Natural diversity in daily rhythms of gene expression contributes to phenotypic variation

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Daily rhythms of gene expression provide a benefit to most organisms by ensuring that biological processes are activated at the optimal time of day. Although temporal patterns of expression control plant traits of agricultural importance, how natural genetic variation modifies these patterns during the day and how precisely these patterns influence phenotypes is poorly understood. The circadian clock regulates the timing of gene expression, and natural variation in circadian rhythms has been described, but circadian rhythms are measured in artificial continuous conditions that do not reflect the complexity of biologically relevant day/night cycles. By studying transcriptional rhythms of the evening-expressed gene *GIGANTEA* (*GI*) at high temporal resolution and during day/night cycles, we show that natural variation in the timing of *GI* expression occurs mostly under long days in 77 *Arabidopsis* accessions. This variation is explained by natural alleles that alter light sensitivity of *GI*, specifically in the evening, and that act at least partly independent of circadian rhythms. Natural alleles induce precise changes in the temporal waveform of *GI* expression, and these changes have detectable effects on *PHYTOCHROME INTERACTING FACTOR 4* expression and growth. Our findings provide a paradigm for how natural alleles act within day/night cycles to precisely modify temporal gene expression waveforms and cause phenotypic diversity. Such alleles could confer an advantage by adjusting the activity of temporally regulated processes without severely disrupting the circadian system.

diurnal | circadian | rhythms | Arabidopsis | GIGANTEA

In plants, many aspects of physiology and development, including metabolism, growth, flowering, and plant defense, are controlled by genes whose expression pattern oscillates on a daily basis (1, 2). These genes usually show peaks of expression around the time at which their function is required to regulate downstream processes. The timing of expression of most temporally regulated genes is at least partly determined by the circadian clock, an endogenous time-keeping mechanism that generates internal rhythms of ∼24 h (3). When synchronized to the external day/night cycle, circadian clocks confer an advantage to plants and other organisms by improving fitness (4, 5). Importantly, circadian rhythms are generally studied under conditions of continuous light (LL) or continuous dark (DD), in which they are not influenced by environmental transitions. These constant conditions, however, do not reflect the complexity of biologically relevant day/night cycles that organisms experience in nature. During the day, fluctuations in external cues such as light and temperature also contribute to defining the timing and amplitude of biologic processes. These cues influence rhythms of gene expression either indirectly, by synchronizing endogenous circadian rhythms to the external day/night cycle (6–8), and/or directly, by activating signaling pathways that regulate transcription (9–11). Thus, the precise timing and amplitude of daily gene expression patterns are defined by a combination of endogenous and external signals.

Temporal rhythms of expression control plant traits of ecological and agricultural importance (12–16), and understanding how precisely these rhythms vary and how this variation influences phenotypes has broad implications for plant biology. Natural diversity in daily transcriptional rhythms has mostly been analyzed by comparing gene expression between limited numbers of selected genotypes and by using temporal resolutions of relatively low precision (14, 17). To date, there has been no extensive survey describing how rhythms of expression vary at the intraspecies level, at more informative temporal resolutions, and during biologically relevant day/night cycles. The latter point is of particular relevance because natural variation in rhythms has mainly been studied in artificial continuous conditions that are used to determine certain circadian parameters. Natural variation of period length, defined as the length of the circadian cycle, was quantified in constant environmental conditions by measuring rhythms of leaf movements or oscillations of gene expression (4, 18–21). Phase, or the time at which an event occurs within a cycle, also varies extensively when determined in constant conditions (4, 22). Although changes in period length would be expected to influence phase, the relationship between both parameters is still unclear in natural genotypes (4). In summary, it is not known how much daily rhythms of expression vary in natural genotypes, what mechanisms generate this variation, and to what extent this variation influences phenotypic outputs.

Significance

Daily rhythms of gene expression ensure that biological processes occur at the optimal time of day. In plants, temporally regulated processes include traits of ecological and agricultural importance, and understanding how changes in daily rhythms of expression modify such traits has broad implications. We find that natural genetic variation can accurately modify temporal gene expression waveforms during the day by influencing light signaling pathways, rather than circadian rhythms. We further show that changes in transcriptional patterns induced by natural alleles are sufficient to affect downstream molecular outputs and cause phenotypic diversity. Such natural alleles could provide an advantage by adjusting the activity of temporally regulated processes while avoiding the pleiotropic effects associated with severe disruptions of the circadian system.

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These questions were addressed by using *GIGANTEA* (*GI*) as a model temporally regulated gene. *GI* is conserved within the plant kingdom and regulates a variety of phenotypes such as growth of the hypocotyl, flowering time, cold resistance, and starch accumulation (23–29). The peak of *GI* expression occurs in the evening in various plant species and is regulated by the circadian clock (12, 25, 26, 30–32). To monitor *GI* expression at high temporal resolution and in a large number of genotypes, we fused a 2.5-Kb fragment of the *GI* promoter to the luciferase (*LUC*) marker gene. Similar *GI::LUC* fusions had already been shown to faithfully track the rhythmic expression pattern of the endogenous transcript (33–35). With the luciferase system, we could accurately determine the timing of *GI* expression during day/night cycles and detect genetic loci that cause precise changes in the *GI* expression waveform. This genetic information was then exploited to create lines that precisely differ in their *GI* expression patterns and to test whether changes in these patterns affect downstream phenotypes.

Results and Discussion

Natural Genetic Variation Regulates the Timing of *GI* Expression Within Long-Day Cycles. Natural variation in the waveform of *GI* transcription was tested for by introducing *GI::LUC* into 77 *Arabidopsis* accessions. Temporal patterns of luciferase activity were recorded under five day lengths and used to determine the peak time of *GI::LUC* expression (*GI* peak time, or sidereal phase) in each accession and condition. *GI* peak time varied in all day lengths, but the range of peak times was broader in long photoperiods, and the genetic contribution to peak time variation was more significant in long days (LDs) compared with in short days (SDs) (Fig. 1*A* and *SI Appendix*, Fig. S1 and Tables S1 and S2). *GI* peak times measured in LDs of 16 h strongly correlated with peak times measured in LDs of 14 h and LDs of 12 h, but not as strongly with peak times measured in SDs (Fig. 1*B*). These data suggest the existence of mechanisms that cause variability in the timing of *GI* expression specifically in LDs.

Daily patterns of gene expression are controlled by endogenous and environmental inputs. Pathways that convey information from these internal or external signals could therefore contribute to the day length-dependent variation of *GI* peak time observed in the accessions. External light signals, on the one hand, directly influence the timing of *GI* expression in the evening because an extension of the light period after dusk in SDs is sufficient to cause an immediate delay in *GI* peak time (*SI Appendix*, Fig. S2*A*). The earlier onset of darkness in SDs might explain why *GI* expression and other rhythms are advanced in SDs compared with LDs (Fig. 1*A*) (9, 26, 36) and might also explain why natural variation of *GI* peak time is limited under SDs (Fig. 1*A* and *SI Appendix*, *SI Discussion*). Endogenous circadian rhythms, on the other hand, did not seem to be related to *GI* peak time variation in any of the photoperiods (*SI Appendix*, Fig. S2 *B* and *C* and *SI Discussion*). Circadian rhythms measured in LL do not correlate with phase in *Arabidopsis* accessions (4), and we report a similar trend for *GI* peak time and period length measured in DD, where circadian rhythms are not influenced by light (Fig. 1*B*). Although these results do not exclude that period length influences phase in particular accessions (*SI Appendix*, Fig. S2*D*), they do suggest that natural variation of *GI* peak time in LDs might generally be determined by natural alleles that regulate light signaling, rather than endogenous rhythms. Searching for such alleles was the goal of this study.

A cluster analysis identified Lipowiec (Lip-0) as belonging to a group of accessions that showed a late peak of *GI::LUC* expression under LDs (Fig. 1*C* and *SI Appendix*, Tables S3 and S4). Lip-0 *GI::LUC* was crossed to Columbia (Col-0), and extensive phenotyping of the Col-0 X Lip-0 F2 population in different photoperiods confirmed that maximum variability of *GI* peak time was observed in LDs of 16 h (*SI Appendix*, Fig. S3*A*). The F2 population (135 individuals) and subsequently selected F3, F4,

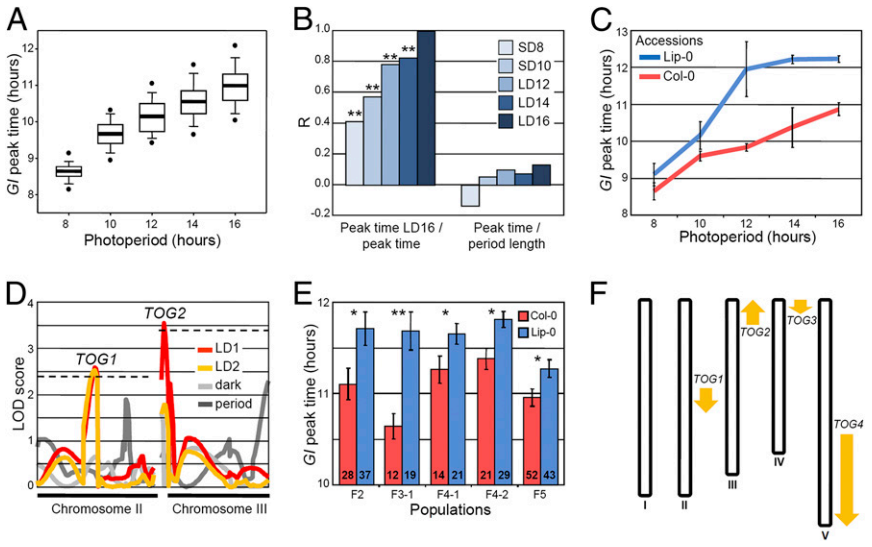

Fig. 1. Natural variation and genetic basis of the timing of *GI* expression during day/night cycles. (*A*) Box plots representing the variation and average (horizontal bars) of *GI* peak time of expression in 77 accessions. (*B*) Correlations between *GI* peak time measured in LDs of 16 h, with *GI* peak time measured in other photoperiods, and between *GI* peak time and period length (DD) measured after entrainment in the same photoperiod. The Pearson correlation coefficient (R) indicates the strength of the correlations, with 1 and −1 indicating perfect positive and negative correlations, respectively. $^{**}P \leq 0.01$. (*C*) *GI* peak time in Col-0 and Lip-0 accessions (mean ± SD of two biological replicates). (*D*) QTL mapping in Col-0 × Lip-0 *GI::LUC* F2 progenies. QTLs were detected for *GI* peak time in two consecutive LDs of 16 h (LD1 and LD2), for *GI* peak time in the first day in darkness after the shift to constant conditions (dark), and for period length in constant darkness (DD). Dashed lines represent LOD thresholds. (*E*) Allelic effect of the *TOG1* QTL in F2, F3, F4, and F5 progenies that were Col-0 or Lip-0 homozygous at *TOG1*. Seedlings were grown in LDs of 16 h. The populations are described in *SI Appendix*, Fig. S4*A* (mean ± SEM; *n* is indicated inside the bars; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ with a two-tailed Student *t* test). (*F*) Location of the *TOG* QTLs. Upward and downward arrows indicate that the Lip-0 allele advances or delays *GI* peak time, respectively.

and F5 families were used to detect and confirm four *TIMING OF GI* (*TOG*) quantitative trait loci (QTL) of moderate effect that precisely regulated the timing of *GI* expression during the LD 16-h cycle but had no significant effect on period length or on *GI* peak time in darkness (Fig. 1 *D–F* and *SI Appendix*, Figs. S3 and S4 and Table S5). The size and direction of the *TOG* effects were consistent with the phenotype of the Lip-0 parent. Allelic variation at the *TOGs* modified the timing of *GI* expression by ∼30 min, and the Lip-0 alleles of all *TOGs* except one (*TOG2*) delayed *GI* peak time (Fig. 1*F* and *SI Appendix*, Fig. S4). Together with the confirmation of the *TOGs* in near isogenic lines (NILs), these data collectively demonstrate the existence of natural alleles of moderate effect that precisely regulate the timing of *GI* expression within LD day/night cycles (*SI Appendix*, Fig. S5). Previous studies had reported natural variation of daily transcriptional rhythms in the range of hours (14, 17), but our experiments reveal that natural alleles can cause significant variation of a higher level of precision.

The Waveform of *GI* Expression Is Regulated by Light Signaling During LD Cycles. The timing of *GI* expression is influenced by light signaling (*SI Appendix*, Fig. S2*A*), and the related mechanism might explain part of the *GI* peak time variation observed between accessions in LDs. Consistent with this idea, the gene encoding the red light photoreceptor PHYTOCHROME B (PHYB) was present in the *TOG1* region and was a candidate for this QTL. The Lip-0 allele of *PHYB* contains a deletion in the N-terminal part of the protein that is associated with longer hypocotyls and reduced PHYB activity (37) (*SI Appendix*, Fig. S6). Light signaling was previously shown to regulate *GI* (38, 39), but how changes in PHYB activity could modify the timing of *GI* expression in LD day/night cycles was not known (35, 40).

Detailed analysis of *GI* expression in *phyB* mutants revealed that PHYB activity shapes the *GI* waveform by mediating light signals that activate *GI* transcription in the evening of a LD (Fig. 2 and *SI Appendix*, Figs. S7–S9 and *SI Discussion*). On the basis of the results of a mathematical modeling study in which rapid responses to light were predicted to modulate the phase of circadian outputs (36), we first tested how *GI* expression responded to 30 min white or red light pulses applied in darkness after entrainment in LDs. The light pulses triggered an immediate response of *GI::LUC* that was maximal in the evening of the subjective day and that was significantly reduced in the *phyB-9* mutant (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S8 *A* and *B*). During LD cycles, substitution of white light by darkness in the evening suppressed the evening peak of *GI::LUC*, whereas substitution by red light was sufficient for full activation of *GI* (*SI Appendix*, Fig. S8*C*). Importantly, reduced activation of *GI* expression in *phyB* mutants was accompanied by a rightward shift of the *GI* waveform (negative skewness) and by a delay of *GI* peak time that was consistent with *TOG1* Lip-0 delaying *GI::LUC* expression (Fig. 2 *C–E* and *SI Appendix*, Figs. S7 *A*, *D*, *F* and S8 *D* and *E*). This effect was specific to LDs of 16 h (*SI Appendix*, Figs. S7 *B* and *C* and S10) and had been reported for rhythms of cytosolic Ca^{2+} (36), but was not detected with a circadian marker that was not regulated by light (Fig. 2*E*). These results provide a mechanistic understanding of how light signaling shapes the waveform of *GI* expression in LDs and support a role for rapid responses to light in determining the phase of circadian outputs (36). The circadian clock is implicated in this mechanism not by modifying endogenous rhythms in DD or in LL but by gating (constraining) light activation of *GI* transcription in the evening (Fig. 2*E* and *SI Appendix*, Figs. S6 *C* and *D* and S7*E* and *SI Discussion*).

We then asked whether natural *TOG* alleles regulate *GI* through the same mechanism. Similar to the *phyB-9* mutation, the less active *TOG1* Lip-0 allele reduced *GI::LUC* evening expression levels in F2 progenies (*SI Appendix*, Fig. S11 *A–C*). *TOG1* Lip-0 also reduced *GI::LUC* expression in segregating populations generated by crossing *phyB-9* with two NILs that carried the *TOG1* Col-0 or Lip-0 alleles (*SI Appendix*, Fig. S11*C*). The effects of the different allelic combinations obtained in these populations were consistent with *PHYB* being the gene underlying *TOG1*. We next combined Lip-0 alleles of *TOG1-4* in the Col-0 background and created a population of 12 NILs that we used to more generally address how the *TOGs* were regulating *GI* (*SI Appendix*, Fig. S12*A*). Again similar to the analysis of *phyB* mutants, *GI* peak time significantly and negatively correlated with maximum *GI* expression levels in the NIL population grown in LDs of 16 h, and changes in peak time occurred at least partly independent of circadian rhythms in DD or in LL (*SI Appendix*, Fig. S12 *B* and *C*, Table S7, and *SI Discussion*). Thus, the detailed description of *GI* expression patterns in various populations supports a role for the *TOGs* in mediating a direct effect of light on the *GI* promoter through a mechanism that involves PHYB activity.

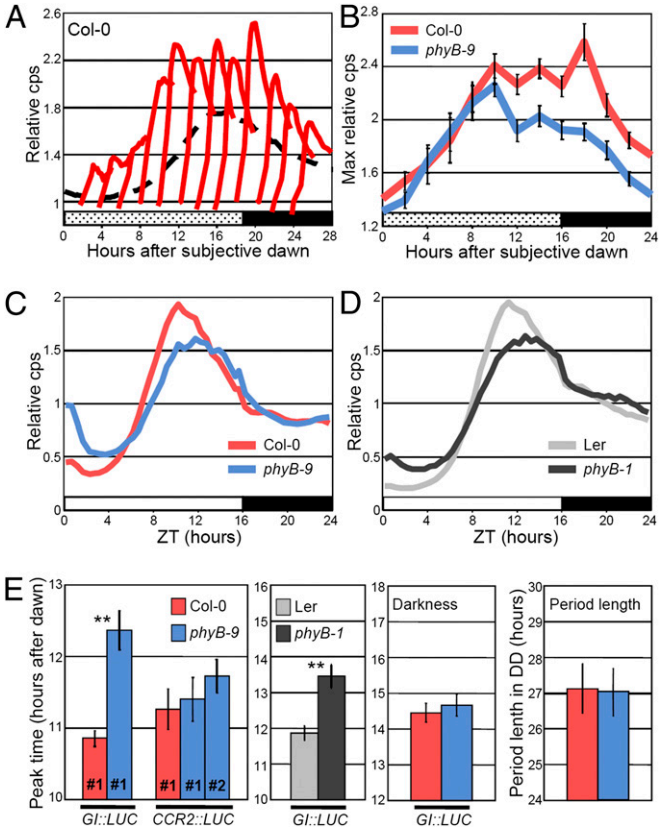

Fig. 2. Light signaling defines the temporal waveform of *GI* expression during LDs. In all experiments, plants were entrained during 9 d in LDs of 16 h and *GI::LUC* expression was monitored on day 10 unless otherwise stated. (*A* and *B*) Col-0 and *phyB-9* plants were entrained in LDs of 16 h, transferred to DD, and exposed to 30-min red light pulses of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ applied every 2 h during the first subjective day. The response of *GI::LUC* expression to each pulse (*A*) is expressed relative to the expression level (cps, counts per second) measured before the pulse. The dashed line represents the nontreated control in DD (not to scale). Maximum relative luminescence after each pulse was plotted in (*B*). (*C* and *D*) Waveform of *GI::LUC* expression in (*C*) Col-0 and *phyB-9* and in (*D*) Ler and *phyB-1* in LDs of 16 h. (*E*) Peak time of *GI::LUC* expression in LDs of 16 h in Col-0 and *phyB-9*, of *CCR2::LUC* expression in Col-0, and in two independent *phyB-9* transgenic lines, of *GI::LUC* expression in the Ler and *phyB-1* background, of *GI::LUC* expression measured in the first day in DD and of period length measured in DD. Confirmation of these results with more transgenic lines is provided in *SI Appendix*, Fig. S7. Mean ± SEM; n = 12–24; $*P \leq 0.05$, $**P \leq 0.01$ with a two-tailed Student t test.

Precise Changes in the Waveform of *GI* Expression Are Sufficient to Alter a Downstream Phenotype. The *TOGs* cause precise changes in the daily pattern of *GI* expression, but it remained to be

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determined whether changes of such magnitude were biologically relevant and could affect overt phenotypes. We used hypocotyl growth as a phenotypic output of GI activity and tested whether alterations of this trait could be a result of precise changes in the *GI* expression waveform. A major advantage of using growth as a trait was that it can be precisely quantified in conditions directly comparable to the ones used for the *GI::LUC* activity assays. GI represses growth of the hypocotyl (27), but how GI function contributes to the molecular network that regulates growth in day/night conditions, and particularly in LDs, was not known.

GI acts in the hypocotyl growth repression pathway activated by PHYB (27), which, according to our results, could at least partially be explained by PHYB-mediated activation of *GI* expression. GI is also known to reduce mRNA levels of the transcription factor *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) during the night when PIF4 contributes to the promotion of growth (41–43). Loss of *GI* function in the Col-0 background also enhanced *PIF4* expression in our conditions, and the long hypocotyl phenotype of *gi-2* required PIF4 activity (Fig. 3 *A* and *B*). We further found that GI and PHYB act synergistically to inhibit growth and repress *PIF4* during the night. Enhanced hypocotyl growth of *phyB-9 gi-2* compared with *phyB-9* required functionally active PIF4 and was associated with increased *PIF4* expression levels (Fig. 3 *A* and *B*). As PHYB also promotes degradation of PIF4 at dawn (41), the synergy between GI and PHYB probably acts at both the transcriptional and posttranscriptional levels (Fig. 3*C*).

In the NILs, growth was affected through the same mechanism. *GI::LUC* expression levels and peak time, but not period length, strongly correlated with hypocotyl length and *PIF4* mRNA levels measured in LDs of 16 h (Fig. 3 *D–F*). The correlations between *GI* expression levels and hypocotyl length or *PIF4* mRNA were negative, which was consistent with GI being a repressor of growth. The data also confirmed the model for the regulation of *GI* transcription by light via the *TOGs*. If the *TOGs* regulate *GI* expression in the evening, phenotypic changes downstream of GI in the NILs should be induced by variations of *GI* expression at this time. As anticipated, the correlations between *GI::LUC* expression levels and *PIF4* mRNA or growth were strongest during the second part of the day, which was also the time when the differences in *GI::LUC* activity between NILs were more pronounced (Fig. 4 *A* and *B*). Thus, natural *TOG* alleles regulate *PIF4* expression and growth in LDs, at least partly by modifying the waveform of *GI* transcription in the evening and in a way that would be enhanced by changes in PHYB activity (Fig. 4*C* and *SI Appendix*, *SI Discussion*).

These data additionally provide novel insights on the function of GI and on the growth regulation model. First, they show that circadian-gated expression of *GI* in the evening contributes to the temporal regulation of hypocotyl length. Second, they reveal how light signaling regulates *PIF4* expression during day/night cycles. The underlying mechanism might involve coexpression and functional interactions of GI with components of the EVENING COMPLEX (EC), a protein complex that directly represses *PIF4* (42–44) (Fig. 4*C*). Interestingly, we detected no significant relationship between *GI* expression and flowering in the NILs, despite an important function of GI being the promotion of flowering through the regulation of *CONSTANS* (*CO*) (45, 46). GI regulates diverse traits through distinct molecular pathways (47–49), and it is possible that these pathways are not all equally sensitive to precise changes in *GI* expression. GI-mediated promotion of flowering might be more robust than growth to small perturbations of *GI* expression and function, an idea supported by a previous study in which an induced mutation of *GI* altered growth but not GI-dependent promotion of *CO* (48). A similar scenario could explain why precise changes in *GI* expression do not alter flowering time in the NILs but do affect growth through the regulation of *PIF4* transcription.

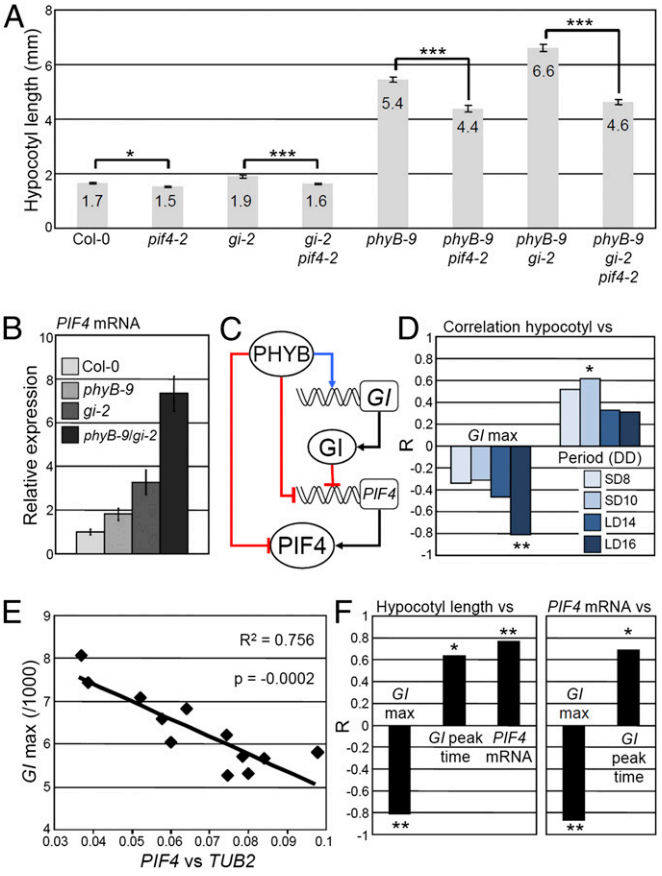

Fig. 3. Precise changes in *GI* expression modify *PIF4* expression and growth. (*A*) Hypocotyl length and (*B*) *PIF4* mRNA levels at zeitgeber time (ZT) 20 h, quantified by quantitative RT-PCR (qRT-PCR) in LDs of 16 h in the indicated mutant backgrounds. (*C*) Working model for how PHYB and GI interact to regulate growth. Red lines indicate repression, blue lines activation, and black lines translation. Rectangles and circles represent genes and proteins, respectively. (*D*) Correlation of hypocotyl length of the NILs grown in LDs of 16 h with *GI::LUC* expression level at peak time (*GI* max) and period length in DD after entrainment in four photoperiods. (*E*) Correlation between *GI::LUC* expression level at peak time (*GI* max) and *PIF4* mRNA levels quantified by qRT-PCR in the NILs entrained in LDs of 16 h and sampled at ZT 20 h. (*F*) Correlation among growth, *GI::LUC* expression level at peak time (*GI* max), *GI* peak time, and *PIF4* mRNA levels at ZT 20 h. The Pearson correlation coefficient (R) indicates the strength of the correlations, with 1 and −1 indicating perfect positive and negative correlations, respectively. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$, with (*A*) a two-tailed Student *t* test or (*D* and *F*) the Pearson test. The correlations were also tested with the Spearman test and yielded similar results.

Conclusions and Perspectives

Collectively, our findings provide a paradigm for how natural alleles cause phenotypic diversity by precisely altering daily waveforms of gene expression. We also show that natural variation in temporal rhythms of expression during the day can be determined by changes in sensitivity to input signals, and not only by changes in circadian rhythms. The LD-specific mechanism of *GI* regulation we describe is part of a more general external coincidence model for the global control of phase in day/night conditions (36). The model predicts that the evening phase of processes dual-regulated by light and by the circadian clock adjusts to seasonal changes by responding predominantly to rapid light inputs. Natural alleles implicated in the perception of input signals that influence rhythms might explain why period length and phase generally do not correlate in *Arabidopsis* accessions. Such natural alleles could confer an advantage by

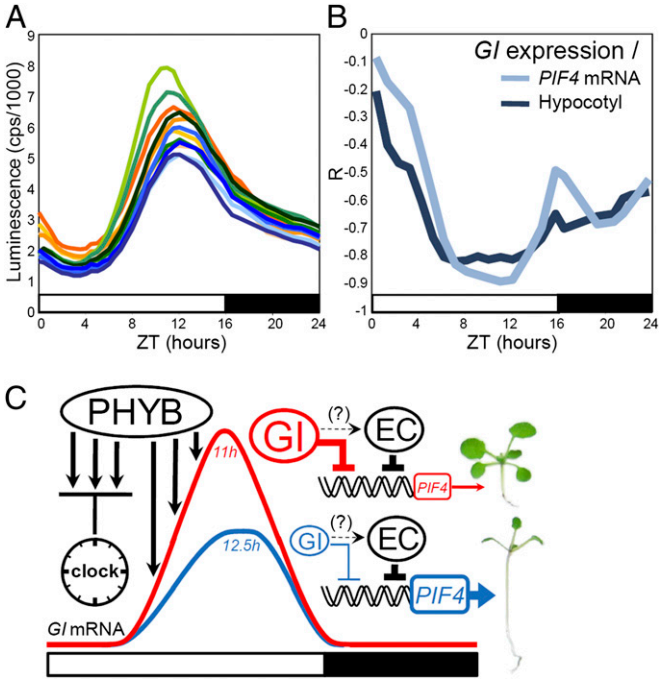

Fig. 4. GI regulates growth by acting predominantly in the evening. (*A*) Waveforms of *GI::LUC* expression in the NILs in LDs of 16 h. (*B*) Pearson coefficient (R) of correlations between *GI::LUC* expression at different times of the day with hypocotyl length and *PIF4* mRNA levels at ZT 20 h in the NILs. R indicates the strength of the correlations, with 1 and −1 indicating perfect positive and negative correlations, respectively. (*C*) A model for the regulation of *GI* expression by natural alleles during the day, and how this affects *PIF4* expression and growth. Light signaling mediated by PHYB is repressed by the circadian clock in the morning of a long day. Clock repression is released later during the day, so that light activates *GI* expression until it reaches its peak in the evening. GI then contributes to the repression of *PIF4* early in the night, so that growth is less efficiently promoted. Weak PHYB alleles cause less GI accumulation (blue line), more *PIF4* transcription, and more growth. GI could hypothetically regulate *PIF4* transcription by interacting with the EC, as represented by the dashed lines. Blue and red lines represent the *GI* waveform when influenced by weak or strong *PHYB* alleles, respectively. Numbers in italics indicate representative *GI* peak times.

optimizing the activity of temporally regulated processes while avoiding the pleiotropic effects associated with severe disruptions of the circadian system (5).

Rhythms of gene expression were analyzed within day/night cycles, at high temporal resolution, and in a population of natural accessions large enough to estimate the range of variation that exists at the intraspecies level. We then revealed the precision with which natural alleles modify daily expression patterns and demonstrated that these modifications have detectable effects on growth, a complex quantitative trait known to be under the control of many small effect loci (50, 51). Theoretical models predict that loci of small effect are a major source of phenotypic variation (52), but understanding how these loci modify phenotypes has been limited by the technical difficulties of their detection and validation. The exploitation of marker gene technology such as luciferase to identify alleles with small phenotypic effects may represent a general approach to uncovering such variation.

Methods

Plant Material and Growth Conditions. The 77 accessions used in this work were a donation from Thomas Altmann, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany, and a subset of these accessions was previously described elsewhere (53). The *phyB-9* and *gi-2* mutant alleles also were described previously (54, 55). To obtain the *GI::LUC* transgene, a 2,513-bp fragment of the *GI* promoter was amplified, using primer 5′-attB1-accagcatatctctaatcag-3′ and primer 5′-attB2-accgaaactaaaccccaac-3′, and recombined with the pGWLuc vector (GeneBank: AM295157). The *GI::LUC* transgene was inserted into *Arabidopsis* by Agrobacterium-mediated transformation (56). Col-0 and *phyB-9 GI::LUC* lines 2 and 3 were obtained after transformation with a vector containing 2,755 bp of the *GI* promoter and were described elsewhere (35). Transgenic lines were made homozygous before use. The first 39 accessions were scored using at least two transgenic lines per accession. A significant contribution of the genotype (accessions) to variations of *GI::LUC* expression was detected in this data set (*SI Appendix*, Table S2). Only one line per accession was subsequently used to reduce the workload. Seedlings were grown in different photoperiods at 22 °C under 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluorescent white light (Philips TL741), or in a Percival growth chamber equipped with E-30LED for monochromatic light experiments (Percival).

Bioluminescence Imaging. Plants were entrained for 9 d in different photoperiods and light conditions, and measurements were started on day 10. For experiments performed in white light, seedlings were transferred to 96-well black optiplates (Perkin-Elmer Life Sciences) containing Murashige and Skoog medium with 2% (mass/vol) sucrose and 20 μL D-luciferin (5 mM). Luminescence of individual seedlings was monitored in a TopCount Microplate Scintillation Counter (Perkin-Elmer) by manual feeding, which allowed the study of *GI* expression in white light conditions that contained the whole spectrum of wavelengths. In constant darkness (DD) conditions, feeding of the plates to the TopCount was automatic. LUC activity in monochromatic red light was monitored with a CCD camera in 24-well plates containing approximately 10 seedlings of the same genotype and supplied with 150 μL of 10 mM Luciferin. The resolution of the assays was 30 min. The images generated by the CCD camera were analyzed with Metamorph (Molecular Devices). Rhythms of *GI::LUC* expression during day/night cycles or in constant conditions were analyzed with BRASS (www.amillar.org).

QTL Detection and Statistical Analyses. The QTLs were detected in 135 F2 progenies scored for *GI::LUC* expression and genotyped by Sequenom Inc. Linkage maps were created using JoinMap 4 (Kyazma B.V.), and QTL analysis was performed with MapQTL 5 (Kyazma B.V.), using the multiple QTL mapping (MQM) procedure. A thousand permutations were used to determine chromosome-specific logarithm of odds (LOD) thresholds. Markers used as cofactors were chosen by backward selection. More detailed information on the phenotyping and genotyping procedures and on the QTL validation in segregating populations and in the NILs is provided in *SI Appendix*, *SI Methods*. Hierarchical clustering was performed using Cluster version 3. The raw data were mean centered and normalized across the different day lengths. Clustering was performed with an uncentered correlation matrix and average linkage clustering. Self-organizing maps were generated before hierarchical clustering to determine the best orientation of the tree nodes. The resulting trees were displayed using Treeview version 3. All other statistical analyses were performed with SigmaStat version 3 or R.

Hypocotyl Measurements. Hypocotyl length of the seedlings grown under different photoperiods and light conditions was measured after 9 days so the data would be directly comparable to the luciferase data. A high-resolution photograph of the seedlings was taken with a digital camera, and hypocotyl length was measured with Image J (National Institutes of Health). In all experiments, ∼20–30 seedlings per genotype were analyzed. Hypocotyl data for the NILs were obtained from five independent biological replicates performed in each of the four photoperiods tested (4,632 seedlings total).

Quantification of mRNA Expression. RNA was isolated from 10-day-old seedlings, using the RNeasy Plant Mini kit (Qiagen), following the recommendations of the manufacturer. Genomic DNA was removed with the DNA-free kit (Ambion). For cDNA synthesis, 1 μg total RNA was primed using the oligodT primer and reverse transcribed with the SuperScript II kit (Invitrogen). The PCR mix was composed of 3 μL diluted cDNA and 7 μL iQ SYBR Green Supermix (Biorad). The thermocycles used for amplification were 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C. *TUB2* (At5g62690) and *IPP2* (At3g02780) were used as housekeeping genes to normalize the expression data and yielded similar results. Primer sequences are provided in *SI Appendix*, Table S8.

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