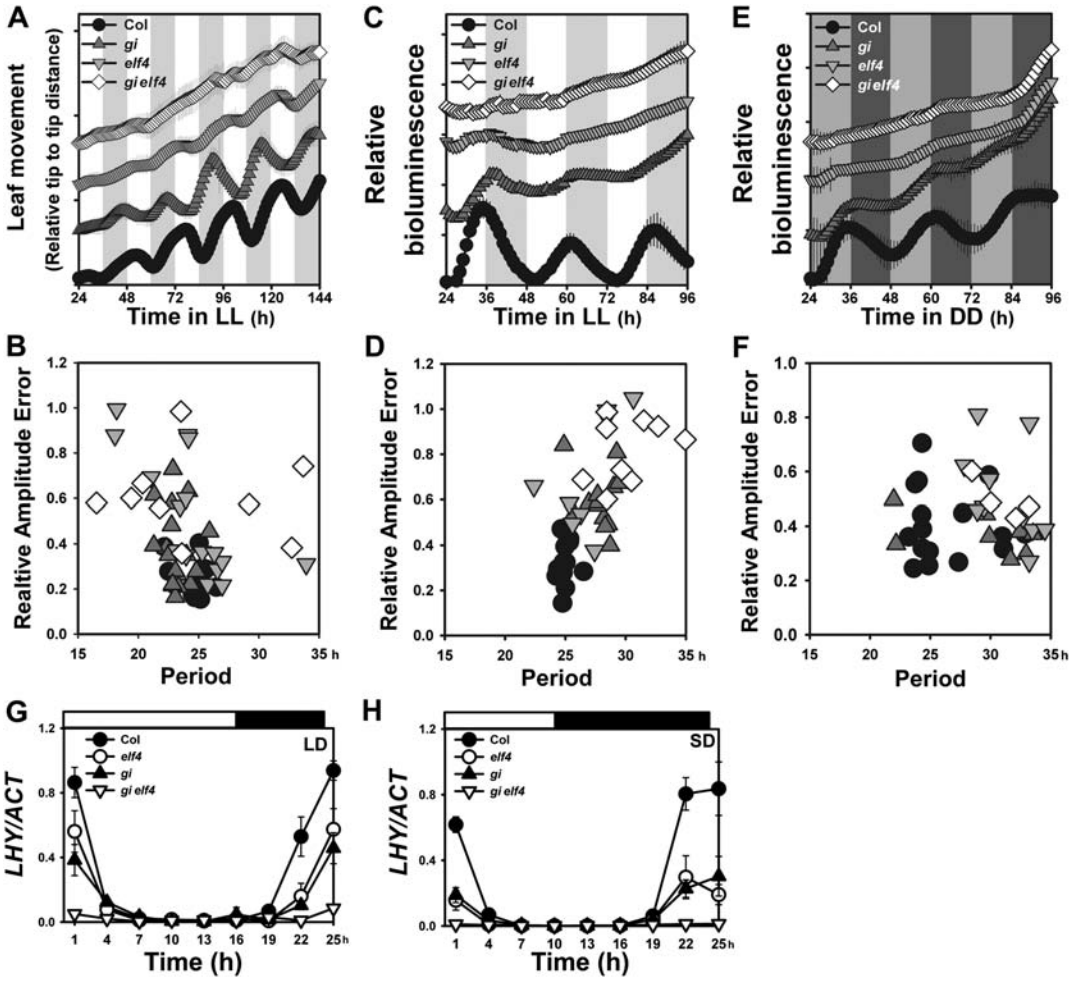

Figure 3. Endogenous Clock Activities in WT and Mutants.

(A) Leaf movements in LL. Tip-to-tip distances between first and second leaves were measured using the leaf movement assay (LMA) program.

(B) Relative amplitude errors (RAE) analyzed by FTT-NLLS.

(C, E) *CCR2* promoter activities in LL (C) and DD (E). Luminescence intensities were measured every hour, and the absolute luminescence intensities were normalized to the mean intensity in each background. Data represent the means ± SE.

(D, F) RAEs analyzed by FTT-NLLS. Period lengths computed from each experiment and statistics are shown in Table 1.

(G, H) *LHY* expression levels in LD (G) and SD (H). Total RNA was isolated from 7-day-old seedlings, and *LHY* mRNA levels were measured by quantitative PCR and then normalized to that of *ACT*. White and black bars represent day and night, respectively. Data represent the means ± SE from experiments performed in triplicate.

genes, relative to each other, might vary throughout a day. To test this hypothesis, we performed gene expression profiling of WT, *gi*, *elf4*, and *gi elf4* plants at dawn (ZT1) and dusk (ZT16). Plants grown under LD for 7 d were used. For each time point, we first identified differentially expressed genes (DEGs; FDR < 0.01) between each type of mutant and WT using a previously reported integrative method (Lee et al., 2010) (see 'Methods'). 1482 and 1766 DEGs at ZT1 and ZT16, respectively, were identified from the three comparisons of (1) *elf4* versus WT (684 and 785 DEGs at ZT1 and ZT16, respectively), (2) *gi* versus WT (570 and 723 DEGs at ZT1 and ZT16, respectively), and (3) *gi elf4* versus WT (918 and 969 DEGs at ZT1 and ZT16, respectively) (Supplemental Figure 1A and Supplemental Table 1). At each time point, the DEGs were categorized into the 26 groups based on their differential expression patterns (Supplemental Figure 1B). Among the 26 groups, three pairs of groups were selected that showed epistatic and synergistic interactions between *gi* and *elf4*. Figure 4 shows the three groups of genes representing *gi* epistasis (Figure 4A), *elf4* epistasis (Figure 4B), and the synergistic effects of *gi* and *elf4* (Figure 4C), respectively. Assessment of the relative epistatic interactions based on differential expression patterns was scored as done for flowering time (Figure 1A), hypocotyl growth (Figure 2A), and circadian rhythmicity (Figure 3A). For example, a group of genes with the same expression changes in *gi* versus WT and *gi elf4* versus WT, but with no expression change in *elf4*

versus WT was considered to indicate that *gi* is epistatic to *elf4* for that particular gene's expression (Figure 4A).

The three groups of genes support the relative genetic relationships between *GI* and *ELF4* as presented above in the regulation of flowering time, hypocotyl growth, and circadian rhythmicity. First, 91 genes were epistatically regulated by *GI* at ZT1 and the number of *GI*-regulated genes is greatly increased up to 301 genes at ZT16, reflecting that the influence of *GI* relative to *ELF4* is more significant at dusk than at dawn. Patterns 1 and 2 at ZT16 contain many genes involved in flowering and photosynthesis pathways, such as *FKF1*, *CO*, *FT*, and *LHCB2.3* (Figure 4A). Enrichment analysis of Gene Ontology Biological Processes (GOBPs) using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al., 2009) also showed that 'flower development' is significantly represented by the genes in Pattern 2 at ZT16 (Figure 5B). These data suggest that *GI* plays a stronger role than *ELF4* in the regulation of dusk-active genes required for flowering.

Second, 193 genes were epistatically regulated by *ELF4* at ZT1 while that number is decreased by about 30% to 129 genes at ZT16. Patterns 3 and 4 include the genes involved in circadian clock and light signaling pathways, such as *TOC1*, *LUX*, *HFR1*, and *PIF4* (Figure 4B). GOBP enrichment analysis also showed that the GOBP term 'response to light stimulus' that is related to hypocotyl growth is significantly represented by the genes in Pattern 3 at ZT1 (Figure 5A).

Table 1. Period Lengths and Relative Amplitude Errors in *gi elf4* Double Mutants.

	Period (h)	RAE	Number of rhythmic (total) plants
Leaf movement assay			
Col	24.64 ± 0.26	0.23 ± 0.02	16 (17)
gi	23.20 ± 0.27	0.38 ± 0.04	18 (18)
elf4	24.50 ± 0.93	0.52 ± 0.07	16 (18)
gi elf4	24.55 ± 2.01	0.61 ± 0.06	9 (17)
P-value	0.49	<0.0001	
CCR2pro::LUC activity (LL)			
Col	24.95 ± 0.13	0.33 ± 0.02	16 (16)
gi	27.85 ± 0.37	0.60 ± 0.03	13 (16)
elf4	26.82 ± 0.88	0.71 ± 0.09	8 (16)
gi elf4	30.12 ± 0.87	0.82 ± 0.05	9 (21)
P-value	<0.0001	<0.0001	
CCR2pro::LUC activity (DD)			
Col	26.34 ± 0.80	0.41 ± 0.03	16 (16)
gi	29.29 ± 1.65	0.37 ± 0.03	8 (16)
elf4	31.22 ± 0.77	0.52 ± 0.06	10 (16)
gi elf4	30.95 ± 1.06	0.5 ± 0.04	4 (19)
P-value	0.003	0.07	

Period lengths and Relative Amplitude Errors (RAEs) were statically analyzed in Col, *gi*, *elf4*, and *gi elf4* using one-way ANOVA and presented in the table. Data represent mean ± 95% CI.

These data suggest that *ELF4* has a stronger influence, relative to *GI*, in the regulation of seedling growth at dawn.

Third, 180 and 119 genes at ZT1 and ZT16 were regulated synergistically/additively by *GI* and *ELF4*, respectively (Figure 4C). These patterns contain clock-regulated genes, such as *PRR5*, *CCL*, *LHY*, *PIF7*, *PIF4*, and *CABs* (Figure 4C). GOBP enrichment analysis further showed that the GOBP term 'response to light stimulus' that is related to circadian rhythm is significantly represented by the genes in Pattern 5 at ZT16 and this biological process is also highly enriched in both Pattern 1 and Pattern 3 (Figure 5B). These data suggest that *GI* and *ELF4* function synergistically at both dawn and dusk in the regulation of circadian rhythmicity. Taken together, the results suggest that the relative functional relationship between *GI* and *ELF4* in the regulation of a variety of physiological processes varies over time along a diurnal cycle. Since expression of both genes is evening-phased (David et al., 2006; Kim et al., 2007; Nusinow et al., 2011), the observation that the mutants affect both evening and morning-phased processes indicates that ELF4 and GI play both repressive and inductive roles in plant development and physiology.

CONCLUSIONS

The endogenous clock in plants regulates diverse circadian physiological outputs. How the clock achieves this diversity still remains elusive. To answer this question, complex genetic interactions between clock genes need to be elucidated. In this study, we investigated genetic interactions between two clock genes, *GI* and *ELF4*, in the regulation of flowering time, hypocotyl growth, and circadian rhythmicity. The results showed that *GI* is epistatic to *ELF4* in the regulation of flowering time, whereas *ELF4* is epistatic to *GI* in the regulation of hypocotyl growth. In addition, *GI* and *ELF4* have a synergistic effect on the regulation of the circadian clock. Gene expression profiling of *gi*, *elf4*, and *gi elf4* mutants further confirmed our qPCR results (Supplemental Figure 2) and extended our analysis to elucidate the differential regulation of *GI* and *ELF4* on circadian outputs at the systems level. Our results further indicate that the relative differential influences of *GI* and *ELF4* on a wide range of processes are achieved in a time-dependent manner: (1) *GI* is epistatic to *ELF4* predominately at dusk, (2) *ELF4* is epistatic to *GI* predominately at dawn, and (3) *GI* and *ELF4* collectively regulate circadian rhythmicity at both dawn and dusk. In summary, our results suggest that the differential relative influences of *GI* and *ELF4* over a diurnal cycle may provide a means to coordinate the regulation of diverse circadian outputs.

METHODS

Plant Materials

We generated *gi-2* mutants as previously described (Koornneef et al., 1991). To generate *elf4* mutants, *elf4-209*

Figure 4. Differential Expression Patterns between Col-0 and Each of *elf4*, *gi*, and *gi elf4* Mutants.

(A) Gene expression patterns representing the relative dominance of *gi* to *elf4*.

(B) Gene expression patterns representing the relative dominance of *elf4* to *gi*.

(C) Gene expression patterns reflecting the synergistic effects of *gi* and *elf4* mutations. Red and green colors represent $\log_2$-fold-changes for up- and down-regulation in *gi*, *elf4*, and *gi elf4* mutants relative to WT, respectively. Patterns were numbered from one to six according to their differential expression. The number of genes in each pattern is denoted in the box. In each pattern, the genes associated with the regulations of flowering time, hypocotyl growth, and circadian rhythmicity.

(Col) (Kolmos et al., 2009) was backcrossed three times to remove other possible mutations with the standard *A. thaliana* laboratory strain, Col-0 (WT), and selected based on long hypocotyl phenotype. We then introduced *elf4-209* into the *gi-2* background and selected *gi-2 elf4-209* double mutants from F3 segregating lines using PCR. The *elf4-209* mutation was identified using primers 5′-AGG CAG AGC AGG GAG AGC CAC CGG CGA T-3′ and 5′-CTT CCA TGG AGC TCT AGT TCC GGC AGC-3′, followed by digestion with *Xcm*I; in this reaction, the *elf4-209* mutation remained undigested. The *gi-2* mutants were isolated using PCR as described previously (Park et al., 1999).

Flowering Time Measurement

To measure day-length-dependent flowering time, seeds were sown on soil following stratification (4°C for 2 d). Plants were grown under either long-day (LD) (16L/8D) or short-day (SD) conditions (10L/14D). Flowering time was measured by counting the number of rosette and cauline leaves when the first flower opened. The data represent the means ± 95% confidence interval (CI) from over 10 plants.

Seedling Growth Measurement

To measure photoperiod-dependent seedling growth, seeds were sown on soil following stratification (4°C for 2 d). Plants were grown under either LD or SD for 7 d. Hypocotyl lengths were measured by Scion Image software (Scion Corp. Frederick, MD, USA). The data represent the means ± 95% CI from over 15 seedlings.

Measurements of Endogenous Clock Activity

To measure leaf movement as a metric of endogenous clock activity, 10-day-old seedlings grown under 12L/12D were transferred to constant light (LL), and images were taken every hour for 7 d. Leaf movement was determined by measuring the tip-to-tip distance between the first and second leaves using Leaf Movement Analysis (LMA) software. The data represent the means ± 95% CI from plants described in Table 1. Alternatively, we measured the clock activity by measuring activities of the *COLD-CIRCADIAN RHYTHM-RNA BINDING* (*CCR2*) gene promoter. Seven-day-old seedlings were transferred to 96-well microplates containing 500 μM luciferin (SYNCHEM, Felsberg/Altenburg, Germany), and images were taken every hour for 4 d. Luminescence intensities from each plant were imported into the Biological Rhythms Analysis Software System (BRASS) (Southern and Millar, 2005), and periods were computed using the FFT-NLLS suite (Plautz et al., 1997).

Measurement of mRNA Expression Levels

To measure *CO* and *FLOWERING LOCUS T* (*FT*) mRNA expression levels, 7-day-old seedlings, grown under LD or SD, were harvested in liquid nitrogen every 3 h. Total mRNA was extracted using WelPrep™ (JOIN BIO-INNOVATION, Daegu, Korea), and DNA was digested by treatment with DNase I (Ambion, Austin, TX). For each sample, 0.75 μg of total mRNA was reverse-transcribed using ImProm II Reverse Transcriptase (Promega, Madison, WI). The amounts of the transcripts were measured by Real-Time PCR, using SYBR Premix Extaq (Takara,

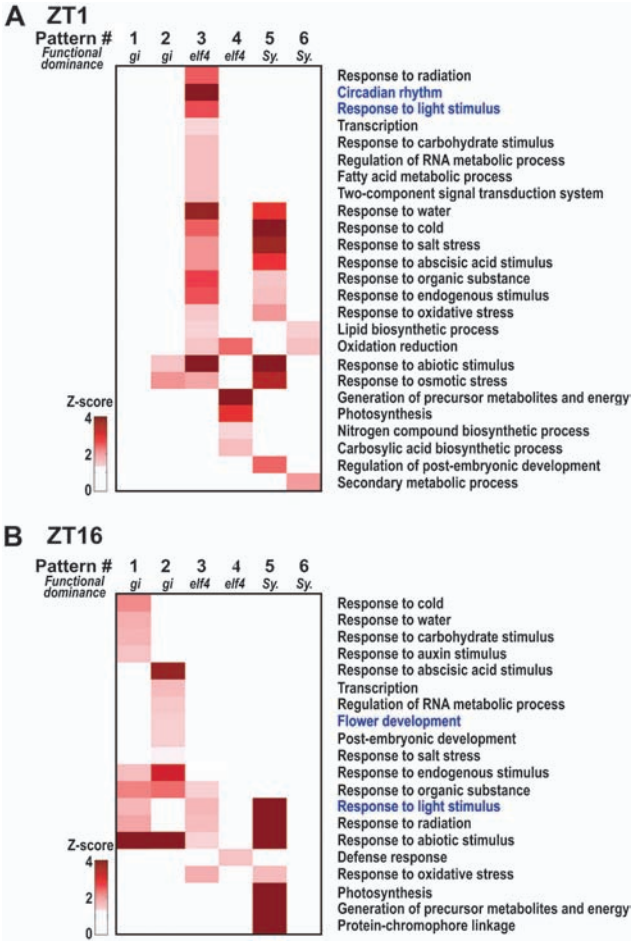

Figure 5. Biological Processes Represented by the Genes in the Six Patterns at Dawn and Dusk.

(A, B) The heat maps of the enrichment scores. A high enrichment score (see 'Methods') in a coordinate (*x*-axis and *y*-axis) of the heat map indicates that the corresponding GOBP (*y*-axis) is significantly represented by the genes in the corresponding pattern (*x*-axis). The red color represents the enrichment score as described in the color bar. Blue letters indicate GOBPs associated with the regulations of flowering time, hypocotyl growth, and circadian rhythmicity.

Shuzo, Kyoto, Japan) and the ABI 7300 Real-Time PCR system (Applied Biosystems, Foster city, CA). The following primers previously reported were used: *CO*, *FT*, *LHY*, and *PIF4* primers (Mockler et al., 2004); *GI* primers (Edwards et al., 2006); *ELF4* primers (Kim et al., 2008); and *ACT* primers (Hall et al., 2003).

Microarray Experiments

Seven-day-old seedlings grown under LD were harvested at ZT1 and ZT16. Total RNA was isolated and used for microarray experiments. The integrity of total RNA was evaluated using the Bioanalyzer 2100 (Agilent, Santa Clara, CA). The RNA integrity in all samples was sufficient for gene expression analysis (RNA integrity number $\geqslant$ 9.5). RNA was reverse-transcribed, amplified, and then hybridized into Customized Arabidopsis Gene Expression Microarrays, containing 43 803 probes corresponding to 25 945 annotated genes, according to the standard Agilent protocols. The levels of mRNAs were measured for three biological replicates of each transgenic plant (wild-type (Col-0), *gi*, *elf4*, and *gi elf4*) at ZT1 and ZT16. Log_2-intensities were normalized using quantile normalization (Bolstad et al., 2003).

Identification of Differentially Expressed Genes

Using the normalized intensities, differentially expressed genes (DEGs) between Col-0 and each mutant were determined using the previously reported integrative method: (1) two independent tests were performed: *t*-test and log_2-median-ratio test; (2) false discovery rates (FDRs) from each test were computed using an empirical distribution of the null hypothesis that the means of the genes are not different, which was obtained from random permutations of the samples; (3) the individual FDRs were combined to compute the overall FDR using Stouffer's method (Hwang et al., 2005); and (4) DEGs were selected as the genes with the overall FDR $<$ 0.01. Finally, potential false positives were further removed by selecting the DEGs fold-change $\geqslant$ 97.5 percentiles or $\leqslant$ 2.5 percentiles of fold-changes obtained from the randomly permuted samples (i.e. the empirical distribution of fold-changes).

Functional Enrichment Analysis

For the genes identified for each pattern, we performed the enrichment analysis of GO biological processes (GOBPs) using DAVID software (Huang da et al., 2009). Among all the resulting GOBPs, we selected those that were significantly enriched ($P < 0.05$) in at least one of the six patterns at ZT1 or ZT16. The enrichment scores were computed as $-\log_{10}(P)$ where P is the P-value from the enrichment analysis.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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