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The Molecular Basis of Temperature Compensation in the *Arabidopsis* Circadian Clock [W]

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Circadian clocks maintain robust and accurate timing over a broad range of physiological temperatures, a characteristic termed temperature compensation. In *Arabidopsis thaliana*, ambient temperature affects the rhythmic accumulation of transcripts encoding the clock components *TIMING OF CAB EXPRESSION1* (*TOC1*), *GIGANTEA* (*GI*), and the partially redundant genes *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*). The amplitude and peak levels increase for *TOC1* and *GI* RNA rhythms as the temperature increases (from 17 to 27°C), whereas they decrease for *LHY*. However, as temperatures decrease (from 17 to 12°C), *CCA1* and *LHY* RNA rhythms increase in amplitude and peak expression level. At 27°C, a dynamic balance between *GI* and *LHY* allows temperature compensation in wild-type plants, but circadian function is impaired in *lhy* and *gi* mutant plants. However, at 12°C, *CCA1* has more effect on the buffering mechanism than *LHY*, as the *cca1* and *gi* mutations impair circadian rhythms more than *lhy* at the lower temperature. At 17°C, *GI* is apparently dispensable for free-running circadian rhythms, although partial *GI* function can affect circadian period. Numerical simulations using the interlocking-loop model show that balancing *LHY/CCA1* function against *GI* and other evening-expressed genes can largely account for temperature compensation in wild-type plants and the temperature-specific phenotypes of *gi* mutants.

INTRODUCTION

The circadian clock is an endogenous 24-h timer found in most eukaryotes and in photosynthetic bacteria. The clock drives rhythms in the physiology, biochemistry, and metabolism of the organisms (reviewed in Hall and McWatters, 2006). Circadian rhythms are defined as rhythms that persist in constant conditions with a periodicity of ~24 h, that can be entrained to local time, and that are capable of maintaining a constant periodicity over a broad range of physiological temperatures. Intriguingly, although these characteristics of the clock are shared across taxonomic groups, there is little conservation of the molecular components used (reviewed in Harmer et al., 2001).

In *Arabidopsis thaliana*, a transcription feedback loop model has been proposed for the clock, similar to the architecture of the clock in other organisms. The loop comprises two *myb* transcription factors, *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), which are proposed to function by repressing the expression of *TIMING OF CAB EXPRESSION1* (*TOC1*), which encodes a pseudoresponse regulator of unknown biological function. *TOC1*, in turn, activates *LHY/CCA1* (Alabadi et al., 2001). It is clear that this model is not sufficient to explain current experimental data, as abnormal rhythms persist in plants lacking both *LHY* and *CCA1* or in those lacking *TOC1* (Alabadi et al., 2002; Mizoguchi et al., 2002; Locke et al., 2005). Mathematical simulations incorporating current experimental data have led to the proposal of a model comprising two interlocking feedback loops, with *GIGANTEA* (*GI*) and *TOC1* as candidates for components of a second loop (Locke et al., 2005).

One of the key characteristics of all circadian rhythms is that they are able to maintain robust rhythms with a period close to 24 h over a broad range of physiological temperatures; this property is termed temperature compensation. In *Drosophila*, studies identified a role for the key clock component *PERIOD* (*PER*) in temperature responses, including temperature-dependent variation in Per protein properties (Sawyer et al., 1997) and differential splicing of the *Per* RNA (Majercak et al., 1999). In *Neurospora*, two isoforms of the central clock component *Frequency* (*FRQ*) have been implicated in maintaining rhythm robustness over a

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physiological range of temperatures: mutants lacking either form are rhythmic in only a limited temperature range (Liu et al., 1997). Both studies suggest the hypothesis that temperature compensation is an intrinsic feature of the central clock components, rather than a result of additional regulators.

To identify components of the temperature compensation mechanism in the model plant *Arabidopsis*, a quantitative genetic approach has been used. This exploits accession-specific variations in the pattern of temperature compensation for rhythms of leaf movement between Columbia (Col) and Landsberg *erecta* (L*er*) and between L*er* and Cape Verde Islands (Cvi). This strategy uncovered a number of quantitative trait loci (QTL) across the genome. The genes underlying some of these, such as the flowering regulator *FLOWERING LOCUS C* (Edwards et al., 2006), had no known function in the *Arabidopsis* clock mechanism. By contrast, further characterization of one of these QTL, *PerCv1b*, identified *GI* as a candidate for the *PerCv1b* trait (Edwards et al., 2005).

The *gi* alleles were originally identified as supervital mutants having increased fitness as measured by high fecundity, resulting in increased seed yield per plant. The basis for this was the large rosettes and late flowering of the mutants (Rédei, 1962). More recently, *GI* has been identified as acting upstream of *CONSTANS* in the photoperiodic regulation of flowering (Suarez-Lopez et al., 2001). The *GI* gene encodes a 127-kD nuclear protein of 1173 amino acids (Fowler et al., 1999; Park et al., 1999; Huq et al., 2000). *GI* is conserved in plants; however, *GI* homologs appear to be absent from the genomes of the microalga *Chlamydomonas* (Mittag et al., 2005) and those of human, mouse, and *Drosophila.* *GI* is expressed throughout the plant and is regulated by the circadian clock, with a maximal level of expression 12 h after dawn (Fowler et al., 1999). Mutations in the clock-affecting genes *LHY*, *CCA1*, and *TIME FOR COFFEE* alter *GI* RNA expression levels (Mizoguchi et al., 2002; Hall et al., 2003). In addition to its role in flowering, *GI* has also been phenotypically characterized as affecting the circadian clock, as the *gi-1* mutant causes a short-period phenotype for both leaf movement and *CAB*:*LUCIFERASE* (*LUC*) expression. Another allele, *gi-2*, causes a similar short-period phenotype for leaf movement, although *CAB*:*LUC* expression oscillates with a long period (Park et al., 1999). The *gi* mutants cause a reduction in the expression of *CCA1* and *LHY* (Fowler et al., 1999). Recent investigations (Mizoguchi et al., 2005), together with this study, suggest that the flowering and circadian functions of *GI* are distinct.

In this study, we demonstrate that the gene *GI* plays a critical role in increasing the temperature range permissive for rhythmicity, extending the temperature range over which both the robust rhythms of *CAB* expression and leaf movement rhythms can be maintained. We go on to investigate, first, how key components of the wild-type circadian mechanism (*GI*, *LHY*, *CCA1*, and *TOC1*) change in response to altered temperature. Second, we describe how this temperature-specific regulation is altered in the *gi* mutant, identifying a mechanism for the temperature-specific phenotype of *GI*. We conclude that a dynamic balance between *LHY* and *GI* is key for the effective temperature compensation of the circadian clock at high temperatures, whereas at low temperature *LHY* appears to be substituted by *CCA1*. In addition to this role in temperature compensation, *GI* also plays a critical role in extending the temperature range over which robust and accurate rhythmicity can be maintained.

RESULTS

GI Plays a Critical Role in Maintaining Rhythmicity in Leaf Movement at Higher Temperatures

The QTL *PerCv1b* was identified as a locus involved in temperature compensation of the circadian clock from a screen of Cvi × L*er* recombinant inbred lines. Further mapping of this QTL using near isogenic lines localized the QTL to an ~900-kb region of chromosome 1. Within this region was the flowering-time gene *GI*. Sequencing *GI* from L*er* and Cvi revealed a number of polymorphisms, two of which resulted in amino acid substitutions (Edwards et al., 2005). This, together with the period effects of the *gi* mutant (Park et al., 1999), confirmed *GI* as a strong candidate for a gene involved in the temperature compensation mechanism.

To identify a role for *GI* in the temperature buffering of the circadian clock, circadian rhythms of leaf movement in the *gi* null mutant *gi-11* over a range of temperatures (12, 17, 22, and 27°C) were characterized. The *gi-11* null mutant is a T-DNA insertion mutation and has been described previously (Richardson et al., 1998; Fowler et al., 1999); furthermore, the late-flowering phenotype of the *gi-11* mutation could be complemented with a wild-type *GI* cDNA (see Supplemental Figure 1 online). Both *gi-11* and wild-type plants were grown under 12-h-light/12-h-dark cycles (12L:12D) at 22°C to entrain the clock and were then transferred to constant light at 12, 17, 22, or 27°C. The free-running periods of the leaf movement rhythms were measured. Figure 1A shows that at 17°C, the *gi* mutant (open triangles) and the wild type (closed squares) had no significant period difference. However, as the temperature increased, the period of *gi* became significantly shorter than that of the wild type. A significant period shortening was also observed in response to a decrease in temperature. This temperature-dependent effect on the free-running period is consistent with *GI* playing a function in the temperature compensation mechanism of the *Arabidopsis* clock.

In addition to a shortening of the period, a temperature-dependent increase in the variance of period was seen for the *gi* mutant, with the variance increasing from 3.1 at 17°C to 18.0 at 27°C (*F* test, $P = 1.8 \times 10^{-9}$). Although an increase in the variance of period with temperature was also observed for the wild type, this was not of the same magnitude (from 1.9 at 17°C to 3.8 at 27°C; *F* test, $P = 4.1 \times 10^{-3}$). The huge increase in the variance of period in the *gi* mutant at 27°C demonstrates a breakdown in the precision of the clock. This reduced precision could clearly be seen when period estimates for each leaf movement rhythm were plotted against the relative amplitude error (Figure 1B), a measure of rhythm robustness varying from 0 (a perfect fit to the cosine wave) to 1 (not statistically significant). For wild-type period estimates, the associated relative amplitude errors remained low, with all 60 points clustering tightly at each temperature (Figure 1B). However, for the *gi* mutant, as temperature increased, both the relative amplitude error and the range of period estimates increased. Similarly, as temperature decreased

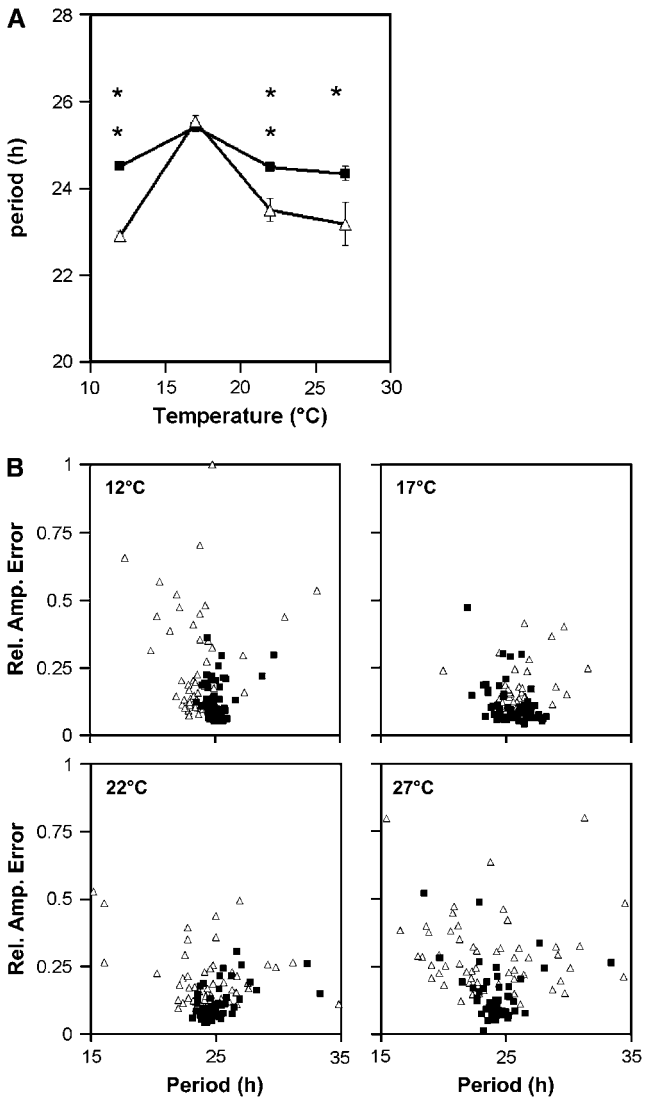

Figure 1. Temperature Compensation of Leaf Movement Rhythms for Wassilewskija (Ws) Wild Type and Ws *gi-11*.

Seedlings were entrained under 12L:12D cycles for 7 d, after which they were transferred to constant light at 12, 17, 22, or 27°C, at which rhythms of leaf movement were assayed. For each temperature, 25 to 30 wild-type plants and 25 to 30 *gi* mutant plants were assayed, corresponding to 50 to 60 leaf movement rhythms.

(A) Variable-weighted mean of period estimates plotted against temperature. Closed squares, wild type; open triangles, *gi-11*. Error bars represent variance-weighted SE. Asterisks indicate *t* test P values: * $P < 0.05$, ** $P < 0.01$.

(B) Period estimates for individual leaves plotted against their relative amplitude errors (Rel. Amp. Error). Closed squares, wild type ($n = 50$ to 60); open triangles, *gi-11* ($n = 50$ to 60). This experiment was performed independently three times at each of the four temperatures; the results shown are representative.

to <17°C, the variability and relative amplitude errors increased (Figure 1B). Similar results were seen for the *gi-3* allele in the L*er* background, with temperature-dependent effects on both period and rhythm robustness observed (see Supplemental Figure 2 online). However, unlike the null mutant, the period of *gi-3* was not wild type at 17°C. The *gi-3* mutation introduces a stop codon at the 3′ end of the gene; therefore, a truncated GI protein can be produced. Furthermore, the expression of *GI* mRNA in the *gi-3* mutant had only been reduced by half (Fowler et al., 1999). Similarly, another *gi* allele isolated in our laboratory, *gi-611*, which lacked the characteristic flowering phenotype of the standard *gi* mutants, also had a temperature-dependent period phenotype, although it did not have a reduced robustness phenotype (see Supplemental Figure 3 online). Together, the leaf movement analysis suggests a role for *GI* not only in buffering the period of the clock against temperature change but also in extending the range of temperature over which rhythmicity can be maintained.

GI Plays a Comparable Role Maintaining the Rhythmicity of Expression of *CAB:LUC* at High and Low Temperatures

It is clear that *GI* functions to extend the temperature range at which accurate leaf movement rhythms can be maintained. To investigate whether this extended to other rhythms, the *CAB*:*LUC* marker was used to assay the rhythmic expression of *CAB* in the *gi-11* background in plants entrained at 22°C and then free run in constant light at 12, 17, or 27°C. After transfer to 17°C, the rhythmic expression patterns of *CAB* in the wild type and *gi-11* were almost identical (Figure 2A), with no significant difference in period and >98% of *gi-11* seedlings producing periods within the circadian range (15 to 35 h). The only difference in the circadian phenotype between *gi-11* and the wild type at 17°C was the low amplitude of *CAB* expression in *gi-11* and an increase in the variance of period (period variance of 0.16 for the wild type and 1.14 for *gi*; *F* test, $P = 3 \times 10^{-12}$). Thus, at 17°C, the circadian clock is able to sustain rhythms of *CAB* expression even in the absence of *GI*. However, upon transfer of *gi-11* seedlings from 22 to 27°C, *CAB* expression rapidly dampened, and by 48 h expression was arrhythmic (Figure 2A). This was reflected by the fact that only 30% (8 of 26) of *gi-11* seedlings produced periods for *CAB* expression within the circadian range, all with relative amplitude errors of >0.5, indicative of weak rhythms. By contrast, 100% (31 of 31) of wild-type seedlings were scored as producing robust rhythms in *CAB* expression at 27°C. This response was mirrored when *gi-11* plants were transferred from 22 to 12°C, with *CAB* expression rapidly becoming arrhythmic (Figure 2A) and only 35% (15 of 43) of seedlings producing rhythms within the circadian range. These observations are consistent with the leaf movement analysis in that *GI* functions to extend the temperature range permissive for rhythmicity. Furthermore, its similar effect on these two outputs is consistent with *GI* acting on the central clock.

Temperature Regulation of Clock-Related Gene Expression

To investigate how key molecular clock components respond to ambient temperature in wild-type plants, the accumulation of transcripts that encoded *CCA1*, *LHY*, and *TOC1* was measured.

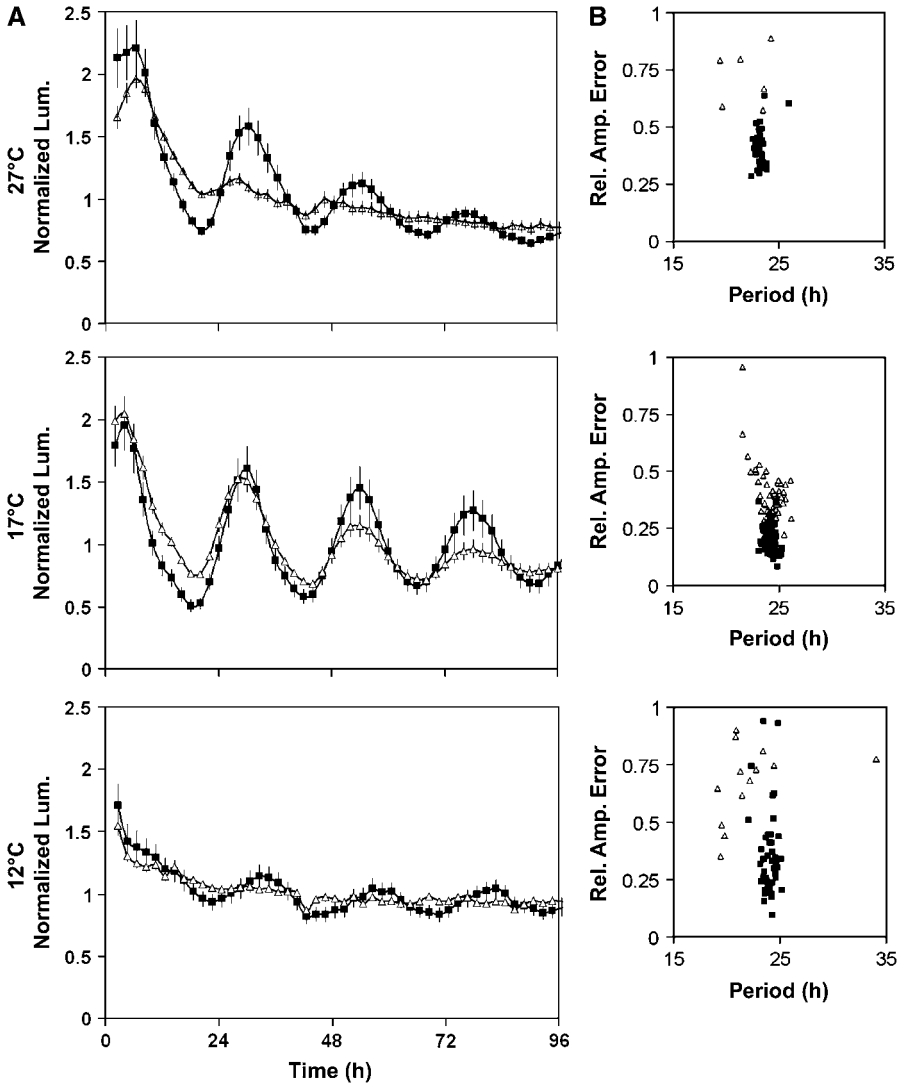

Figure 2. *GI* Is Required for the Rhythmicity of CAB at 12 and 27°C.

Transgenic seedlings carrying the *CAB*:*LUC* reporter gene were entrained under 12L:12D cycles for 7 d, after which the seedlings were transferred to 12, 17, or 27°C and constant red and blue light. Luminescence was monitored in the wild-type Ws (closed squares) and *gi-11* (open triangles). For both the wild type and *gi-11*, expression was monitored in at least three independently transformed lines.

(A) Plots represent the normalized average expression of 8 to 27 single seedlings of one representative transformed line. Error bars indicate SE.

(B) Summary of the mathematical analysis of the expression rhythms of the seedlings, represented by plots of period estimates plotted against the respective relative amplitude errors (Rel. Amp. Error) for wild-type Ws and *gi-11*. At 27°C, wild-type $n = 31$, *gi-11* $n = 26$; at 17°C, wild-type $n = 116$, *gi-11* $n = 51$; at 12°C, wild-type $n = 52$, *gi-11* $n = 43$.

Plants were entrained for 7 d with 12L:12D cycles before transfer to 12, 17, or 27°C and constant light. Transcript abundance was then assayed, starting at 72 h after transfer, every 4 h over a 24-h period. Figures 3A to 3C compare transcript abundance and expression patterns of plants free-running at 17 and 27°C. At 27°C, the amplitude of the *TOC1* expression rhythm was almost double that at 17°C, with the trough level of expression remaining constant but the peak level increased. Also, the shoulder seen after the peak of expression was lost at 27°C. A temperature-dependent increase in expression was also observed for *GI* (Figure 4). The reciprocal was observed for *LHY* levels, with the amplitude of the oscillation of *LHY* decreasing at 27°C. *CCA1* expression showed no significant change with increased temperature. The difference in response of *LHY* and *CCA1* was consistent with the two genes not playing completely redundant roles. A temperature-dependent increase in clock components, similar to that seen for *TOC1*, has also been observed in *Neurospora*, in which the amount/amplitude of FRQ protein increased with temperature (Liu et al., 1998). A comparison of the free-running clock at 17 and 12°C revealed no significant differences

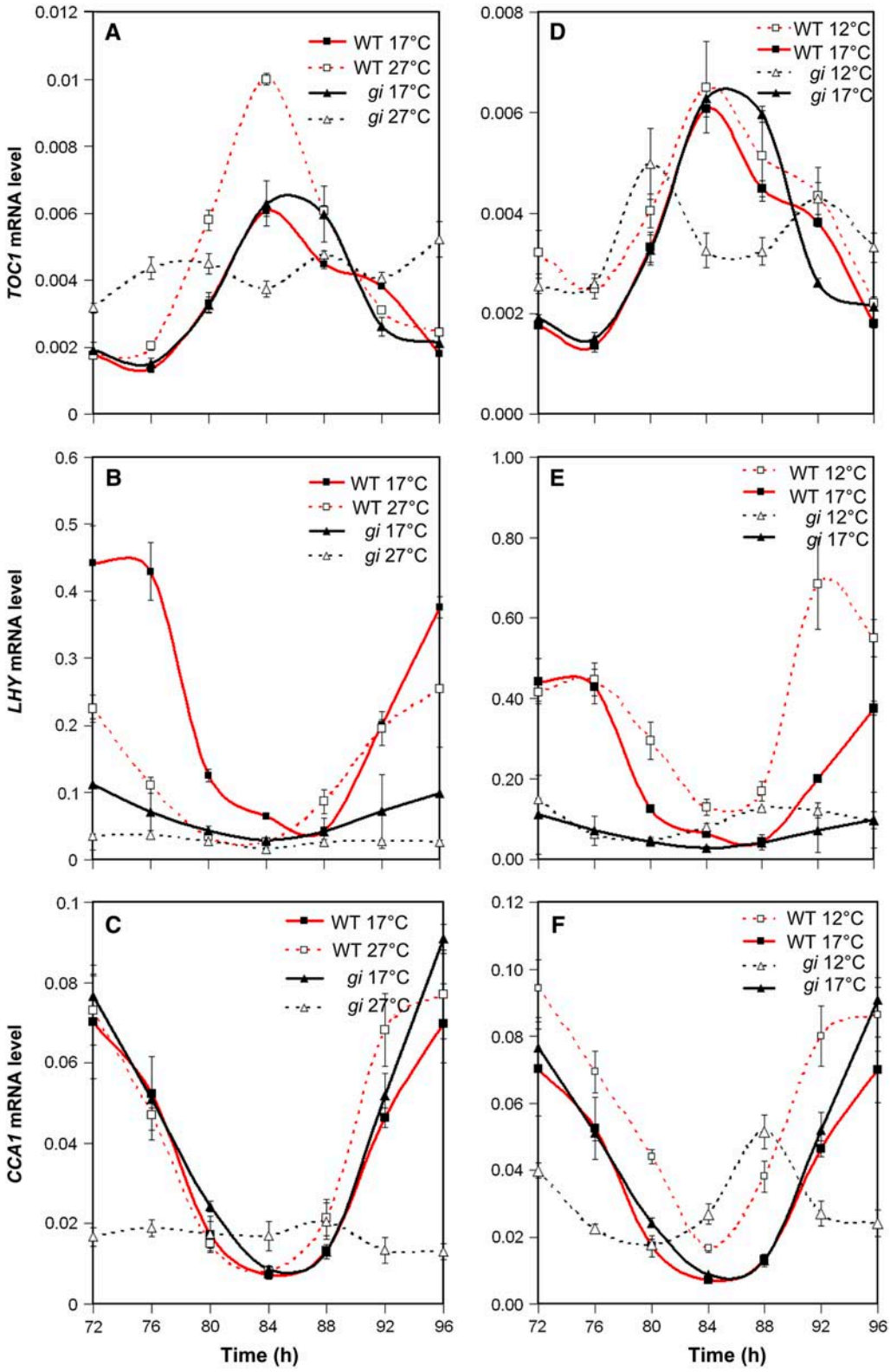

Figure 3. The Clock Components *TOC1*, *LHY*, and *CCA1* Have Altered Amplitudes in Response to Temperature and *GI* Function.

Seedlings were grown at 22°C in 12L:12D conditions for 7 d; the seedlings were then transferred to constant light and 12, 17, or 27°C. They were harvested after 72 h and every 4 h thereafter for the next 24 h. Total RNA was assayed by real-time quantitative PCR, and the accumulation of *TOC1* (**[A]** and **[D]**), *LHY* (**[B]** and **[E]**), and *CCA1* (**[C]** and **[F]**) was measured relative to an internal *UBIQUITIN* (*UBQ*) control. The plots compare the accumulation of mRNAs at 27°C (open symbols) with that at 17°C (closed symbols) (**[A]** to **[C]**) or at 17°C (closed symbols) with that at 12°C (open symbols) (**[D]** to **[F]**). Expression in the *gi-11* background is represented by triangles, and that in the wild type is represented by squares. Error bars indicate SE. Each point represents the average of three biological repeats, with each biological repeat made up of three technical repeats.

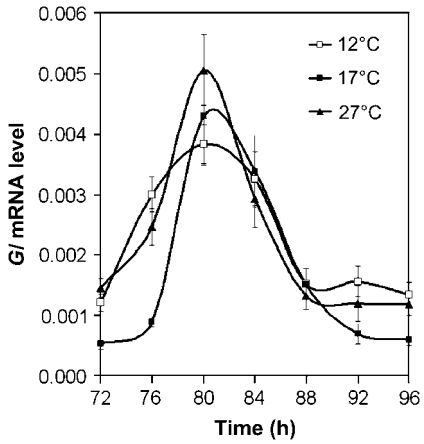

Figure 4. *GI* mRNA Expression Is Weakly Temperature-Regulated.

Wild-type seedlings were grown at 22°C in 12L:12D conditions for 7 d, and the seedlings were then transferred to constant light and 12°C (open squares), 17°C (closed squares), or 27°C (closed triangles). They were harvested after 72 h and every 4 h thereafter for the next 24 h. Total RNA was assayed by real-time quantitative PCR, and the accumulation of *GI* was measured relative to an internal *UBQ* control. Error bars indicate SE. Each point represents the average of three biological repeats, with each biological repeat made up of three technical repeats.

in the expression of *TOC1*. However, although less pronounced than the effect of high temperature, peak levels of *CCA1* and *LHY* did increase at 12°C compared with 17°C, and the peak level of *GI* decreased (Figure 4). Similarly, a reciprocal decrease in *GI* was also seen (Figures 3D to 3F).

Although only subtle changes in the regulation of clock components were observed between 17 and 12°C, in a microarray experiment comparing seedlings grown at 22 and 4°C, transcripts encoding many of the known clock components rapidly dampened to a high level upon transfer to 4°C (NASCArrays experiment reference number NASCARRAYS-138; see Supplemental Figure 4 online). Although this microarray analysis is consistent with a loss of clock function at 4°C, a longer time course will be required to confirm this finding. However, this apparent loss of rhythmicity has also been observed in chestnut (*Castanea sativa*), in which the levels of Cs *LHY* and Cs *TOC1* dampened to a high level upon transfer to 4°C, with *LHY* failing to repress *TOC1* levels (Ramos et al., 2005).

Temperature-Dependent *GI* Function at the Molecular Level

Both our leaf movement experiments and *CAB*:*LUC* expression data suggest that *GI* functions to maintain accurate and robust rhythmicity at high and low temperatures. Moreover, the effect on multiple outputs would support the hypothesis that *GI* acts directly on the central clock at higher and lower temperatures. Previous studies investigating *GI*'s role in the clock found that *CCA1* and *LHY* levels are reduced in plants grown under long days at 22°C in the *gi-3* mutant. This finding supports a function for *GI* in the regulation of *CCA1* and *LHY* (Fowler et al., 1999). To determine whether *GI*'s effects on the key clock components are temperature-dependent, we compared the expression profiles of *TOC1*, *CCA1*, and *LHY* in wild-type and *gi-11* seedlings free-running in constant light at 27 and 17°C, as described above (Figure 3). At 17°C, *TOC1* expression levels were identical in both the *gi-11* and wild-type plants. At 27°C, *TOC1* levels failed to increase in the *gi-11* background and were expressed at an almost constant level. At 17°C, the expression of *LHY* was lower in the *gi* mutant, and this may contribute to the reduction in amplitude of the *CAB*:*LUC* oscillations observed in *gi-11* at 17°C. At 27°C, the expression level and amplitude of *LHY* were further significantly reduced. For *CCA1* expression, in contrast with *LHY*, the level, amplitude, and expression profile were identical in the *gi* mutant and the wild type at 17°C, demonstrating a clear difference in the regulation of the *LHY* and *CCA1* genes by *GI*. At 27°C in the wild type, *CCA1* expression was temperature-independent, whereas in the *gi* mutant, expression became temperature-dependent, with levels and amplitude of *CCA1* decreasing at 27°C. Together, our expression analysis of key clock components suggests a mechanism whereby *GI* maintains rhythmicity and accuracy at higher temperatures by the temperature-dependent regulation of *TOC1*, thereby maintaining sufficient levels/amplitude of *CCA1* and *LHY* to sustain accurate and robust clock function.

We also tested changes in gene expression in *CCA1*, *LHY*, and *TOC1* in the wild type and the *gi* mutant at 12°C (Figure 3). Little change in the expression of clock components in response to decreases in temperature was observed in the wild type; however, in the *gi* mutant, the expression of both *CCA1* and *TOC1* decreased, with the expression of *TOC1* becoming biphasic, with two peaks of expression only 12 h apart. This was not observed for *CCA1* or *LHY*, consistent with a breakdown of the feedback regulatory loop composed of *CCA1*/*LHY* and *TOC1* described by Alabadi et al. (2001). The expression of *LHY* remained at a similar level at both 17 and 12°C, although both *CCA1* and *LHY* expression had an altered phase, suggesting that the period length of *CCA1* and *LHY* is shorter at 12°C, which is consistent with the short-period phenotype of rhythms of leaf movement (Figure 1A).

The Interlocking Loop Model Supports *GI* Function in Temperature Compensation

GI is proposed to contribute to a key component, *Y*, of the interlocking loop model for the *Arabidopsis* clock mechanism (Locke et al., 2005). Therefore, we tested whether the observed temperature-dependent effects of *GI* were consistent with the function of *Y* in this model. Without explicit modeling, it would be difficult to determine whether this was the case or whether our experimental data reflected additional temperature-dependent functions of *GI*. mRNA expression profiles for *TOC1* and *LHY*/*CCA1* at 27, 17, and 12°C for both wild-type and *gi* genotypes were simulated (see Methods; see also Supplemental Methods and Supplemental Figures 5 and 6 online). As temperature effects were not explicitly included in the model, a qualitative fit was obtained by varying only the transcription rates of the model components *LHY* and *Y*. *LHY* in the model represents both *LHY* and *CCA1*. *Y* represents *GI*, although unidentified genes with functions that overlap with *GI* may also contribute to *Y*.

The wild type at 27°C was modeled as having reduced *LHY* expression (Figure 3), which would shorten the circadian period if this were the only effect of temperature. Higher *GI* expression in the model (following the trend in Figure 4) compensated for the reduced *LHY* levels, substantially restoring the period length and also recapitulating several features of the molecular data in Figure 3 (see Supplemental Methods and Supplemental Figure 5 online). In particular, a *gi* mutation in the model had a more severe low-amplitude phenotype at 27°C compared with the standard temperature (see Supplemental Figure 5 online), consistent with the experimental data (Figures 1 and 2). The wild type at 12°C was modeled by increasing the transcription rate of *LHY*, as *LHY* RNA levels are increased at lower temperatures (see Supplemental Figure 3C online). *GI* RNA levels were little reduced at 12°C (Figure 4), but reducing *Y* transcription rates in the model again compensated the circadian period. The simulated *gi* mutation again recapitulated the observed effects of the *gi* mutant at 12°C (see Supplemental Figure 6 online). We propose that the reduced *Y* function that was required to simulate the wild type at 12°C might represent downregulation of another gene that contributes to *Y*, overlapping with *GI* function (see Supplemental Methods online). Altering only the *LHY* and *Y* transcription rates was sufficient to recreate the major effects of temperature on clock gene expression, showing that the temperature-dependent effects of *GI* proposed here are consistent with *GI*'s location as a component of *Y* in the two-feedback-loop model.

LHY and *CCA1* Contribute Differentially to Temperature Compensation

Our data suggest that a dynamic balance between *LHY* and *GI* functions maintains robust and accurate rhythmicity at higher temperatures and that *LHY* function is distinct from *CCA1* in this respect. To confirm the function of *LHY* in this mechanism, we tested temperature compensation in the *lhy* and *cca1* loss-of-function mutants. Both *lhy* and *cca1* maintained robust rhythms of *CAB*:*LUC* expression at 17 and 27°C (Figure 5). Both mutants had the same short-period phenotype at 17°C (wild type, 23.9 h; *cca1*, 22.8 h; *lhy*, 22.8 h). At 27°C, a period difference of ~1 h was maintained between the wild type (23.0 h) and *cca1* (22.2 h), whereas in the *lhy* mutant, the period difference increased to nearly 2 h (wild type, 23.0 h; *lhy*, 21.1 h) (Figure 5). The *lhy* mutant, therefore, has a shorter period than *cca1* (Student's *t* test, $P < 0.01$) at 27°C, supporting a role for *LHY* in the temperature compensation mechanism. This was suggested previously by analysis of natural genetic variation (Edwards et al., 2005), as *LHY* was noted as a candidate gene for the temperature-specific QTL *PerCv1a* and *PerCola*. At 12°C, the period of the wild type (23.3 h) differed from that of *lhy* (21.6 h) by 1.7 h. In *cca1*, however, the rhythm of *CAB*:*LUC* expression was dampened (Figure 5), and the period difference between the wild type (23.3 h) and *cca1* (20.0 h) was 3.3 h. This temperature-dependent shortening of the circadian period and the reduction in rhythm robustness almost phenocopied the effect of *gi-11* at 12°C (Figures 1 and 2). At lower temperatures, our data indicate that *CCA1* plays a greater role than *LHY* in temperature compensation and the maintenance of rhythm robustness, confirming that *CCA1* and *LHY* functions are not completely overlapping.

DISCUSSION

Our characterization of key components of the circadian clock over a range of temperatures has uncovered temperature-dependent changes in their gene expression. We found that levels of *LHY* mRNA decrease with temperature increases and that this was counterbalanced by increases in *TOC1* and *GI*. By contrast, *CCA1* levels changed little between 27 and 17°C; however, we did see an increase at lower temperatures (Figures 3 and 4). This balance between clock components is consistent with the antagonistic balance model of temperature compensation put forward by Ruoff et al. (1997). We tested whether these new data could fit within the constraints of a proposed interlocking-loop model of the circadian clock, incorporating *GI* (Locke et al., 2005).

First, we considered compensation of the clock in response to increasing temperature. The effects of increases in temperature could be simulated by reducing *LHY* transcription rates, causing a shortening of the period; however, this could be balanced by increasing *GI* transcription levels. Although the experimental increases in *GI* expression were not pronounced, there was a gradual increase in peak expression with temperature (Figure 4). It has been shown that the GI protein oscillates and that this is regulated at the posttranscriptional level (David et al., 2006). Therefore, it is possible that levels of GI could also be modulated by temperature. This is consistent with the temperature compensation mechanisms of other organisms (Rutila et al., 1996; Liu et al., 1997; Lahiri et al., 2005). By altering the transcription parameters of *GI* and *LHY* alone, the period of the model clock system was balanced and there was little difference in the phase of either *LHY* or *TOC1* mRNA with temperature (see Supplemental Figure 5 online). Together, the simulation and experimental data are supportive of the conclusion that *LHY* and *GI* form a dynamic counterbalance to temperature-compensate the clock at high temperature.

In support of this dynamic counterbalance hypothesis, we show that a null *gi* mutant (*gi-11*) has a temperature-dependent circadian phenotype, affecting both robustness and period (Figures 1 and 2). In *gi-11*, the counterbalance between *GI* and *LHY* is lost and *LHY* RNA levels decrease at 27°C. A similar temperature compensation phenotype was also observed in the *gi-611* and *gi-3* alleles; however, the partial *gi-611* mutation does not display the loss of rhythm robustness with temperature (see Supplemental Figure 3 online). The *lhy* loss-of-function mutant likewise shows a strong period alteration at high temperature (Figure 5).

This proposal can account for temperature compensation at 27°C, but our experimental data show that different processes are involved at low temperature. Specifically, between 17 and 12°C, *GI* expression is little altered in the wild type, and the *gi* mutation reduces the expression of *CCA1* rather than *LHY*. Furthermore, at 12°C, the *cca1* loss-of-function mutant alters period more severely than the *lhy* mutation and also reduces rhythmic robustness, the reverse of the situation at 27°C. At low temperature, our data are consistent with *CCA1* replacing *LHY* in temperature compensation of the clock. The switch in the apparent importance of these two transcription factors may reflect unknown differences in temperature-dependent biochemical properties of the two proteins. *GI* participates by regulating the relative expression levels of *CCA1* and *LHY* in a

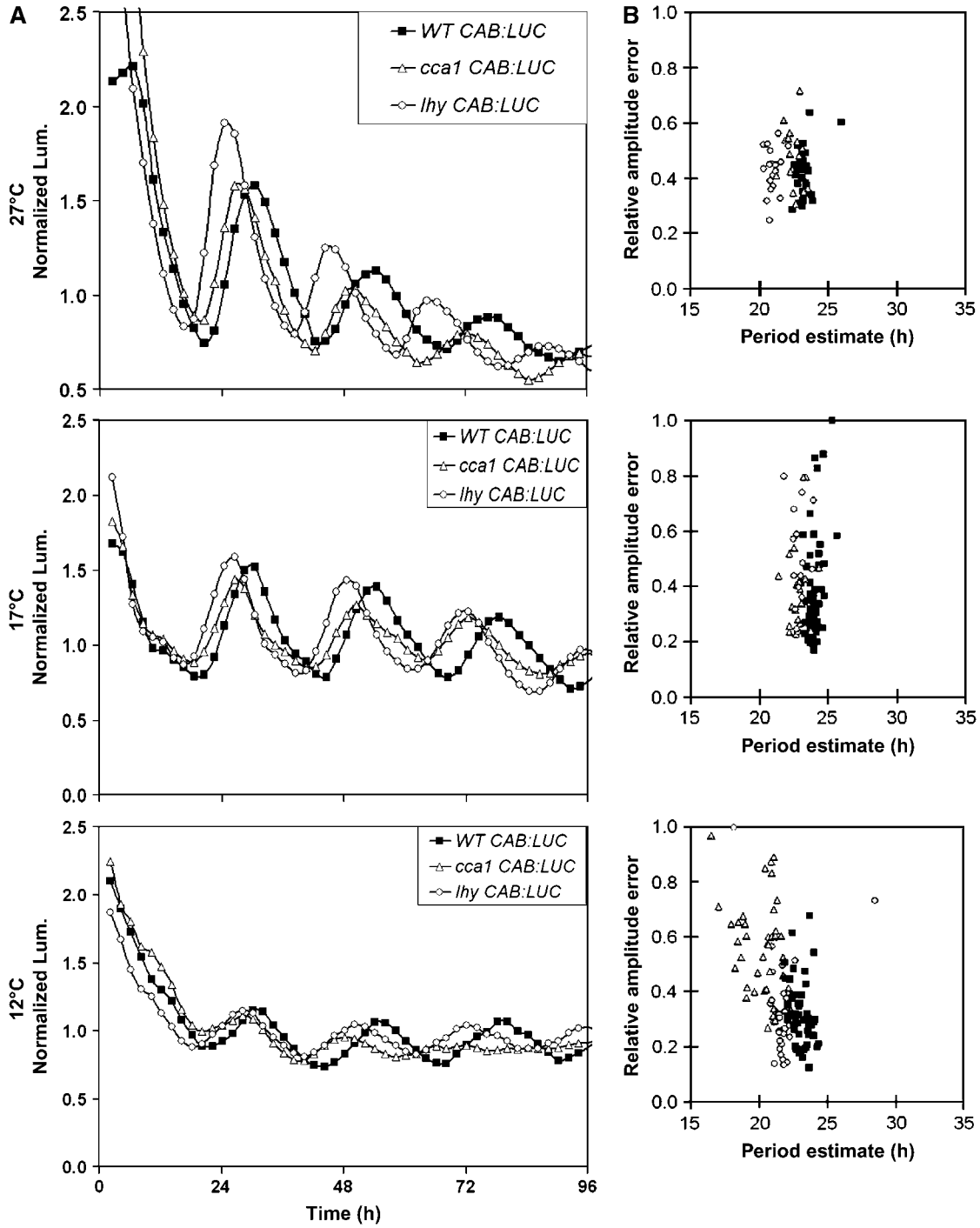

Figure 5. The *lhy* Mutant Is Compromised in Its Ability to Buffer the Clock against Increases in Temperature, and the *cca1* Mutant Is Compromised in Its Buffering Capacity at Lower Temperatures.

Transgenic seedlings carrying the *CAB*:*LUC* reporter gene were entrained under 12L:12D cycles for 7 d, after which the seedlings were transferred to 12, 17, or 27°C and constant red and blue light. Luminescence was monitored over the subsequent 96 h.

(A) Plots represent average normalized expression of *CAB* in wild-type Ws (closed squares), the *cca1* mutant (open triangles), and the *lhy* mutant (open circles) at 12, 17, or 27°C. Mutant and wild-type expression was monitored in at least four independently transformed lines, and the plots show average expression in representative *CAB*:*LUC* lines.

(B) Summary of the mathematical analysis of the expression rhythms of the seedlings, represented by plots of period estimates plotted against the respective relative amplitude errors for wild-type Ws (closed squares), *cca1* (open triangles), and *lhy* (open circles).

temperature-dependent manner, as demonstrated by the effects of the *gi* mutation, thus contributing to the stable period over a wide range of temperatures. The qualitative effect of decreases in temperature could be simulated by increasing *LHY* transcription rates, causing an increase in the period, which could be balanced by decreasing the transcription rates of components of *Y* redundant to GI (see Supplemental Methods Figure 6 online). In addition to its effect on period, the *gi* null mutant affects the robustness of circadian rhythms, an effect not shared by the partial *gi-611* allele. The *lhy* mutant at 27°C affects only the circadian period, not rhythmic robustness, but *cca1* at 12°C affects both, phenocopying the *gi* mutant. This difference between the *gi* and *lhy* mutants suggests that the effect of *GI* on rhythm robustness at high temperature functions via a mechanism that is less dependent on *LHY* than the effect on circadian period.

Simulating the effects of temperature by altering *LHY* and *Y* expression showed that these counterbalancing effects were largely sufficient to replicate the observed effects of temperature on clock gene expression in the wild type. The simulations also confirmed that the effects of the *gi* mutation on the clock at all temperatures are consistent with the identification of *GI* as part of *Y*. Temperature, in fact, will affect many processes in addition to *LHY* and *Y* transcription rates (potentially, all 61 kinetic parameters in the model). Obtaining simulations that exactly fit the data would likely require all of the parameters to be estimated afresh for each temperature. To constrain the estimation, most of the experimental results that were used in developing the original model would also have to be replicated at 12 and 27°C. The current clock model must also be extended to account for our temperature-specific data. For example, *LHY* and *CCA1* were modeled as a single gene and must be separated to explore the differences in their functions at high and low temperature.

This role of *GI* in extending the temperature range over which robust rhythms can be maintained is analogous to that of the two alternatively spliced forms of the *Neurospora* clock gene *FRQ*, $FRQ^{1\text{-}989}$ and $FRQ^{100\text{-}989}$ (Garceau et al., 1997). Both the long and short forms of FRQ are capable of restoring rhythmicity in a *frq* null strain; however, the long form is unable to sustain rhythmicity at low temperatures, and the short form is unable to sustain rhythmicity at high temperatures (Liu et al., 1997). Thus, the two spliced forms together extend the range of temperatures over which rhythms can be maintained. From our data, it is not clear how *GI* extends the range of rhythmicity. However, it is possible that the *GI*/*TOC1* loop of the interlocking-loop model proposed by Locke et al. (2005) plays an important function in stabilizing the clock at higher temperatures.

Previously, it was demonstrated that the flowering phenotype of *gi* mutants is temperature-independent, with the exception of *gi-2* (Araki and Komeda, 1993). Here, we demonstrate that the circadian phenotype of *gi* alleles is temperature-dependent. Together, these two observations suggest that the circadian and flowering phenotypes of *gi* mutants result from distinct functions of GI. This conclusion is further supported by the isolation of two new *gi* alleles, *gi-611* and *gi-596* (see Supplemental Figure 1 online). These have short- and long-period circadian phenotypes, respectively, but neither shows the striking late-flowering phenotype of other *gi* alleles under long photoperiods. Indeed, *gi-611* mutants flowered early in short photoperiods, which might be predicted from their short-period phenotype (Yanovsky and Kay, 2002). A similar conclusion has been drawn by Mizoguchi et al. (2005) based on the characterization of a *GI* overexpressor and *gi-3*. They demonstrated a disproportionate effect of both *GI* overexpression and the *gi-3* mutation on the control of circadian clock–regulated flowering genes compared with other circadian clock–regulated genes.

Here, we have shown that *GI* plays a critical role in temperature compensation of the clock and in extending the range of temperatures at which rhythmicity can be maintained. We recently demonstrated that having a robust and accurate clock increases photosynthesis and productivity in *Arabidopsis* (Dodd et al., 2005); thus, our new insight into how the clock maintains robustness and accuracy at high temperatures is likely to have implications for enhancing the performance of plants at higher and lower temperatures. It is feasible that this could be used to extend the geographical range of crops and potentially allow the development of new varieties able to cope better with global climate change.

METHODS

Plant Material

Arabidopsis thaliana gi-11 was isolated in a screen of T-DNA insertion lines described by Richardson et al. (1998) and Fowler et al. (1999). The *CAB*:*LUC*+ transgene, in the Ws background, was as described by Hall et al. (2002). The *cca1-11* and *lhy-21* mutants were isolated from the Arabidopsis Functional Genomics Consortium population (Krysan et al., 1999). Single mutants have been described (Hall et al., 2003). The mutants *gi-611* and *gi-596* were isolated in a screen for mutants with altered temporal expression of *CAB* from an ethyl methanesulfonate population of the Ws *CAB*:*LUC* transgenic line 6A.

Growth Conditions

For luminescence, RNA extraction, and leaf movement analysis, seedlings were first surface-sterilized in 70% ethanol for 2 min, immediately followed by 50% bleach for 10 min. They were then rinsed twice in sterile distilled water and resuspended in 0.15% agar. The seedlings were then sown on Murashige and Skoog medium containing 3% sucrose and 1.5% agar. Seeds were kept at 4°C for 2 d and then grown in 12L:12D cycles of 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a Sanyo MLR350 plant growth chamber. Temperatures both during entrainment and during experiments were logged using Hobo temperature loggers (Onset Computer).

Rhythm Analysis

Leaf movement rhythms were measured using a time-lapse video imaging system as described by Edwards et al. (2005). However, instead of Ultra-track cameras, Sony Exwave HAD cameras (Sovereign International) were used, having high resolution and allowing the number of plants assayed on one plate to be increased from 12 to 15. Luminescence levels were analyzed using an ORCA-II-BT 1024 16-bit camera cooled to −80°C (Hamamatsu Photonics). The camera was housed on top of a Sanyo MIR-553 cooled incubator maintaining a uniform temperature ±0.5°C (Sanyo Gallenkamp). Illumination was provided by four red/blue light-emitting diode arrays (MD Electronics). Image acquisition and light control were driven by WASABI imaging software (Hamamatsu Photonics). The images were processed using Metamorph 6.0 image-analysis software (Molecular Devices). Individual period estimates were generated

by importing data into BRASS (available from www.amillar.org) and using BRASS to run fast Fourier transform nonlinear least-squares analysis programs (Plautz et al., 1997) on each data trace to generate period estimates and relative amplitude errors.

RNA Analysis

Plants entrained in 12L:12D for 7 d at 22°C were transferred to constant light at 12, 17, 22, and 27°C. After 72 h of free-running conditions, ~100 seedlings were harvested every 4 h for 24 h and frozen in liquid nitrogen. Total RNA was extracted and treated with DNase using RNeasy plant mini kits (Qiagen) according to the manufacturer's instructions. A 2-μg portion was reverse-transcribed using the Advantage-for-PCR kit (BD Bioscience) with random hexamer primers according to the manufacturer's instructions. *TOC1*, *LHY*, *CCA1*, and *GI* transcript abundance was measured in each sample relative to UBQ10 using quantitative real-time PCR in a Rotor-Gene 3000 real-time PCR machine (Corbett Research). Each reaction contained 2 μL of cDNA product and 6 μL of QuantiTect SYBR Green PCR mix (Qiagen) together with gene-specific primers: *UBQ10* forward primer, 5′-CACACTCCACTTGGTCTTGCGT-3′; *UBQ10* reverse primer, 5′-TGGTCTTTCCGGTGAGAGAGTCTT-3′; *TOC1* forward primer, 5′-TCTTCGCAGAATCCCTGTGAT-3′; *TOC1* reverse primer, 5′-GCTGCACCTAGCTTCAAGCA-3′; *LHY* forward primer, 5′-ACGAAACAGGTAAGTGGCGACA-3′; *LHY* reverse primer, 5′-TGGGAACATCTTGAACCGCGTT-3′; *CCA1* forward primer, 5′-GATGATGTTGAGGCGGATG-3′; *CCA1* reverse primer, 5′-TGGTGTTAACTGAGCTGTGAAG-3′; *GI* forward primer, 5′-GGTCGACGGTTTCTCCAATCTA-3′; *GI* reverse primer, 5′-CGGACTATTCATTCCGTTCTTC-3′.

The *UBQ10* and *LHY* primers have been described previously (Czechowski et al., 2004), as have the *CCA1* and *GI* primers (Hall et al., 2003) and the *TOC1* primers (Farre et al., 2005). The efficiency value of amplification for each set of primers was determined beforehand by measuring the abundance of transcripts from a cDNA dilution series. Efficiency values were computed for each primer set using REST (http://www.wzw.tum.de/gene-quantification/; Pfaffl et al., 2002). Each RNA sample was assayed in triplicate, and RNAs were assayed from three biological repeats. The *TOC1*, *LHY*, *CCA1*, and *GI* transcript abundance levels were normalized to *UBQ* using Q-gene software (http://www.biotechniques.com/softlib/qgene.html; Muller et al., 2002).

Computational Methods

Simulations were performed in MATLAB and Circadian Modeling, a user-friendly simulation interface that is freely available from www.amillar.org/downloads.html. Starting parameter values and model equations were as described (Locke et al., 2005). Parameter values were modified to replicate experimental data at high and low temperatures, as described in the Supplemental Methods online.

Accession Numbers

Sequence data for *GI*, *LHY*, *CCA1*, and *TOC1* can be found in the GenBank/EMBL data libraries under the following accession numbers: At1g22770 (*GI*), At1g01060 (*LHY*), At2g46830 (*CCA1*), and At5g61380 (*TOC1*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The Late-Flowering Phenotype of the *gi-11* Mutation Can Be Complemented with a Wild-Type *GI* cDNA.

Supplemental Figure 2. Temperature Compensation of Leaf Movement Rhythms for L*er* Wild Type and L*er gi-3*.

Supplemental Figure 3. Amino Acid Substitutions in the N-Terminal Half of the GI Protein Can Lengthen or Shorten Circadian Period but Do Not Result in Late Flowering.

Supplemental Figure 4. At 4°C, the Oscillations of Key Clock Genes Dampen Rapidly.

Supplemental Figure 5. Modeling the Temperature Compensation Mechanism at High Temperatures.

Supplemental Figure 6. Modeling the Temperature Compensation Mechanism at Low Temperatures.

Supplemental Methods.

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