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

Peter D. Gould,^a James C.W. Locke,^{b,c,d} Camille Larue,^a Megan M. Southern,^c Seth J. Davis,^e Shigeru Hanano,^e Richard Moyle,^{f,g} Raechel Milich,^f Joanna Putterill,^f Andrew J. Millar,^{d,h,1} and Anthony Hall^{a,1}

^a School of Biological Sciences, University of Liverpool, Liverpool L69 7ZB, United Kingdom

^b Department of Physics, University of Warwick, Coventry CV4 7AL, United Kingdom

^c Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom

^d Interdisciplinary Programme for Cellular Regulation, University of Warwick, Coventry CV4 7AL, United Kingdom

^e Max Planck Institute for Plant Breeding, Cologne D-50829, Germany

- ^fUniversity of Auckland, School of Biological Sciences, Private Bag 92019, Auckland, New Zealand
- ⁹ Botany Department, School of Integrative Biology, University of Queensland, Brisbane Qld 4072, Australia

^h Institute of Molecular Plant Sciences, University of Edinburgh, Edinburgh EH9 3JH, United Kingdom

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 *Ihy* 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, 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 \sim 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

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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

¹To whom correspondence should be addressed. E-mail andrew. millar@ed.ac.uk or anthony.hall@liverpool.ac.uk; fax 44-0131-6505392 or 44-0151-7954403.

The authors responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) are: Andrew J. Millar (andrew.millar@ed.ac.uk) and Anthony Hall (anthony.hall@liverpool. ac.uk).

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* (*Ler*) and between *Ler* 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; Hug 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*.

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 \times *Ler* 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 *Ler* 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 *qi-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 wildtype 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 freerunning 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 temperaturedependent 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 = 1.8×10^{-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 Ler 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.

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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 *qi-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 × 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 temperaturedependent 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 freerunning 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 freerunning 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 *qi* 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 temperatureindependent, 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 shortperiod 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 Ihy and cca1 loss-offunction mutants. Both Ihy 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 \sim 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 Ihy (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 temperaturedependent 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 *Ihy* 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 *Ihy* 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*¹⁻⁹⁸⁹ and *FRQ*¹⁰⁰⁻⁹⁸⁹ (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 *Gl* overexpressor and *gi*-3. They demonstrated a disproportionate effect of both *Gl* 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 μ mol·m⁻²·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 $\pm 0.5^{\circ}$ 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 realtime PCR in a Rotor-Gene 3000 real-time PCR machine (Corbett Research). Each reaction contained 2 μ L of cDNA product and 6 μ L 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'-ACG-AAACAGGTAAGTGGCGACA-3'; LHY reverse primer, 5'-TGGGAACAT-CTTGAACCGCGTT-3'; CCA1 forward primer, 5'-GATGATGTTGAGG-CGGATG-3'; CCA1 reverse primer, 5'-TGGTGTTAACTGAGCTGTG-AAG-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 userfriendly 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 Ler Wild Type and Ler 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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