

Ambient Temperature Signal Feeds into the Circadian Clock Transcriptional Circuitry Through the EC Night-Time Repressor in *Arabidopsis thaliana*

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An interlocking multiloop model has been generally accepted to describe the transcriptional circuitry of core clock genes, through which robust circadian rhythms are generated in *Arabidopsis thaliana*. The circadian clock must have the ability to integrate ambient temperature signals into the clock transcriptional circuitry to regulate clock function properly. Clarification of the underlying mechanism is a longstanding subject in the field. Here, we provide evidence that temperature signals feed into the clock transcriptional circuitry through the evening complex (EC) night-time repressor consisting of EARLY FLOWERING 3 (ELF3, ELF4) and LUX ARRHYTHMO (LUX; also known as PCL1). Chromatin immunoprecipitation assays showed that PSEUDO-RESPONSE REGULATOR7 (PRR7), GIGANTEA (GI) and LUX are direct targets of the night-time repressor. Consequently, transcription of PRR9/PRR7, GI and LUX is commonly regulated through the night-time repressor in response to both moderate changes in temperature ($\Delta 6^{\circ}\text{C}$) and differences in the steady-state growth-compatible temperature ($16\text{--}28^{\circ}\text{C}$). A warmer temperature inhibits EC function more, whereas a cooler temperature stimulates it more. Consequently, the expression of these target genes is up-regulated in response to a warm temperature specifically during the dark period, whereas they are reversibly down-regulated in response to a cool temperature. Transcription of another EC target, the PIF4 (PHYTOCHROME-INTERACTING FACTOR 4) gene, is modulated through the same thermoregulatory mechanism. The last finding revealed the sophisticated physiological mechanism underlying the clock-controlled output pathway, which leads to the PIF4-mediated temperature-adaptive regulation of hypocotyl elongation.

Keywords: *Arabidopsis thaliana* • Circadian clock • Hypocotyl elongation • Pseudo-response regulator • Response to temperature • Transcription circuitry.

Abbreviations: CCA1, CIRCADIAN CLOCK ASSOCIATED 1; ChIP, chromatin immunoprecipitation; EC, evening complex; ELF3, EARLY FLOWERING 3; ELF4, EARLY FLOWERING 4; GFP, green fluorescent protein; GI, GIGANTEA; LBS, LUX-binding site; LD, light/dark; LL, continuous light; LHY, LATE ELONGATED HYPOCOTYL; LUX, LUX ARRHYTHMO; PCL1, PHYTOCLOCK 1; PIF4, PHYTOCHROME-INTERACTING FACTOR 4; PMSF, phenylmethylsulfonyl fluoride; PRR, PSEUDO-RESPONSE REGULATOR; qRT-PCR, quantitative real-time PCR; TOC1, TIMING OF CAB EXPRESSION 1; YFP, yellow fluorescent protein; ZT, Zeitgeber time.

Introduction

In the flowering plant *Arabidopsis thaliana*, significant progress has been made in defining the molecular mechanism of circadian clock operation (McClung 2011, Nagel and Kay 2012, Carre and Veflingstad 2013, Sanchez and Yanovsky 2013). The central oscillator that has been uncovered is composed of mainly three classes of transcriptional regulators (**Supplementary Fig. S1**), comprising (i) Myb-related proteins CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL) (Mizoguchi et al. 2002, Schaffer et al. 1998, Wang and Tobin 1998), (ii) pseudo-response regulators (PRR9, PRR7 and PRR5) including TOC1 (TIMING OF CAB EXPRESSION 1; also known as PRR1) (Makino et al. 2000, Matsushika et al. 2000, Strayer et al. 2000, Eriksson et al. 2003, Farre et al. 2005, Nakamichi et al. 2005a, Nakamichi et al. 2005b); and (iii) the so-called evening complex (EC) (Helfer et al. 2011, Nusinow et al. 2011), which is composed of LUX (LUX ARRHYTHMO; also known as PCL1) (Hazen et al. 2005b, Onai and Ishiura 2005), ELF3 (EARLY FLOWERING 3) and ELF4 (Doyle et al. 2002, Kikis et al. 2005, Kolmos et al. 2009, Thines and Harmon 2010, Dixon et al. 2011). According to a current model of the clock transcriptional circuitry (Nagel and Kay

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2012, Pokhilko et al. 2012, Carre and Veflingstad 2013), (i) the morning gene products CCA1 and LHY repress the transcription of evening genes *LUX*, *ELF3* and *ELF4*; (ii) in turn, the daytime gene products *PRR9*, *PRR7* and *PRR5* repress the morning genes; (iii) and eventually the EC night repressor represses the trio of day genes; (iv) another evening gene, *TOC1*, is also repressed by CCA1 and LHY, and it plays widespread roles through repressing the morning gene CCA1, the day genes *PRR9*, *PRR7* and *PRR5*, and the evening genes *LUX* and *ELF4*. GIGANTEA (*GI*) together with ZEITLUPE (*ZTL*) is also crucially implicated in the clock transcriptional circuitry by negatively regulating *TOC1* and *PRR5* post-translationally (Kiba et al. 2007, Kim et al. 2007).

The clock transcriptional circuitry must have the capacity to integrate the external cues of light in order not only to maintain the central oscillator functions accurately, but also to control a variety of output pathways properly. It has been postulated that CCA1/LHY, *PRR9* and *GI* are implicated in light responses in the clock transcriptional circuitry (Pokhilko et al. 2010, Pokhilko et al. 2012). Ambient temperature is as important as light for the circadian clock to measure the time accurately (Penfield 2008, McClung and Davis 2010, Boikoglou et al. 2011, Wigge 2013). Temperature has two mechanistic impacts on the plant oscillator system. On the one hand, in a process referred to as temperature compensation, the oscillator resists changes in ambient temperature. This ensures a constant oscillation period of about 24 h within a wide range of ambient temperatures. On the other hand, in a process termed entrainment, temperature can act as a resetting cue. Indeed, temperature fluctuations as small as $\Delta 4^{\circ}\text{C}$ within a day can reset the plant circadian oscillator. How does circadian oscillation resist differences in growth temperature? Paradoxically, how does the phase respond to small changes in ambient temperature to reset the circadian rhythm? The answers to both of these fundamental questions are largely unknown, despite the fact that they have been addressed in various studies of *A. thaliana* (Edwards et al. 2005, Salome and McClung 2005a, Edwards et al. 2006, Gould et al. 2006, Salome et al. 2010, Gould et al. 2013).

Ambient temperature is as crucial as light for the circadian clock to control output pathways properly. For example, Arabidopsis seedling morphogenesis is controlled through the circadian clock so as to modulate the length of hypocotyls in a manner dependent on both photoperiod and temperature (Breton and Kay 2007, Nozue et al. 2007, Niwa et al. 2009). Specifically, the circadian clock and photoreceptors act in concert in regulation of hypocotyl elongation by regulating the basic helix–loop–helix transcription factor PHYTOCHROME-INTERACTING FACTOR 4 (*PIF4*), which promotes the elongation of hypocotyls preferentially in warm short days (Koini et al. 2009, Franklin et al. 2011, Kunihiro et al. 2011, Nomoto et al. 2012a). To understand the underlying molecular mechanism, a model has been proposed based on demonstrating that diurnal expression profiles of *PIF4* are modulated in response to both photoperiod and temperature (Nomoto et al.

2012b, Nomoto et al. 2013, Yamashino et al. 2013). However, little is known about how the circadian clock integrates the temperature signal to modulate diurnal expression profiles of the output *PIF4* gene.

On the bases of this background, here we asked the following general and specific questions. (i) How does the circadian clock integrate growth-compatible (or moderate) temperature signals into the clock transcriptional circuitry? (ii) How does the circadian clock modulate the diurnal expression profile of *PIF4* in response to differences in growth-compatible temperature, thereby leading to a temperature-adaptive output pathway? To address these issues, here we show that temperature signals feed into the clock transcriptional circuitry through the EC night-time repressor to regulate the transcription of *PRR9*/*PRR7*, *GI* and *LUX*, as well as *PIF4* by a common mechanism in response to both changes in temperature and differences in steady-state growth temperature. It is tempting to speculate that these findings are relevant to the longstanding issues of temperature compensation and entrainment. The findings also explain well the molecular mechanism underlying the clock-dependent and *PIF4*-mediated temperature-adaptive control of hypocotyl elongation.

Results

Transcription of certain core clock genes is regulated in response to changes in ambient temperature

We focused on the transcriptional circuitry of core clock genes including CCA1, LHY, *PRR9*, *PRR7*, *PRR5*, *LUX*, *TOC1*, *GI*, *ELF3* and *ELF4*, with special interest in their responses to ambient temperature (Fig. 1). Wild-type seedlings (accession Columbia, Col-0) were grown at 22°C in light/dark cycles, and the temperature was increased to 28°C at different Zeitgeber time (ZT) points. After 3 h, RNA samples were prepared and quantified (Fig. 1, red). As a control, samples were prepared from plants grown continuously at 22°C (Fig. 1, green). The transcription of both *PRR9* and *PRR7* was up-regulated following the temperature upshift, specifically before dawn (red arrows in Fig. 1A, B), while *PRR5* did not respond at any time (Fig. 1C). In contrast, the expression of LHY was down-regulated (Fig. 1D). Among the five evening genes, *LUX* and *TOC1* were up-regulated specifically during the early night (Fig. 1E, F), while *GI*, *ELF3* and *ELF4* did not respond at any time (Fig. 1G–I).

To examine these phenomena more closely, seedlings grown at 22°C were upshifted to 28°C during either the night-time or daytime, and the temperature responses were followed at 1 h intervals (Fig. 2, shaded and white panels, respectively). CCA1 as well as LHY were down-regulated by the temperature upshift before dawn, but not after dawn (Fig. 2A–D). *PRR9*, *PRR7* and *LUX* were up-regulated during the night (Fig. 2E, G, I), but not during the daytime (Fig. 2F, H, J). The up-regulation of *TOC1* was subtle (Fig. 2K, L). Interestingly, *GI* was also up-regulated significantly in response to the temperature upshift after

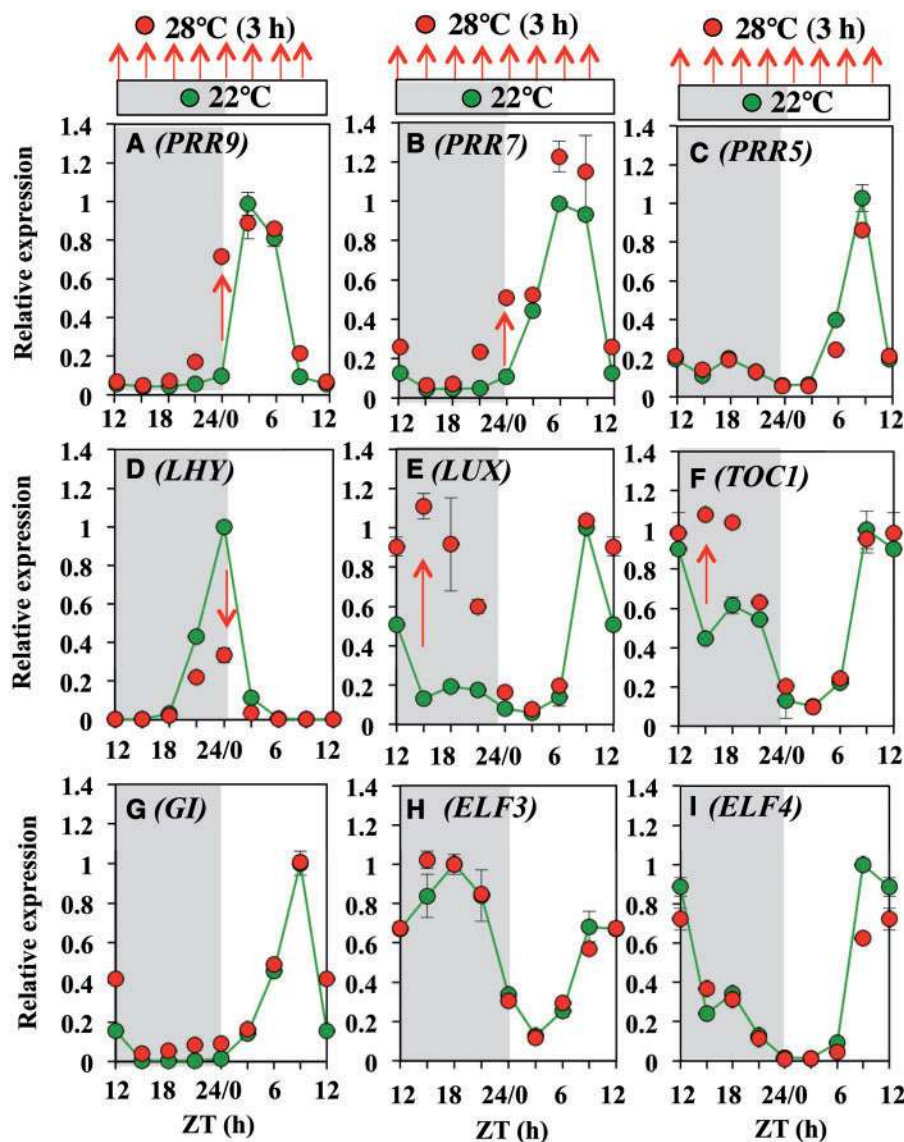


Fig. 1 Temperature responses of a set of clock genes. Seedlings (Col-0) were grown at 22°C for 8 d in light/dark cycles, and the growth temperature was upshifted to 28°C at different Zeitgeber time (ZT) points, as indicated schematically. RNA samples were prepared after incubation for 3 h, and levels of transcripts were determined by qRT-PCR (red). RNA samples were also prepared from control plants grown continuously at 22°C (green). Relative expression levels are shown as mean values \pm SD ($n = 3$). The values were normalized to the maximum value of the samples at 22°C. The shaded period corresponds to the dark.

lights-off, but not during the daytime (Fig. 2M, N). This effect on *GI* had previously been overlooked, because a linear scale for the y-axis was adopted for Fig. 1 (see Fig. 6F, G). Other genes (*PRR5*, *ELF3* and *ELF4*) appeared to be insensitive to changes in temperature, although subtle and/or indirect effects of temperature changes on their expression were seen (Fig. 2O–T).

In summary, the transcription of *LHY* (and its homolog *CCA1*) is repressed in response to a temperature upshift from 22 to 28°C, whereas *PRR7* (and its homolog *PRR9*), *GI* and *LUX* are markedly induced in response to the temperature upshift, specifically during the night. To elucidate these phenomena further, we focused on *LHY*, *PRR7*, *GI* and *LUX*. We characterized *CCA1* and *PRR9* simultaneously, but, for clarity, redundant data

are not presented in the main text, unless otherwise noted as **Supplementary data**.

Temperature responses of the clock genes in question are gated through the clock function

As shown above, *LHY* (*CCA1*), *PRR7* (*PRR9*), *GI* and *LUX* respond to a temperature upshift only during the dark period. This suggested that their temperature responses are gated in a time of day-specific manner through the clock function. To address this issue, we compared the temperature responses of these genes in seedlings grown under light/dark (LD) cycles with those grown in continuous light (LL) (Fig. 3, first and second columns, respectively). First, a control experiment was

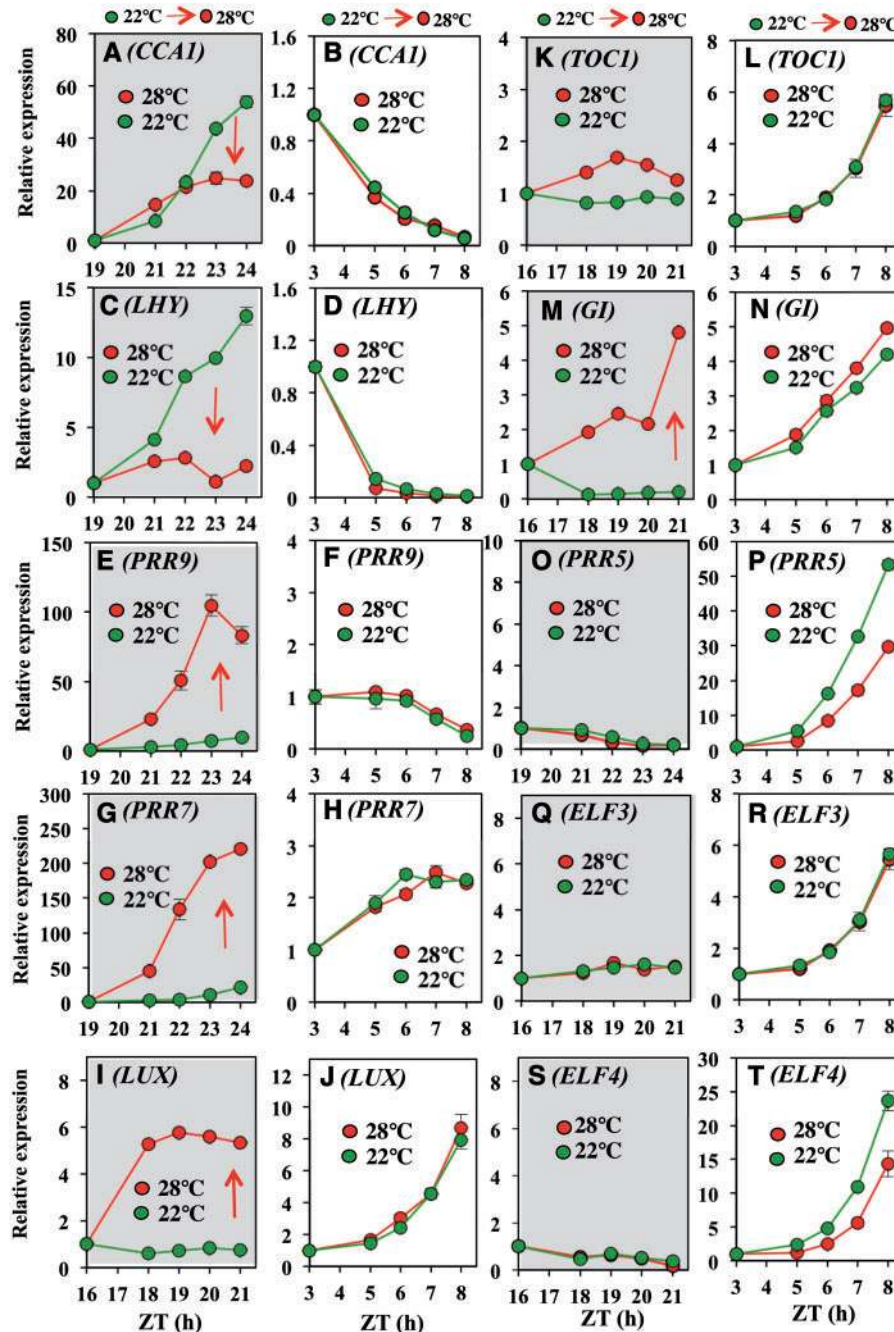


Fig. 2 Expression of a set of clock genes following temperature upshift. Seedlings (Col-0) grown at 22°C under light/dark cycles were upshifted to 28°C at the indicated ZT points, and the temperature responses of a set of indicated clock genes were followed at 1 h intervals. Values were normalized to the initial values, and relative expression levels are shown as mean values \pm SD ($n = 3$). The shaded period corresponds to the dark.

replicated biologically in LD for *LHY*, *PRR9*, *PRR7*, *GI* and *LUX* (Fig. 3, first column). On the other hand, when seedlings grown at 22°C under LD were transferred to LL, then exposed to 28°C with appropriate timing during the first subjective midnight, the genes in question also responded to changes in temperature in LL (Fig. 3, second column). These results suggested that the free-running circadian clock gates temperature signals in such a manner that the timing of temperature responses is confined strictly to during the dark period.

Temperature responses of the clock genes in question are not due to a heat stress response

To rule out the possibility that the observed temperature responses were merely due to non-physiological heat stress responses, we adopted an alternative temperature upshift from a relatively low temperature of 16°C to an optimal growth temperature of 22°C. Essentially the same transcriptional responses were observed as those following an upshift from 22 to 28°C

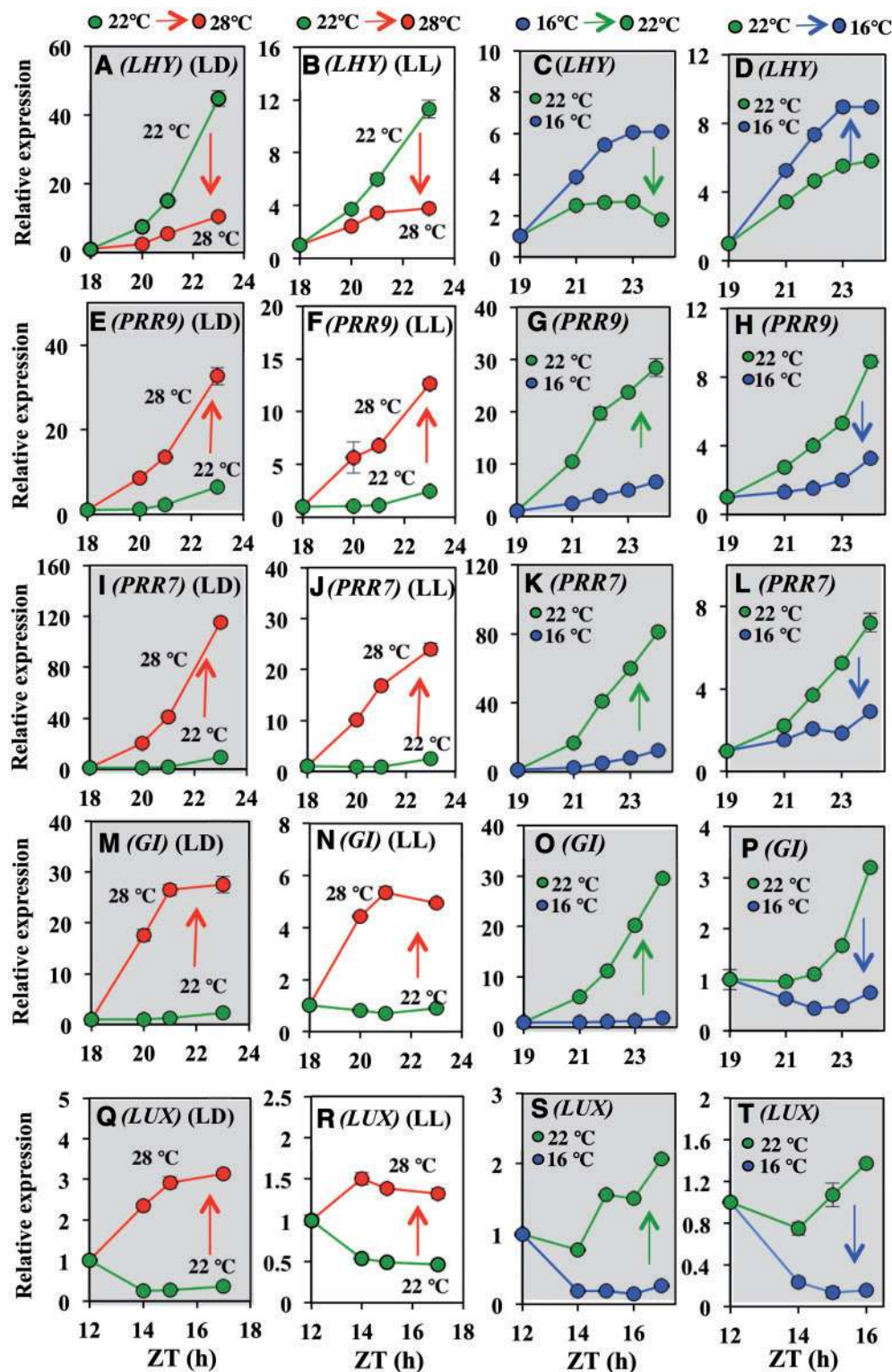


Fig. 3 Expression of a set of clock genes following either temperature upshift or downshift. (A, E, I, M, Q) Seedlings (Col-0) grown at 22°C under light/dark (LD) cycles were upshifted to 28°C at the indicated ZT points, and the temperature responses of a set of indicated clock genes were followed at 1 h intervals. (B, F, J, N, R) Seedlings (Col-0) grown at 22°C under LD cycles were released into continuous light (LL), and then upshifted to 28°C during the first subjective night at the indicated ZT points. (C, G, K, O, S) Seedlings (Col-0) grown at 16°C under LD cycles were upshifted to 22°C at the indicated ZT points. (D, H, L, P, T) Seedlings (Col-0) grown at 22°C under LD cycles were downshifted to 16°C at the indicated ZT points. Values were normalized to the initial values, and relative expression levels are shown as means \pm SD ($n = 3$). The shaded period corresponds to the dark.

(Fig. 3, third column). These results showed that *LHY* (*CCA1*), *PRR7* (*PRR9*), *GI* and *LUX* respond to changes across a wide range of growth-compatible temperatures (for *CCA1*, see [Supplementary Fig. S2A](#)).

Temperature responses of the clock genes in question are reversible

Next, seedlings grown at 22°C were downshifted to 16°C (Fig. 3, fourth column). The expression of *PRR7* (*PRR9*), *GI* and *LUX* was down-regulated in response to the temperature downshift, whereas the expression of *LHY* (*CCA1*) was up-regulated (for *CCA1*, see [Supplementary Fig. S2B](#)). These observations indicated that the temperature responses are reversible events. This view was confirmed with an alternative temperature downshift from 28 to 16°C (see Fig. 5L–N).

Detailed characteristics of the temperature responsiveness of clock genes

From these results (Figs. 1–3), a common point revealed was that the expression of *LHY* (*CCA1*), *PRR7* (*PRR9*), *GI* and *LUX* is reversibly regulated in response to changes across a wide range of growth-compatible temperatures, specifically during the dark period. However, a detail to point out is that *LHY* (*CCA1*) and *PRR7* (*PRR9*) tend to respond to changes in temperature preferentially after midnight and before dawn, whereas *LUX* responds sensitively during the early night (Fig. 1; [Supplementary Fig. S3](#)). *GI* responds throughout the dark period. Therefore, the temperature responsiveness of *LUX* was examined mainly at the onset of lights-off, and that of other genes was characterized at midnight, unless otherwise noted. Temperature effects on *TOC1* were subtle (most probably indirect), while *PRR5*, *ELF3* and *ELF4* appear to be insensitive to changes in temperature at any time ([Supplementary Fig. S4](#)). Hence, these genes were not further characterized, unless otherwise noted.

Temperature responsiveness of *LHY* (*CCA1*) was not compromised in any clock mutant tested

LHY (*CCA1*) is unique in that this gene is down-regulated in response to a temperature upshift, while others are up-regulated. The temperature responsiveness of *LHY* was examined through employing a comprehensive set of loss-of-function clock mutants, namely *prp9 prr7* (double mutant), *prp9 prr7 prr5* (triple mutant), *toc1*, *gi*, *elf3*, *elf4* and *pcl1* (or *lux*) (see the Materials and Methods). Surprisingly, none of these mutations compromised the temperature responsiveness of *LHY*, although its level of expression varied significantly depending on the mutation ([Supplementary Fig. S5](#); see also [Supplementary Fig. S6](#) for *CCA1*). The simplest explanation would be that the promoter activity of *LHY* (*CCA1*) itself is regulated directly by ambient temperature. Although this is likely, verification must await further studies. In any case, the temperature responsiveness of *LHY* (*CCA1*) was not further characterized in this study.

The EC night-time repressor is implicated as a common factor in temperature responsiveness of *PRR7* (*PRR9*), *GI* and *LUX*

The temperature responses of *PRR7*, *GI* and *LUX* were also examined through employing a set of appropriate mutants, namely *cca1 lhy*, *prp9 prr7*, *toc1* and *gi*. The temperature response of *PRR7* was not compromised in these mutants, although its expression level and profile in the mutant seedlings were considerably different from those in the wild-type seedlings (Fig. 4, first row). Hence, it was suggested that mutations of these clock genes (i.e. *CCA1*, *LHY*, *TOC1* and *GI*) appear to affect the temperature response of *PRR7* indirectly. The same was the case for the temperature responses of *GI* and *LUX* (Fig. 4, second and third rows, respectively).

We then focused attention on the EC night-time repressor, which is a transcriptional regulator composed of *ELF3*, *ELF4* and *LUX* (or *PCL1*). We employed a set of evening gene mutants, namely *elf3*, *elf4* and *pcl1*. These mutant seedlings were upshifted from 22 to 28°C. In *elf3*, the expression of *PRR9*, *PRR7*, *GI* and *LUX* was already constitutively high both before and after the temperature upshift (Fig. 5, first column). As a result, the temperature responsiveness of these genes was apparently abolished in this mutant. Essentially the same phenotypes were seen in both *elf4* and *pcl1* mutant seedlings (Fig. 5, second and third columns, respectively). Hence, we concluded that the EC night-time repressor is crucially and commonly implicated in the mechanism underlying the temperature responses of *PRR7* (*PRR9*), *GI* and *LUX* (see also [Supplementary Fig. S7](#)). To support this conclusion, *elf3* seedlings were downshifted from 28 to 16°C. The expression of *PRR7*, *GI* and *LUX* was again constitutively high before and after the temperature downshift (Fig. 5, fourth column). As negative controls, the expression of *TOC1* and *PRR5* was also examined in *elf3*, demonstrating that the expression of these genes was not affected as in the case in Col-0 ([Supplementary Fig. S8](#)).

Diurnal expression profiles of the clock genes in question in response to differences in steady-state growth temperature

Thus far, we have characterized expression of *PRR7* (*PRR9*), *GI* and *LUX* in response to changes in ambient temperature. However, we also needed to characterize the effects of differences in steady-state growth temperature on the diurnal expression profiles of these genes, because both conditions are equally crucial to understand the effects of ambient temperature on plant physiology in natural habitats.

First, the data for *PRR7*, *GI* and *LUX* shown in Fig. 1 were re-examined through adopting a logarithmic scale for the y-axis (Fig. 6, first column). In addition, the effects of differences in steady-state growth temperature on the diurnal expression profiles of *PRR7*, *GI* and *LUX* were examined. Wild-type seedlings were grown continuously at two different temperatures (i.e. 22 and 28°C) for 7 d in LD cycles, and then their diurnal expression profiles were examined throughout the next day

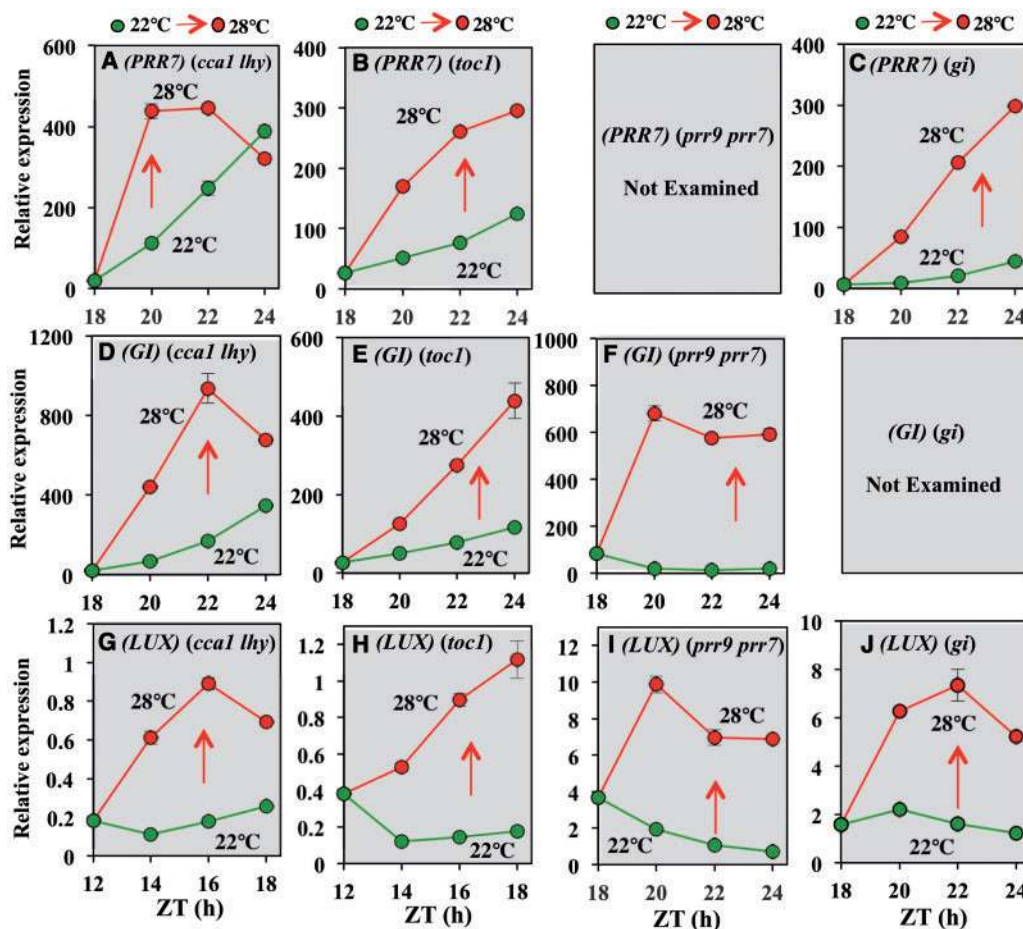


Fig. 4 Responses of *PRR7*, *GI* and *LUX* to a temperature upshift in a set of clock mutants. The set of indicated mutant seedlings, together with wild-type seedlings, were grown at 22°C, upshifted to 28°C at the indicated ZT points, and the temperature responses of a set of indicated clock genes were followed at 1 h intervals. Values were normalized to the initial values of wild-type seedlings, which are not shown for clarity. Their relative expression levels are shown as mean values \pm SD ($n = 3$). The shaded period corresponds to the dark.

(Fig. 6, second column). When the profile of *PRR7* was compared between these two temperatures (Fig. 6A, B), they were very similar, in that a warm temperature of 28°C up-regulated the expression of *PRR7*, specifically before dawn. The same temperature effect was seen for *GI* (Fig. 6E, F). In the case of *LUX* (Fig. 6I, J), both profiles were also similar, in that a warm temperature of 28°C up-regulated the expression of *LUX* preferentially during the early night. As negative controls, the diurnal expression profiles of *TOC1*, *ELF3* and *ELF4* were also examined by showing that the growth temperature does not affect their expression profiles (Supplementary Fig. S9). Then, we replicated experiments by employing wild-type and *elf3* seedlings grown at both 16 and 28°C, respectively. The results were reproducible for the wild-type seedlings (Fig. 6, third column), whereas the *elf3* seedlings showed high levels of constitutive expression of *PRR7*, *GI* and *LUX*, regardless of temperature (Fig. 6, fourth column). Hence, we concluded that an EC night repressor-mediated common mechanism is responsible for the temperature responses of *PRR7*, *GI* and *LUX* to both changes in temperature and differences in steady-state growth

temperature. It should be noted that such temperature effects on the diurnal expression profiles of *PRR7* and *LUX* were seen also for seedlings released into free-running LL conditions (Supplementary Fig. S10).

A biochemical approach to clarify EC function in connection with our proposal

There are conflicts between current general knowledge and our findings, based on which we hypothesized that the EC night-time repressor directly regulates *PRR7*, *GI* and *LUX* transcription in response to temperature. (i) It has been reported that *LUX* (i.e. the EC night-time repressor) does not bind to the *PRR7* promoter, although it binds to the *PRR9* promoter (Chow et al. 2012). (ii) There is no solid evidence that *GI* is also a target of the EC night-time repressor. (iii) There is no evidence that *ELF3* binds to the *LUX* promoter, although *LUX* binds to its own promoter (Dixon et al. 2011, Helfer et al. 2011). Hence, we needed to reassess whether the EC night-time repressor does indeed bind to the *PRR7*, *GI* and *LUX* promoters. We conducted chromatin immunoprecipitation (ChIP) assays by employing a

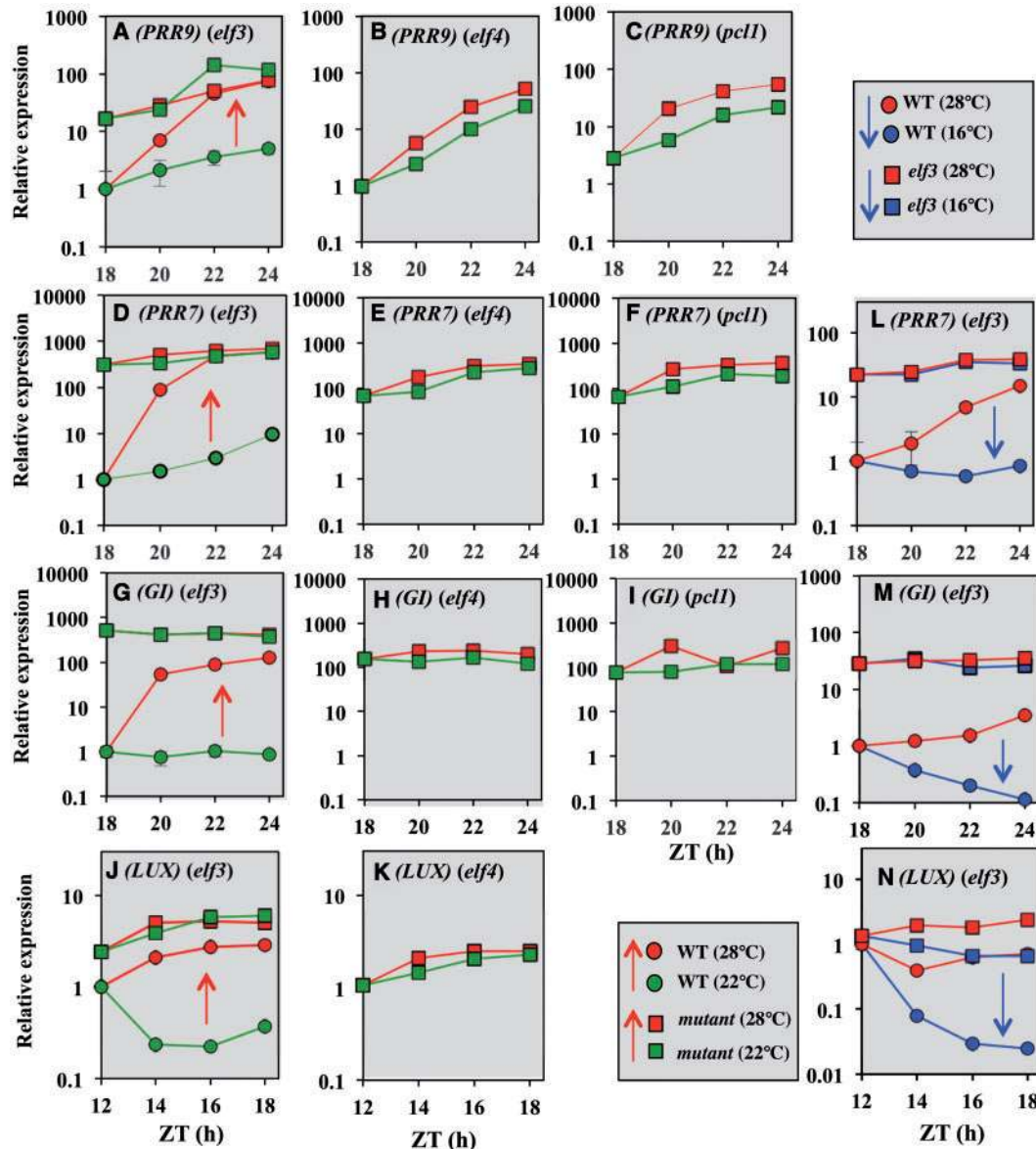


Fig. 5 Responses of *PRR9*, *PRR7*, *GI* and *LUX* to a temperature upshift in a set of clock mutants. (A–K) The set of indicated mutant seedlings (*elf3*, *elf4*, *pcl1*), together with wild-type seedlings, were grown at 22°C, upshifted to 28°C at the indicated ZT points, and the temperature responses of a set of indicated clock genes were followed at 1 h intervals. (L, M, N) Wild-type and *elf3* seedlings were grown at 28°C, and then downshifted to 16°C at the indicated ZT points. Values were normalized to the initial values of wild-type seedlings. The design of the figures is shown in the boxes. Their relative expression levels are shown as mean values \pm SD ($n = 3$). The shaded period corresponds to the dark.

set of transgenic lines each carrying an appropriate composite transgene, namely *LUX^{pro}-LUX-GFP* (Helfer et al 2011), *ELF3^{pro}-ELF3-YFP* (Dixon et al 2011) and *35S^{pro}-ELF3-HA* (see the Materials and Methods). First, we checked whether our ChIP procedures were reliable. We carried out ChIP assays to see whether we could successfully identify the best-established EC-binding site within the *PIF4* promoter (Nusinow et al. 2011). ChIP samples from transgenic lines carrying *LUX^{pro}-LUX-GFP* and *ELF3^{pro}-ELF3-YFP* were assayed (Supplementary Fig. S11). We succeeded in identifying the known EC-binding region, which is located about 150 bp downstream of the transcription start site of the *PIF4* gene. The result was quite

consistent with that reported previously (Nusinow et al. 2011). Then, we carried out a series of ChIP experiments with special reference to the *PRR7*, *GI* and *LUX* promoters (Figs. 7–9, respectively).

The consensus LUX-binding site (designated LBS) is 5'-GATT/ACG-3' (Helfer et al. 2011). The promoter sequence of *PRR7* contains a few perfect LBSs (Fig. 7, filled triangles). In addition, it contains a few near-perfect LBSs (open triangles). We conducted ChIP assays with special reference to these candidate regions (or amplicons of them). The results suggested that both LUX and ELF3 efficiently and preferentially bind to the same region, confined by amplicon d, which is located

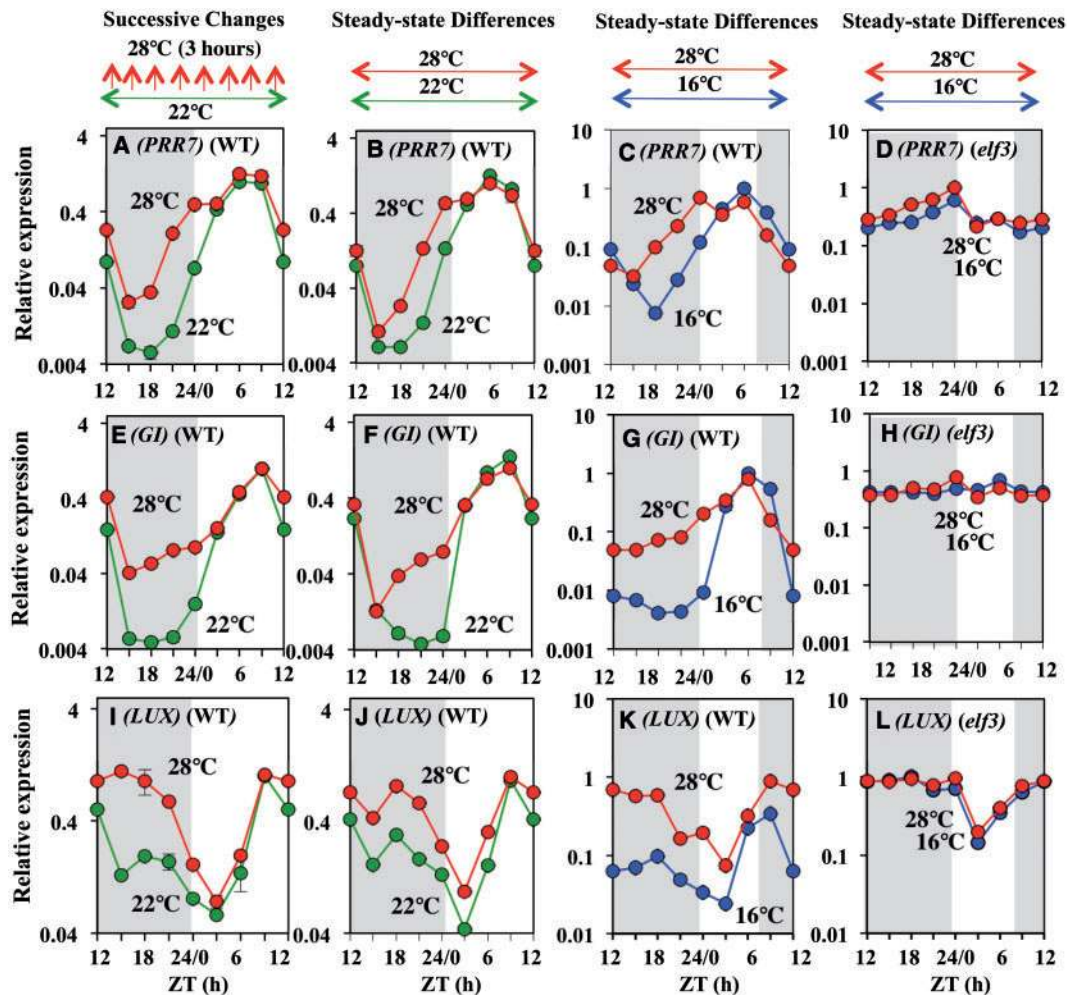


Fig. 6 Evidence for a common mechanism responsible for the temperature responses of *PRR7*, *GI* and *LUX* to both changes in temperature and differences in steady-state growth temperature. (A, E, I) The data shown in Fig. 1 were modified using the same logarithmic scale, as indicated. (B, F, J) Wild-type seedlings were grown at 22 and 28°C, as indicated at the top. The effects of differences in growth temperature on the diurnal expression profiles of *PRR7*, *GI* and *LUX* were analyzed. (C, G, K) Wild-type seedlings were grown at either 16 or 28°C, as indicated. (D, H, L) Similarly, the effects of differences in growth temperature on the diurnal expression profiles of *PRR7*, *GI* and *LUX* were analyzed in *elf3* mutant seedlings. Relative expression levels are shown as mean values \pm SD. Values were normalized to the maximum values of the samples at 28°C. The shaded period corresponds to the dark.

about 150 bp upstream of the *PRR7* transcription start site (Fig. 7A–C). This region contains two near-perfect LBSs in the fashion of an inverted repeat. This region within the *PRR7* promoter is most probably a previously unrecognized EC-binding site, one distinct from the weak ELF3-binding site located about 1,000 bp upstream of the *PRR7* gene (Dixon et al. 2011).

An upstream sequence of the *GI* promoter also contains several perfect or near-perfect LBSs (Fig. 8). The results of ChIP assays suggested that both *LUX* and *ELF3* preferentially bind to both amplicons a and b extending upstream from the *GI* transcription start site (Fig. 8A, B). This region is most probably a previously unrecognized EC-binding site within the *GI* promoter.

The *LUX* gene has a perfect LBS about 400 bp upstream of the transcription start site, and *LUX* binds to this region (Helfer et al. 2011) (Fig. 9). We repeated essentially the same ChIP assay

on the transgenic line carrying *LUX*^{pro}-*LUX*-GFP, and confirmed that *LUX* efficiently binds to this LBS-containing region (Fig. 9A). We then showed that *ELF3* is capable of binding to the same LBS-containing region (Fig. 9B, C).

In summary, these ChIP assay results supported our proposal in which we postulated that the *PRR7*, *GI* and *LUX* genes are direct targets of the EC night-time repressor, although ChIP assays with an appropriate *ELF4* probe must still be performed. In general, biochemical results of ChIP assays alone are not formally conclusive, unless additional genetic and/or biological evidence is provided concomitantly. In this study, such genetic evidence was already provided in Fig. 5, which showed that *LUX*, *ELF3* and *ELF4* are all essential for efficient repression of their transcription. Taken together, we concluded that the *PRR7*, *GI* and *LUX* genes are direct targets of the EC night-time repressor.

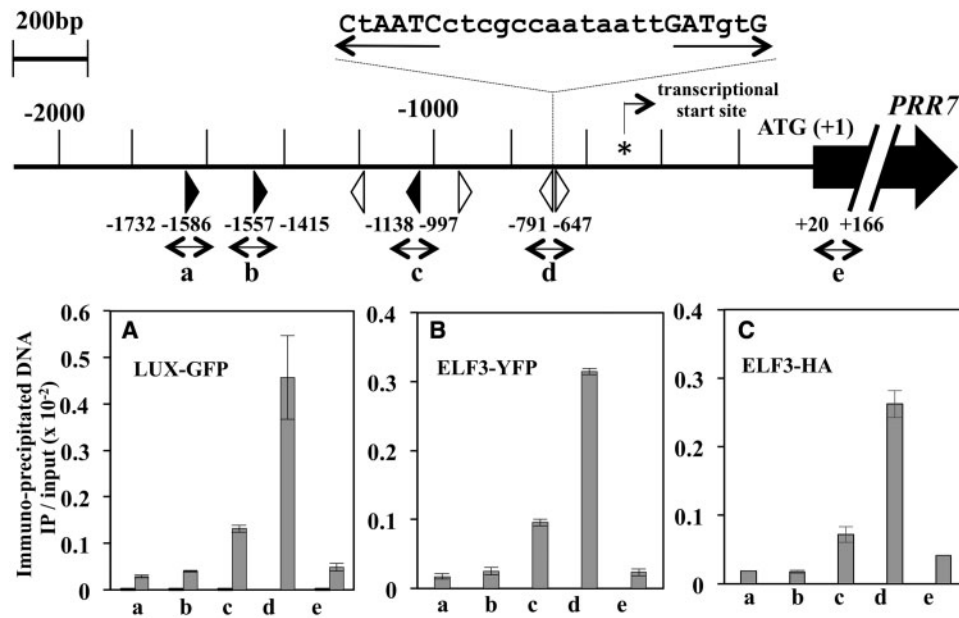


Fig. 7 ChIP assays with the *PRR7* promoter. An upstream sequence of the *PRR7* promoter is schematically depicted at the top. It contains a few perfect LBSs (LUX-binding sites, filled rectangles). In addition, there are a few near-perfect LBSs (open rectangles). ChIP assays were carried out with reference to the indicated amplicons denoted by a–e by employing a set of transgenic lines carrying (A) *LUX*^{PRO}-*LUX*-GFP, (B) *ELF3*^{PRO}-*ELF3*-YFP and (C) *ELF3*^{PRO}-*ELF3*-HA. As a negative control, Col-0 seedlings were also analyzed, but the values obtained were too small to show. These experiments were biologically replicated at least twice with essentially the same results; representative results are shown.

The *PIF4* gene is also regulated through the EC night-time repressor in response to ambient temperature

Finally, we examined the physiological impact of our findings. The best established target of the EC night-time repressor is the clock-controlled output gene, *PIF4*, which is involved in temperature-adaptive hypocotyl elongation (see [Supplementary Fig. S11](#), and the Introduction). If our proposal with regard to the EC-mediated temperature responses of *PRR7*, *GI* and *LUX* is correct, another EC target, *PIF4*, should display essentially the same temperature responses as those observed for *PRR7*, *GI* and *LUX*. Such experimental evidence is collectively shown in [Figs. 10](#) and [11](#). Expression of *PIF4* was rapidly up-regulated by a temperature upshift within growth-compatible temperatures (i.e. 16–28°C) specifically and reversibly before dawn ([Fig. 10](#), first row). The temperature response of *PIF4* was compromised in *elf3*, *elf4* and *pcl1* mutants in such a manner that the expression of *PIF4* in these mutants was constitutively high even at 22°C ([Fig. 10](#), second row). This is exactly what was observed earlier for *PRR7*, *GI* and *LUX* in response to changes in temperature.

A physiological approach to clarify EC function in connection with the *PIF4*-mediated output pathway

If our idea was correct, differences in steady-state growth temperatures should also affect the diurnal expression profile of

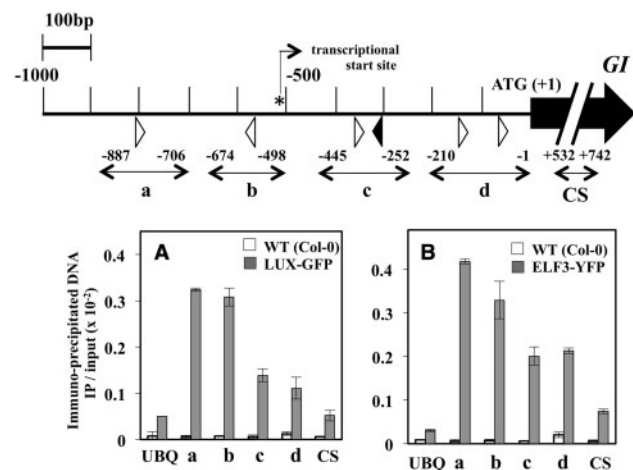


Fig. 8 ChIP assays with the *GI* promoter. An upstream sequence of the *GI* promoter is schematically depicted at the top. It contains a few perfect LBSs (LUX-binding sites, filled rectangles). In addition, there are a few near-perfect LBSs (open rectangles). ChIP assays were carried out with reference to the indicated amplicons denoted by a–d, together with appropriate negative references [an amplicon from the *UBQ10* gene and an amplicon from the *GI* coding sequence (CS)], by employing a set of transgenic lines carrying (A) *LUX*^{PRO}-*LUX*-GFP and (B) *ELF3*^{PRO}-*ELF3*-YFP. As a negative reference, Col-0 seedlings were also analyzed, as indicated. These experiments were biologically replicated at least twice with essentially the same results; representative results are shown.

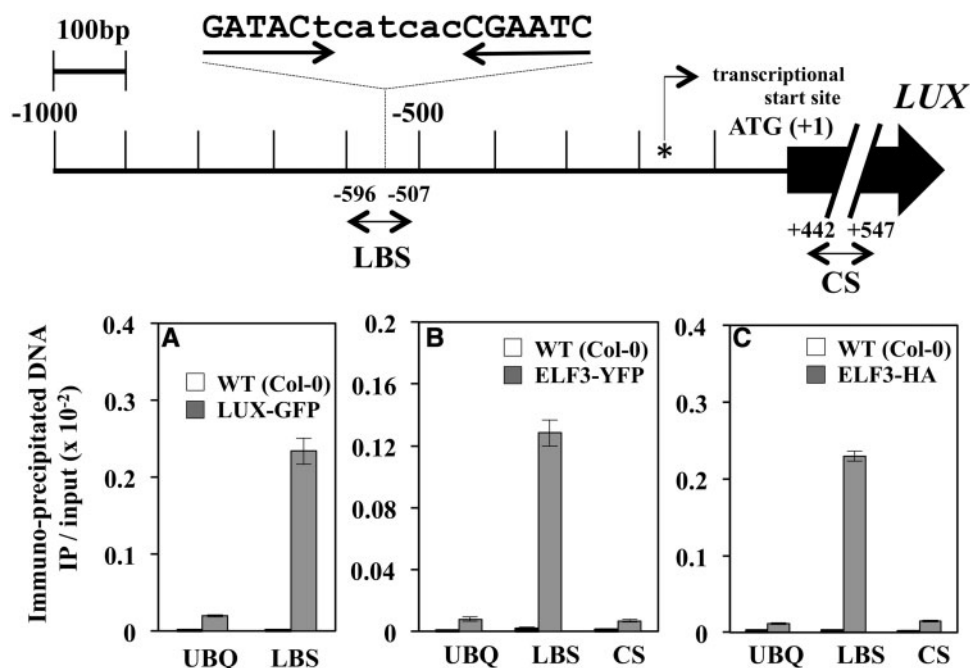


Fig. 9 ChIP assays with the *LUX* promoter. An upstream sequence of the *LUX* promoter is schematically depicted at the top. It contains a perfect LBS, as indicated. ChIP assays were carried out with reference to the LBS amplicon, together with an appropriate negative reference (an amplicon from the *UBQ10* gene), by employing a set of transgenic lines carrying (A) *LUX*^{PRO}-*LUX*-GFP, (B) *ELF3*^{PRO}-*ELF3*-YFP and (C) *ELF3*^{PRO}-*ELF3*-HA. As a negative reference, Col-0 seedlings were also analyzed. These experiments were biologically replicated at least twice with essentially the same results; representative results are shown.

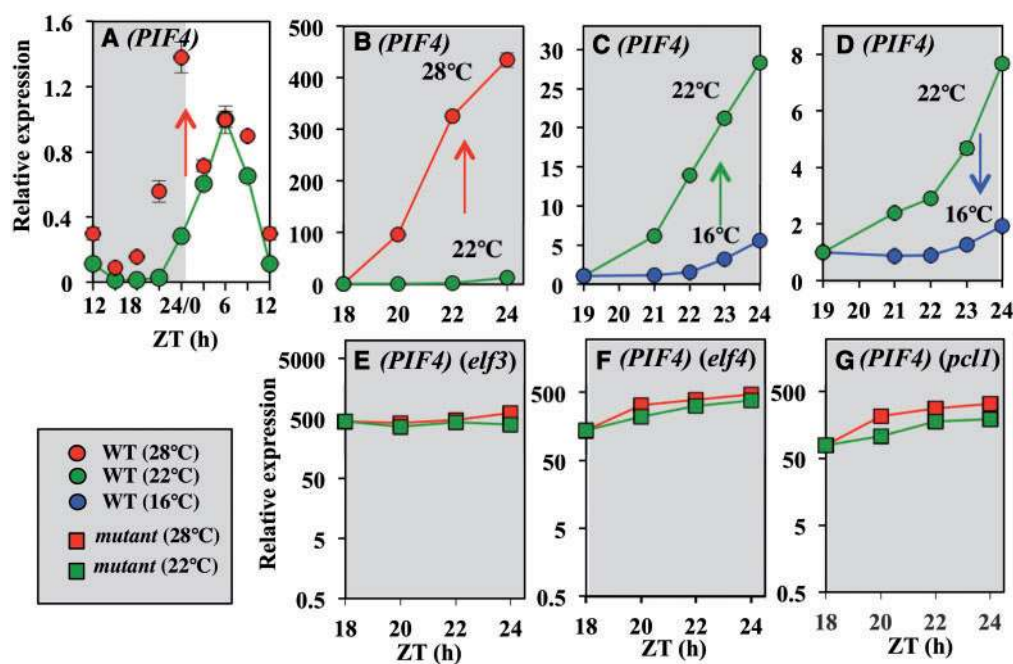


Fig. 10 *PIF4* response to changes in temperature through the same EC-mediated mechanisms as those revealed for *PRR7*, *GI* and *LUX*. These results were obtained using the same experimental details given in the legends to Figs. 1, 3, and 5.

PIF4. We addressed this issue on the basis of the pathway proposed for the *PIF4*-mediated temperature-adaptive control of hypocotyl elongation, as schematically shown (Fig. 11A; Nomoto et al. 2012b). Indeed, the diurnal expression of *PIF4*

was enhanced at a warm temperature of 28°C during the dark period before dawn in wild-type seedlings (Fig. 11B). This experiment was biologically replicated not only to confirm the critical result for *PIF4* (Supplementary Fig. 12A), but also to

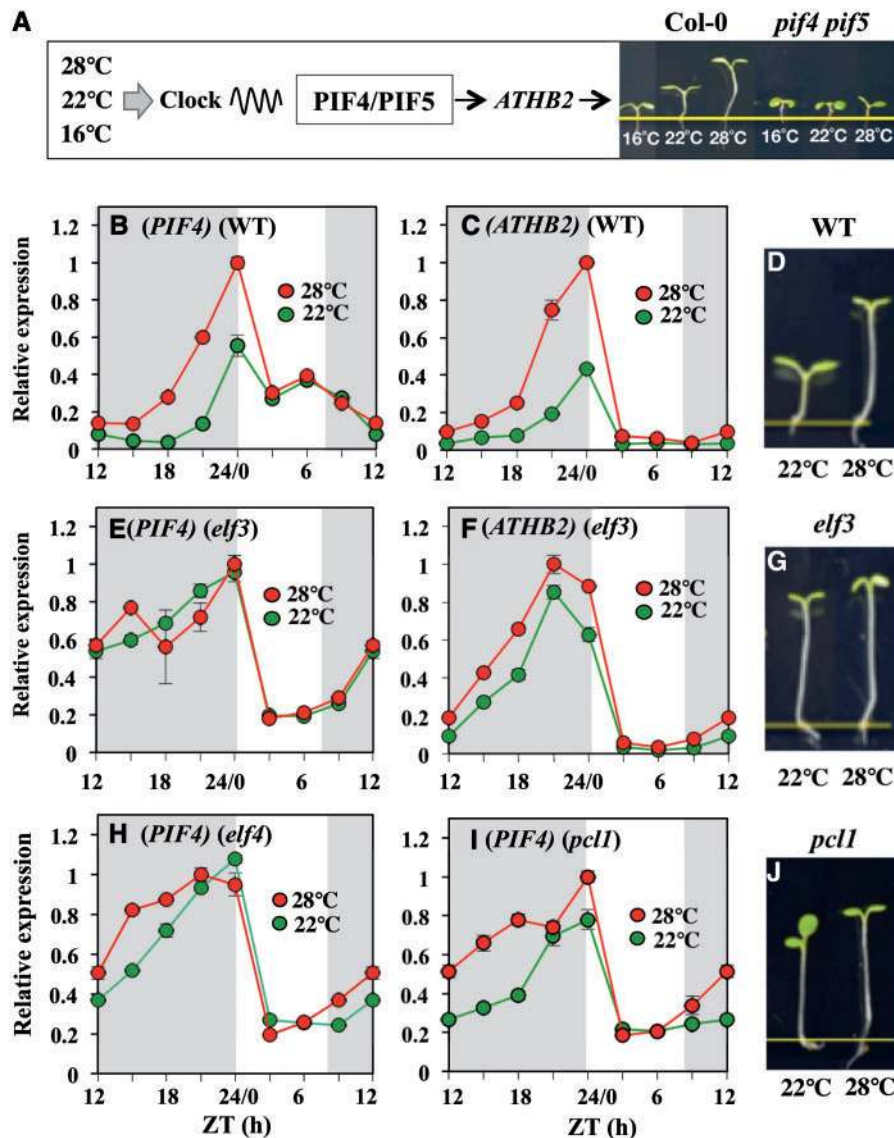


Fig. 11 Diurnal expression profiles of *PIF4* are modulated in response to differences in steady-state growth temperatures. (A) A schematic representation of the pathway of *PIF4*-mediated temperature-adaptive control of hypocotyl elongation. Wild-type (B, C), *elf3* (E, F), *elf4* (H) and *pcl1* (I) seedlings were grown at different temperatures of 22 and 28°C under light/dark cycles, and RNA samples were prepared at 3 h intervals throughout one day. Diurnal expression profiles of *PIF4* (B, E, H, I) and *ATHB2* (B, F) were examined, as indicated. Relative expression levels are shown as mean values \pm SD ($n = 3$). The maximum value of the samples at 28°C was taken as 1.0. The shaded period corresponds to the dark. (D, G, J) During the course of these experiments, representative seedlings were photographed.

examine the temperature response of *PIF5*, which plays a redundant role with *PIF4* (Nusinow et al. 2011, Nomoto et al. 2012b). As expected, the expression of *PIF5* was also enhanced before dawn in seedlings grown at 28°C, while the expression of *STO* employed as a negative control was not (Supplementary Fig. 12B). The temperature-dependent alteration of the *PIF4* profile could also be seen in free-running LL conditions (Supplementary Fig. 13). Then, we showed that the enhanced derepression of *PIF4*/*PIF5* at 28°C in the dark coincidentally resulted in a temperature-dependent enhancement of the target *ATHB2* gene (Fig. 11C), which results in the elongation

of hypocotyls at a higher temperature (Fig. 11D). These findings were in good agreement with the observation that the *PIF4*/*PIF5* proteins are stably and actively accumulated only in the dark (Kunihiro et al. 2011).

This temperature-dependent regulation of the diurnal *PIF4* profile was compromised in *elf3*, *elf4* and *pcl1* mutant seedlings (Fig. 11E, H, I). Constitutive *PIF4* expression in the dark in *elf3* resulted in a high level of *ATHB2* expression, regardless of temperature (Fig. 11F). These results nicely accounted for the EC mutant phenotype of constitutively long hypocotyls (Fig. 11G, J). These results demonstrated that *PIF4* is also

regulated through the EC night-time repressor in the same manner as *PRR7* (*PRR9*), *GI* and *LUX*. Through this clock-dependent mechanism, the diurnal expression profile of *PIF4* is modulated in response to differences in growth temperatures; consequently, the elongation of hypocotyls is controlled by ambient temperature.

Discussion

Based on the results of this study, we propose the following views, shown schematically in Fig. 12. Transcription of *PRR7* (*PRR9*), *GI* and *LUX* is up-regulated in response to a temperature upshift, specifically during the dark period. These phenomena are consistent with those reported previously (Paltiel et al. 2006, Salome et al. 2010, Thines and Harmon 2010). We further showed the following. The *PRR7* (*PRR9*), *GI* and *LUX* clock genes are common targets of the EC night-time repressor. Both types of temperature signals (i.e. changes in temperature and differences in steady-state temperature) feed into the clock transcriptional circuitry through a common pathway in which a warm temperature antagonizes EC activity, whereas a cool temperature stimulates it. Consequently, the *PRR7* (*PRR9*), *GI* and *LUX* clock genes are reversibly regulated at the level of transcription in such a way that a warmer temperature more efficiently induces the transcription of these genes specifically during the dark period, whereas a cooler temperature more strongly represses their transcription. It should be noted that we do not know whether the DNA binding of EC to the target promoters itself is inhibited by a warm temperature, or if a

warm temperature inhibits the repressor ability of EC without affecting its DNA binding ability. It should also be noted that no significant effect on the stabilities of ELF3–yellow fluorescent protein (YFP) and LUX–green fluorescent protein (GFP) was observed in response to changes in temperatures (data not shown). The diurnal expression profile of another EC target, the *PIF4* output gene, is also modulated by temperature through the same mechanism. Consequently, temperature signals feed as far as the *PIF4*-mediated output pathway, thereby leading to the temperature-adaptive control of hypocotyl elongation, as schematically illustrated in Fig. 12B.

First of all, several details should be discussed about the findings of this study. (i) The magnitude of induction by warm temperature varied from gene to gene in the order *PRR7* > *PRR9*/*GI* > *LUX*. This is partly due to the trough level of *LUX* expression being higher than that of the other genes (see Fig. 6, first and second column). (ii) The effect of the *elf3* mutation on the expression of *PRR7*, *GI* and *LUX* was more severe than the effects of the *elf4* and *pcl1* mutations (compare the derepressed values in Fig. 5, second row). This is probably because there are functionally redundant genes in the latter cases (e.g. *NOX* is homologous to *LUX*; Nusinow et al. 2011). (iii) The timing of efficient responses to temperature during the night was also different from gene to gene; for example, for *LUX* it was early night, for *GI*, throughout the night, and for *PRR9*/*PRR7*, before dawn (see Fig. 6). This observation is intriguing, as will be discussed later. (iv) Our quantitative real-time PCR (qRT-PCR) results showing steady-state levels of transcripts at a given time do not necessarily indicate fluctuation in activity of a given promoter (e.g. it may be partly due to changes in mRNA stability or to alternative splicing). To test this possibility, a transgenic line carrying a *PCL1^{Pro}::LUC* fusion gene was employed. This transgene contains only approximately 2,000 bp of *LUX* promoter sequence upstream of its ATG initiation codon (Onai and Ishiura 2005). Expression of the reporter gene was up-regulated following a temperature upshift from 22 to 28°C (Supplementary Fig. S14). (v) It was postulated that a warm temperature inhibits the EC night-time repressor. Nevertheless, the same conditions induce the expression of *LUX*, the protein product of which is one of the EC components. Seemingly, this does not make sense. However, it is conceivable that the induced *LUX* transcription factor may play a specific role in regulating a certain set of output genes without the need to form the EC. It is also conceivable that the induced *LUX* transcription factor may compensate for the EC inactivation by a temperature upshift. (vi) We do not know whether the EC night-time repressor itself is a temperature sensor, or whether an as yet unidentified temperature-sensing factor is implicated as a thermostat. A possible temperature-sensing factor is the histone variant H2A.Z, which generally regulates gene expression in response to changes in ambient temperature through modulating chromatin structure (Deal and Henikoff 2010, Franklin 2010, Kumar and Wigge 2010). H2A.Z-containing nucleosomes might be present exclusively in the EC target promoters. To examine this possibility, we employed an *arp6*

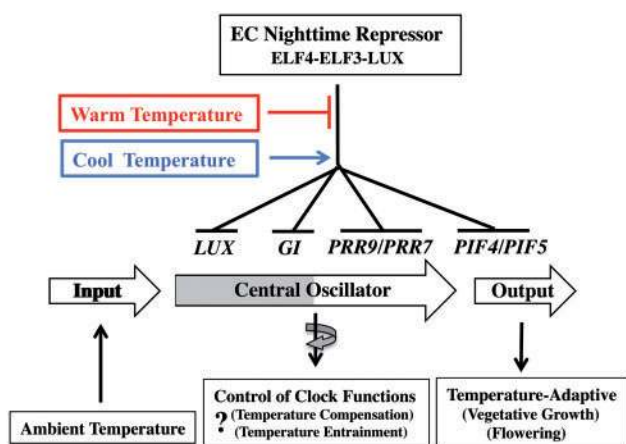


Fig. 12 Schematic representation of the EC night-time repressor-mediated temperature responses of the clock transcriptional circuitry. Details are given in the text. Briefly, both temperature signals (i.e. changes in temperature and differences in growth temperature) feed into the clock transcriptional circuitry through the EC at night-time so as to regulate its direct targets, namely *PRR7* (*PRR9*), *GI* and *LUX*, in such a manner that a warm temperature antagonizes EC activity, whereas a cool temperature stimulates it. These findings may be relevant to the longstanding problems referred to as temperature compensation and entrainment.

mutant, which is defective in the H2A.Z-mediated chromatin thermostat (Deal et al. 2007). The temperature-dependent alterations of diurnal expression profiles of *LUX* and *PRR7* were also seen even in *arp6* seedlings (Supplementary Fig. S15).

We now consider the main issue of the connection of our findings with the longstanding problems with regard to fundamental clock function: temperature compensation and entrainment (Penfield 2008, McClung and Davis 2010, Boikoglou et al. 2011). We do not know whether the findings of this study are closely relevant to these fundamental issues. However, a number of miscellaneous previous reports should be mentioned here. A *prp9 prp7* double loss-of-function mutant fails to maintain oscillation after entrainment to temperature cycles (Salome and McClung 2005a). The effects of temperature on clock speed are also overcompensated in the *prp9 prp7* mutant (Salome et al. 2010). Both *LUX* and *GI* are also implicated in temperature compensation (Edwards et al. 2006, Gould et al. 2006). The diurnal rhythm of *GI^{Pro}::LUC* expression could not be synchronized with temperature cycles in a *lux* mutant (named *pcl1-1* in this study) (Onai and Ishiura 2005). Furthermore, an *elf3* mutant fails to exhibit any indication of entrainment by temperature cues in continuous darkness (Thines and Harmon 2010); the same authors showed that *ELF3* activity appears to be a prerequisite for seedlings to respond correctly to temperature signals. This last notion is consistent with the findings of this study. Taking these preceding notions together, the EC-dependent temperature-responsive signaling within the clock transcriptional circuitry should provide insights into the classical problems in the field of study of the plant circadian clock, as further considered below.

In this respect, we noted earlier that the expression of *LUX* tends to be prolonged by a warm temperature signal in the early night, whereas *PRR7* (*PRR9*) is precociously induced by the same stimulus in the late night (see Fig. 6). In contrast, *LHY* (*CCA1*) is promptly repressed at a warm temperature in the late night (see Fig. 2). In other words, through the EC night-time repressor, a warm temperature at dusk causes a delayed phase of *LUX*, whereas the same stimulus at dawn results in advancing phases of *PRR7* and *LHY*. These make a lot of sense judged on the basis of temperature phase response curves (PRCs), which provides us with the general idea that a warmer temperature present at the beginning or end of the dark period resets the circadian clock (Salome and McClung 2005b). A warmer temperature at dusk is indicative of a prolonged evening (i.e. the evening gene *LUX* should continue being expressed), and the same signal at dawn is indicative of the coming sunrise (i.e. the daytime genes *PRR9* and *PRR7* should start to be expressed, and the morning genes *CCA1* and *LHY* should also be promptly repressed). Hence, it is tempting to speculate that the EC night-time repressor-mediated temperature responses are important for fine-tuning of the clock transcriptional circuitry to adjust clock speed properly and/or to entrain to temperature cycles properly. In other words, the EC-dependent modifications of *GI*, *LUX*, *PRR9* and *PRR7* are implicated in the mechanisms underlying temperature compensation and entrainment. This

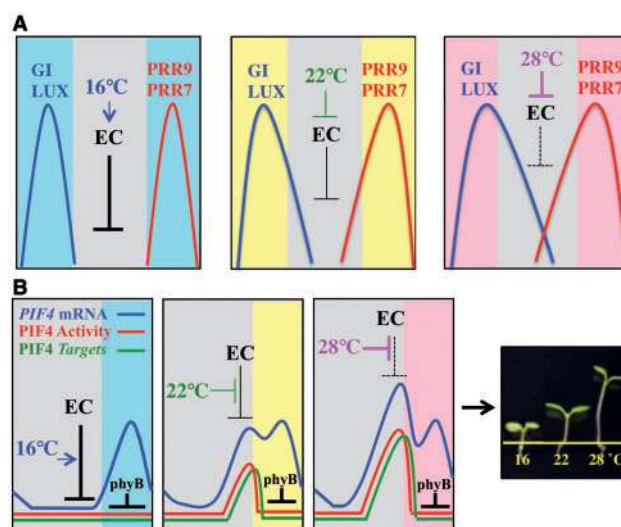


Fig. 13 Schematic representations of views proposed in this study. (A) A simple model of rhythmic expression of EC-sensitive clock genes under different temperature conditions. In this study, it was shown that, through the EC night-time repressor, a warm temperature at dusk causes a delayed phase of *LUX* and *GI*, whereas the same stimulus at dawn results in advanced phases of *PRR9* and *PRR7*, as illustrated. It is tempting to speculate that such EC night-time repressor-mediated temperature responses are important for fine-tuning of the clock transcriptional circuitry to adjust clock speed properly and/or to entrain properly to temperature cycles. Hence, the EC-dependent modifications of *GI*, *LUX*, *PRR9* and *PRR7* might be implicated in the mechanisms underlying temperature compensation and entrainment. (B) Schematic representation of the mechanism underlying the *PIF4*-mediated control of hypocotyl elongation. Briefly, the diurnal expression profile of another EC target, the *PIF4* output gene, is also modulated through a common mechanism in response to differences in growth temperatures (e.g. 16, 22 and 28°C), as schematically shown in each panel. Based on the observation that the *PIF4* protein is stably and actively accumulated only in the dark, the enhanced derepression of *PIF4* at 28°C in the dark coincidentally results in a temperature-dependent enhancement of *PIF4* target genes, the protein products of which more highly promote the elongation of hypocotyls at a higher temperature. Consequently, a temperature signal is fed as far as the *PIF4*-mediated output pathway, thereby leading to the temperature-adaptive control of hypocotyl elongation.

idea is schematically illustrated in Fig. 13A. In fact, through analysis of a temperature phase response curve of a *prp9 prp7* double mutant, it was suggested that the mutant fails to reset the phase of *TOC1* rhythms in response to a cold temperature stimulus after sunrise (Salome and McClung 2005a). We also found that temperature effects on *TOC1*, *PRR5*, *ELF3* and *ELF4* were subtle (most probably indirect) (Supplementary Fig. S4). However, these indirect effects on the clock transcriptional circuitry might also induce a significant consequence of the clock functions including temperature compensation and entrainment.

Next, we would like to point out the implications of our findings to clock-controlled aspects of plant physiology

(Fig. 13B). It is important to understand how the central oscillator integrates ambient temperature signals to control output pathways in order to adapt properly to changes in temperature and/or differences in steady-state growth temperature in natural habitats. Based on this rationale, we have been focusing on the well-characterized temperature-adaptive output pathway, which is mediated by PIF4 (see Fig. 11A) (Niwa et al. 2009, Kunihiro et al. 2011). The diurnal expression profile of PIF4 is regulated through the circadian clock so as to accumulate PIF4 transcripts at the end of night in a short-day-specific manner (Niwa et al. 2009). However, this photoperiodic regulation is conditional on growth temperature such that a warmer temperature more heavily stimulates PIF4 expression before dawn (see Fig. 11) (Nomoto et al. 2012a, Nomoto et al. 2012b, Nomoto et al. 2013, Yamashino et al. 2013). This temperature-dependent regulation of PIF4 is central to the temperature-adaptive control of hypocotyl elongation. Nevertheless, how the diurnal expression profile of PIF4 is modulated in response to temperature has been puzzling. The results of this study revealed that the mode of PIF4 regulation in response to temperature is the same as that of PRR7, GI and LUX (Fig. 12). Through this EC night-time repressor-mediated mechanism, Arabidopsis seedlings are able to regulate their hypocotyl length properly in response to differences in ambient temperature, as schematically shown in Fig. 13B (see also Figs. 10 and 11). It should be noted that PIF4 is also crucial to promote flowering particularly in warm short days (Kumar et al. 2012). This example suggested the intriguing idea that temperature signals feed into the central oscillator through the EC night-time repressor to control a variety of output genes, thereby leading to various temperature-adaptive physiological outputs.

The circadian clock has the unique ability to integrate and unify the environmental cues of both photoperiod and temperature to control plant growth properly. However, the characteristics of ambient temperature are different in principle from those of photoperiod, because the former varies on a time-to-time, day-to-day, season-to-season and habitat-to-habitat basis, whereas the latter is more constant in that photoperiod varies only on a season-to-season and latitude-to-latitude basis. During the distant past, plants evolved sophisticated mechanisms to adapt to such ever-changing temperatures in order to adapt to the lateral and/or horizontal drift of domestication. The likely coming era of global warming in the absence of a changing photoperiod should be a concern. For example, elevated temperatures induce premature flowering in many plant species, and the probable photoperiod-dependent transition to flowering being affected by global warming has been a concern (Samach and Wigge 2005, Wigge 2013). In this respect, it has been suggested that precocious flowering under warm short days is partly mediated by GI (Paltiel et al. 2006), and crucially mediated by PIF4 (Kumar et al. 2012). A general message from this study is that the circadian clock-associated EC night-time repressor might regulate a variety of output genes in a temperature-dependent manner (Helfer et al. 2011, Liu et al. 2013). This type of regulation could be reversible in

response to a small change (e.g. as small as $\Delta 6^{\circ}\text{C}$) across a wide range of growth-compatible temperatures (from 16 to 28°C). Appropriate genetic manipulation of such sophisticated temperature-adaptive outputs, which evolved inevitably during the distant past, would allow not only model plants but also crops to cope with a small but deleterious change in ambient temperature in natural habitats.

Materials and Methods

Plant lines and growth conditions

The *A. thaliana* plants used in this study were all of the Col-0 genetic background except for *elf3-4* ELF3:ELF3-YFP (WS background; Dixon et al. 2011). The *cca1-1 lhy-11* (Niwa et al. 2007), *elf3-8* (Hicks et al. 2001), *elf4-2* (Hazen et al. 2005a), *pcl1-1* (Onai et al. 2004, Onai and Ishiura 2005) *lux-4* (Hazen et al. 2005b), *prr9-10 prr7-11* (Nakamichi et al. 2005a), *prr9-10 prr7-11 prr5-11* (Nakamichi et al. 2005b), *toc1-2* (Ito et al. 2007), *gi-2* (Araki and Komeda 1993) and *arp6-1* (Kumar et al. 2012) mutants were described previously. For ChIP experiments, transgenic lines expressing LUX-GFP under the control of its natural promoter in *lux-4* (LUX:LUX-GFP; Helfer et al. 2011), ELF3-YFP under the control of its natural promoter in *elf3-4* (ELF3:ELF3-YFP; Dixon et al. 2011) and ELF3-HA under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter in *elf3-8* (35S:ELF3-HA, this study) were used. Seeds were surface sterilized and stratified at 4°C , germinated and grown on Petri dishes containing Murashige and Skoog (MS) medium, 1.0 % (w/v) sucrose and 0.3% (w/v) gellan gum, at pH 5.7, in climate-controlled growth chambers at 22°C under neutral white fluorescent light ($70\ \mu\text{mol m}^{-2}\text{s}^{-1}$) for 3 d and further grown in LD photoperiod cycles at different temperatures. Temperature conditions used were 16°C (cool temperature), 22°C (optimum temperature) and 28°C (warm temperature).

Plasmid construction

Synthesized DNA including the *Sall*–*XbaI*–*BamHI*–*SmaI*–*NotI*– $2 \times \text{HA}$ –stop codon–*NotI* sequence (GTGACTCTAGAGGATCCCCGGGTACCGGTGCGCCACCATGGGCTACCTTACGACGTTCCAGATTACGCTGGTTACCTTACGACGTTCCAGATTA CGCTTAAAGCGGCCGC) was cloned into *Sall* and *NotI* sites of pBluescript II SK(+) (pBS-2xHA-C). The coding sequence of *ELF3* was amplified from the Arabidopsis cDNA library by PCR with the AtELF3-*Sall*-*SpeI*-CF primer (GTAGTCGACACTAGTATGAAGAGAGGGAAAGATGAGGAG) and the AtELF3-*BamHI*-CR primer (GGGGATCCGGCTTAGAGGAGTCATAGC G), and digested with *Sall* and *BamHI*. About 2.1 kbp of purified *Sall*–*BamHI* fragments were cloned to *Sall*- and *BamHI*-digested pPBS-2xHA-C. The cloned plasmid was digested by *SpeI* and *NotI*, and about 2.2 kbp of purified *SpeI* and *NotI* fragments including the *ELF3*-HA fusion construct were cloned into *XbaI*- and *NotI*-digested pSK1 (Kojima et al. 1999). The resultant plasmid pSK1-ELF3-HA was used for *Agrobacterium*-mediated transformation of *A. thaliana*.

Preparation of RNA and qRT-PCR

Total RNA was purified from frozen plant materials (the aerial part of 7- or 8-day-old seedlings) with the RNeasy plant mini kit (Qiagen, Venlo). To synthesize cDNA, RNA (1 µg of each) was converted into cDNA with ReverTra Ace (TOYOBO) and an oligo(dT) primer. The synthesized cDNAs were amplified with SYBR Premix Ex Taq II (TAKARA) and the primer set for each target gene, and analyzed by using a StepOne Plus™ Real-Time PCR System (Life Technologies). The primer sets used are summarized in **Supplementary Table S1**. The following standard thermal cycling program was used for all PCRs: 95°C for 120 s, 40 cycles of 95°C for 10 s and 60°C for 60 s. The Ct value for individual reactions was determined by analysis of raw fluorescence data (without baseline correction) using the freely available software PCR Miner (Zhao and Fernald 2005; <http://www.miner.ewindup.info>). Based on the comparative Ct method, the relative expression level was calculated. The APX3 encoding an ascorbate peroxidase isozyme was used as an internal reference. To pre-set our qRT-PCR procedure properly, the following experiments have been carried out (**Supplementary Fig. S16**). Wild-type seedlings (Col-0) were grown in LD cycles, and RNA samples were prepared at 3 h intervals. By means of qRT-PCR with the set of appropriate primers, we examined the diurnal expression profiles of the set of clock genes in question (*CCA1*, *LHY*, *PRR9*, *PRR7*, *PRR5*, *TOC1*, *GI*, *ELF3*, *ELF4* and *LUX*) (red lines). The expression profiles were compared with the corresponding standard profiles retrieved from the public 'Diurnal' database, a web-based tool for accessing the diurnal and circadian genome-wide expression profiles of any gene from a series of DNA microarray experiments conducted on *A. thaliana* (<http://diurnal.mocklerlab.org>) (blue lines). In each case, our results were well superimposed with the standard profiles, indicating that our experimental conditions, particularly the primer specificity and qRT-PCR conditions, have been properly pre-set for this study.

Chromatin immunoprecipitation

The transgenic lines described above were grown on MS gellan gum plates containing 1.0% sucrose under 12 h light/12 h dark cycle conditions at 22°C for 2 weeks after germination. At ZT = 21, about 3 g FW of the aerial parts of the seedlings were harvested in a dark room and cross-linked for 20 min under vacuum in 50 ml of cross-linking buffer [10 mM Tris-HCl, pH 8, 1 mM EDTA, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% formaldehyde]. Cross-linking was quenched in stopping buffer (2 × TBS, 125 mM glycine), under vacuum for 5 min, and seedlings were washed twice in water before snap freezing. Tissues were disrupted in a ball mill in liquid nitrogen. Ground tissues were resuspended with 25 ml of extraction buffer I [0.4 M sucrose, 10 mM Tris-HCl, pH 8, 10 mM MgCl₂, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 50 µM Z-Leu-Leu-Leu-al (MG132), and 1/100 vol. of protease inhibitor

cocktail; Sigma], then filtered through three-layered Miracloth (Calbiochem). The filtrate was centrifuged at 4,000 r.p.m. at 4°C for 20 min. The pellet was resuspended in 1 ml of extraction buffer II (0.25 M sucrose, 10 mM Tris-HCl, pH 8, 10 mM MgCl₂, 1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 50 µM MG132 and 1/100 vol. of protease inhibitor cocktail) and centrifuged at 14,000 r.p.m. at 4°C for 10 min. The pellet was resuspended in 300 µl of extraction buffer III (1.7 M sucrose, 10 mM Tris-HCl, pH 8, 0.15% Triton X-100, 2 mM MgCl₂, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 50 µM MG132 and 1/100 vol. of protease inhibitor cocktail) and loaded on the top of an equal amount of clean extraction buffer III, then centrifuged at 14,000 r.p.m. for 1 h. The crude nuclear pellet was resuspended in 300 µl of nuclear lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 50 µM MG132 and 1/100 vol. of protease inhibitor cocktail) and sonicated by a Bioruptor (Cosmo Bio) with an option setting of high power 30 s on/60 s off 10 times to achieve an average fragment size of 0.3–1.0 kb. The sonicated chromatin was centrifuged (15,000 r.p.m. for 5 min at 4°C), and the insoluble pellet was discarded. The soluble chromatin solution was diluted 10-fold with ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, and 167 mM NaCl). After pre-clearing with a 50 µl bed volume of ChIP dilution buffer-equilibrated Dynabeads protein G (Invitrogen) for 1 h, 5 µl of HA tag-specific monoclonal antibody (clone 3F10; Roche) or 1 µl of AvGFP (EGFP, EYFP and ERFP)-specific monoclonal antibody (JL-8, TAKARA BIO INC.) was added to 1 ml of chromatin solution and incubated overnight at 4°C. The solution was further incubated with a 50 µl bed volume of the ChIP dilution buffer-equilibrated Dynabeads protein G for 1 h at 4°C. After washing with low salt buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA) and high salt buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA), immunocomplexes were eluted from the beads using elution buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% SDS). The samples were incubated with DNase- and RNase-free proteinase K (Invitrogen) at 65°C to remove cross-linking and all proteins, and then treated with 2 µg of RNase A for 30 min (ChIP DNA sample) at 37°C. Another 1 ml of the chromatin solution without any treatment described above was added to 80 µl of 5 M NaCl and the DNase- and RNase-free proteinase K, incubated at 65°C and subjected to the RNase treatment as a control (Input DNA sample). DNA was purified by NucleoSpin (Macherey-Nagel) according to the manufacturer's protocol. The amount of ChIP and input DNA was determined by real-time PCR using specific primers (**Supplementary Table S2**). The rate of the immunoprecipitated chromatin was calculated for each amplicon using the following equation: $2^{Ct[ChIP]} / 2^{Ct[Input]}$.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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